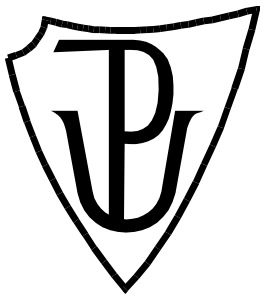

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Effect of brassinosteroids on ethylene production in plants

Ph.D. thesis
P1527 Biology – 1501V019 Experimental biology

Supervisor – Mgr. Jana Oklešťková, PhD.

Olomouc
2019

ACKNOWLEDGEMENTS

I would like to thank my supervisor Mgr. Jana Oklešťková, PhD. for her support, encouragement, patience and supervision during my studies. Prof. Miroslav Strnad, the head of the laboratory for the material support and critical reading of this thesis. All of my colleagues and friends from the Laboratory of Growth Regulators are acknowledged for their endless help, support, scientific and non-scientific discussions and the friendly environment they create.

I would also like to thank Prof. Dominique van der Straeten, Prof. Jenny Russinova and members of their groups for excellent scientific and technical assistance during my research stays in their labs.

My sincerest gratitude goes also to one Steppenwolf, basically for everything, thank you.

And last but not least I would like to express my huge thanks to my parents and my husband for believing in me and for great support.

DECLARATION

I hereby declare that this Ph.D. thesis is my original work. The literature used is listed in the references section.

In Olomouc

Petra Jiroutová

BIBLIOGRAPHICAL IDENTIFICATION

Author's first name and surname	Petra Jiroutová
Title of thesis	Effect of brassinosteroids on ethylene production in plants
Type of thesis	Ph.D. thesis
Department	Laboratory of Growth Regulators
Supervisor	Mgr. Jana Oklešťková, PhD
The year of presentation	2019
Abstract	Brassinosteroids (BRs) are a group of steroidal plant hormones involved in a variety of physiological processes in plants. BR mutants are characterized by dwarf phenotype with curled, dark-green leaf rosette, short internodes, and male sterility. The first and most active BR - brassinolide, was isolated in 1979. Since then, a significant amount of work on their biosynthesis, signaling, biological activity and practical applications, especially in agriculture, has been done. Various crosstalk mechanisms between BRs and other hormones are postulated or have been characterized. In this work, we investigated the interaction between brassinosteroids and ethylene. Using two different bioassays (the Pea inhibition test and Arabidopsis growth test) we investigated the structure–activity relationship of biosynthetic precursors of brassinolide to determine which structural motif is important for BR- induced inhibition of etiolated growth in pea plants as well as ethylene production. The same two assays were used for screening the biological activity of new BR synthetic derivatives.
Keywords	brassinosteroid, ethylene, crosstalk, 1-aminocyclopropane-1-carboxylic acid (ACC)
Number of pages	66
Number of appendices	5
Language	English

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LIST OF PAPERS & CONTRIBUTION REPORT

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    - Co-author – screening of biological activities of new brassinosteroid analogues (performing the pea inhibition test, measurement of ethylene production in treated pea plants)

- **Korinkova P**., Bazgier V., Oklestkova J., Rarova L., Strnad M., Kvasnica M. (2017). Synthesis of novel aryl brassinosteroids through alkene cross-metathesis and preliminary biological study. Steroids 127, 46-55. (Supplement II)
    - First author - screening of biological activities of new brassinosteroid analogues (performing the pea inhibition test, measurement of ethylene production in treated pea plants, Arabidopsis root and hypocotyl sensitivity assays)

- **Jiroutová P**., Oklestkova J., Strnad M., (2018) Crosstalk between Brassinosteroids and Ethylene during Plant Growth and under Abiotic Stress Conditions. Int. J. Mol. Sci 19 (10), 3283. (Supplement III)
    - First author – writing of manuscript

- **submitted paper** - **Jiroutová P**., Mikulík J., Novák O., Oklestkova J., Strnad M., (2019) Brassinosteroids induce strong, dose-dependent inhibition of etiolated pea seedling growth correlated with ethylene production. Biomolecules, special issue – Phytohormones. (Supplement IV)
    - First author - bioassay development, optimization, performing of the experiments, writing of manuscript

<u>Chapter in book</u>

- J. Oklestkova, **P. Korinkova**, M. Strnad: The Crosstalk of Brassinosteroids and Other Hormones in Abiotic Stress Tolerance, in: Mechanisms Behind Phytohormonal Signalling and Crop Abiotic Stress Tolerance, NOVA SCIENCE PUBLISHERS, INC., NY 11788-3619, USA, 2017. (Supplement V)
    - Co-author – participation in writing of the manuscript

ABBREVIATIONS

ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
AOX	alternative oxidase
AtEXP	expansin gene
APX	ascorbate peroxidase
ATP	adenosine triphosphate
BAK1	BRASSINOSTEROID INSENSITIVE1-ASSOCIATED RECEPTOR KINASE 1
BAS1	PHYB ACTIVATION TAGGED SUPPRESSOR 1
BES1	BRI1-EMS-SUPPRESSOR1
BIN2	BRASSINOSTEROID INSENSITIVE2
BKI1	BRI1 kinase inhibitor-1
BL	brassinolide
BRI1	BRASSINOSTEROIDS INSENSITIVE 1
BRs	brassinosteroids
BSK1	BRASSINOSTEROID-SIGNALLING KINASE 1
BSU1	BRI1-SUPPRESSOR 1
BY-2	BRIGHT YELLOW 2
BZR1	BRASSINAZOLE-RESISTANT 1
BZR2	BRASSINAZOLE-RESISTANT 2
CAT	catalase
CDG1	CONSTITUTIVE DIFFERENTIAL GROWTH 1
CMT	cortical microtubules
CN	campestanol
Col-0	Columbia ecotype
CPD	constitutive photomorphogenesis and dwarfism protein
CR	campesterol
CS	castasterone
CT	cathasterone
CTR1	CONSTITUTIVE TRIPLE RESPONSE 1
CYP85A1	cytochrome P450 85A1
CYP85A2	cytochrome P450 85A2
CYP90A1	cytochrome P450 90A1
CYP90B1	cytochrome P450 90B1
CYP90C1	cytochrome P450 90C1

CYP90D1	cytochrome P450 90D1
DET2	DE-ETIOLATED 2
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
DRL1	DWARF AND ROUND LEAF-1
3DT	3-dehydroteasterone
DWF	DWARF
6-deoxoCS	6-deoxocastasterone
6-deoxoCT	6-deoxocathasterone
6-deoxoTY	6-deoxotyphasterol
EIL1	EIN3-LIKE
EIN2	ETHYLENE INSENSITIVE 2
EIN3	ETHYLENE INSENSITIVE 3
EIN4	ETHYLENE INSENSITIVE 4
24-epiBL	24-epibrassinolide
24-epiCS	24-epicastasterone
ERF1	ETHYLENE RESPONSE FACTOR 1
ERS1	ETHYLENE RESPONSE SENSOR 1
ERS2	ETHYLENE RESPONSE SENSOR 2
ER	endoplasmic reticulum
ET	ethylene
eto1	ethylene overproducing
ETR1	ETHYLENE RESPONSE 1
ETR2	ETHYLENE RESPONSE 2
FID	flame ionic detector
GACC	1-(γ-L-glutamylamino)
HLS1	HOOKLESS
JA	jasmonic acid
LRR/RLK	leucine-rich repeat receptor-like kinase
MACC	malonyl-ACC
Met	methionine
MVA	mevalonate
NLS	nuclear localization sequence
6-oxoCN	6-oxocampestanol
P450	P450 monooxygenase
PIN	pin formed
PP2A	PROTEIN PHOSPHATASE 2A
28-norCS	28-norcastasterone
ROS	reactive oxygen species
ROT3	ROTUNDIFOLIA3

SA	salycilic acid
SAM	S-adenosylmethionine
SERK3	SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3
TE	teasterone
TYP	typhasterol
UGT73C	UDP-glycosyltransferase
UPLC-MS/MS	ultra-performance liquid chromatography-tandem mass spectrometry
WT	wild type

INTRODUCTION

Plant hormones affect various aspects of plant development and play key roles in plant resistance to diverse environmental stresses. While extensive research has uncovered the effects of the main phytohormone classes - auxins, cytokinins, gibberellins, abscisic acid and ethylene, newer classes of plant hormones have been discovered like brassinosteroids, jasmonic acid, salicylic acid and polyamines. It is now recognized that plant growth and development are controlled by the mutual interactions among plant hormones. This thesis focuses on plant hormone crosstalk as an emergent area of this research.

Brassinosteroids (BRs) are a family of naturally occurring steroid plant hormones that regulate various processes of growth and development, including cell elongation, cell division, leaf senescence, vascular differentiation, flowering time control, male reproduction, photomorphogenesis and responses to biotic and abiotic stresses. A number of these effects are tightly linked with almost all other classes of plant hormones.

This work looks closer at the relationship between brassinosteroids and ethylene, which is the only gaseous plant hormone with a simple structure. Ethylene plays an important role in a number of developmental processes in plants like opening of flowers, ripening of fruits and abscission of leaves. It is also a stress hormone and such is involved in most plant responses to biotic and abiotic stresses.

The structure-activity relationship of biosynthetic precursors of brassinolide and a series of new brassinosteroid analogues were also studied in this research to elucidate which structural motifs are important for BR induced biological activity. These structures could be then used as a template for synthesis of new BR analogues with growth promoting activity for agricultural usage.

AIMS AND SCOPE

Brassinosteroids (BRs) as an important group of steroidal plant hormones involved in a variety of crucial physiological processes, are interesting compounds for further investigation and potential usage in agriculture. Because plant growth and development is a complex subject in which more than one group of plant hormones is involved, it is important to take the crosstalk between plant hormones into account and think more broadly about the topic.

The overall aims of the work described in this thesis were as follows:

1. To write a review about crosstalk between brassinosteroids and ethylene
2. To examine and assess the effect of new BR synthetic derivatives on ethylene production in plants
3. To evaluate the biological activity of these compounds in different bioassays
4. To investigate the biological activity of BR biosynthetic precursors and their effect on ethylene production

1. LITERATURE REVIEW

1.1 BRASSINOSTEROIDS

1.1.1 Introduction

Brassinosteroids represent a class of plant polyhydroxysteroids playing an essential role in plant growth and development. Characteristically, BRs occur in plants in very low concentrations and have been detected throughout the plant kingdom (including both lower and higher plants). Regarding the distribution of these compounds within plants, brassinosteroids have been found in all organs including roots, stems, leaves, flowers, pollen, anthers and grain. The richest sources of BRs are pollen and immature seeds (1-100 μg/kg of fresh weight). The lowest content is usually found in shoots and leaves (0.01-0.1 μg/kg of fresh weight). BL was discovered in 1979, and since then more than 70 BRs have been isolated, where castasterone (CS) is the most widely distributed followed by brassinolide (BL), typhasterol (TYP), 6-deoxocastasterone (6-deoxoCS), teasterone (TE), 28-norcastasterone (28-norCS) and 6-deoxotyphasterol (6-deoxoTY) (Hayat and Ahmad 2011; Oklestkova et al. 2015). All known natural BRs have a common 5α-cholestane skeleton with structural differences due to type and position of functionality in the A and B rings and also by changes in the side chain. The most active brassinosteroid so far, is brassinolide [(22R,23R,24S)-2α,3α,22,23-tetrahydroxy-24-methyl-homo-7-oxa-5α-cholestan-6-one] its structure consisting of a lactone function at C-6/C-7, cis-vicinal hydroxyls at C-2 and C-3, R configuration of the hydroxyls a C-22/C-23 and a methyl substitution at C-24 appears to be the optimal structure for greatest BR activity (Hayat and Ahmad 2011; Clouse 2011; Tarkowska et al. 2016).

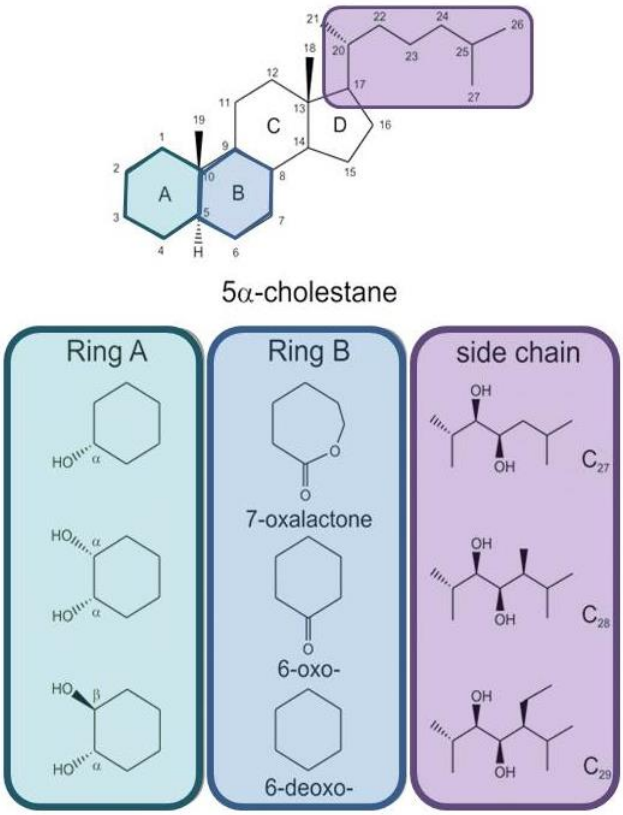

Figure 1. General structure of 5α-cholestane and various substituents on ring A, B and the side chain of naturally occurring brassinosteroids (adapted from Tarkowska et al. 2016).

1.1.2 Biosynthesis

The pathway of BR synthesis was initially established in suspension cultures of *Catharanthus roseus* through feeding experiments with labeled substrates and following their metabolism (Suzuki et al. 1995). Further experiments using Arabidopsis mutants were crucial for the subsequent identification of several BR biosynthetic genes which considerably contributed to elucidating the BR biosynthetic pathway as we know it today (Noguchi et al. 2000; Zhao and Li 2012). Most known BR biosynthetic enzymes (with the exception of DET2 DE-ETIOLATED 2) belong to the cytochrome P450 enzyme family (Wei and Li 2015). P450 enzymes have broad substrate-specificity, enabling conversion of multiple intermediates which results in several parallel biosynthetic pathways creating a highly networked complex (Hayat and Ahmad 2011).

Currently, it is assumed that synthesis of BRs is provided via a secondary metabolic pathway, where Acetyl-CoA is converted to mevalonate (MVA) via a number of steps. MVA is then converted through multiple reactions to campesterol (CR), which is considered to be the first BR-specific biosynthetic precursor (Wei and Li 2015). As mentioned above, the BR biosynthetic pathway is formed more like a grid than a simple linear pathway. This implies that in plants there are several ways in which BL is synthesizes from CR. The most commonly accepted scheme according to the literature offers two main parallel pathways - the early and the late C-6 oxidation pathway. In both cases the biosynthesis starts with conversion of campesterol (CR) to campestanol (CN) and then in the case of the early C-6 oxidation pathway CN is converted into 6-oxocampestanol (6-oxoCN) and then to cathasterone (CT), teasterone (TE), 3-dehydroteasterone (3DT), typhasterol (TYP), and castasterone (CS), respectively. Otherwise the late C-6 oxidation pathway is characterized by initial hydroxylation of CN at C-22 which forms 6-deoxocathasterone (6-deoxoCT). This is then converted to corresponding intermediates similar to those in the early C-6 oxidation pathway but in a C-6 deoxy form. These two pathways converge at CS, which ultimately leads to the biosynthesis of BL and both are known as CN-dependent pathways. Later analyses also discovered an early C-22 oxidation branch called “CN-independent pathway”. In this case, campesterol is directly oxidized at C-22. This branch is linked to the later part of the late C-6 oxidation pathway. The high levels of 6-deoxoCT and 6-deoxoCS in different analyzed species, suggest that the late C-6 oxidation pathway is the predominant branch of BR biosynthesis (Wei and Li 2015; Ohnishi 2018).

As in the case of other plant hormones, not only biosynthesis itself but more likely homeostasis of the hormone level is crucial for proper growth and development. Therefore plants have evolved a strategic feedback loop which can downregulate the biosynthesis of BRs in a concentration dependent manner and consequently control the rate of synthesis. For example, one of the first discovered mechanisms in this loop is the suppression of *CPD* transcription on BR treatment (Mathur et al. 1998). Further experiments showed that BR transcription factors BES1 and BZR1 are directly involved in repressing the expression of several BR biosynthesis genes when endogenous levels

of BRs reach the proper level of hormone to maintain normal plant growth and development (He et al. 2005; Sun et al. 2010; Yu et al. 2011). Not only regulation of BR synthesis but also removal of the bioactive hormone helps to maintain the homeostasis and avoid harmful accumulation of bioactive BRs. Compared to biosynthesis, the mechanisms and enzymes of BR catabolism are still poorly understood. Nevertheless, some enzymes are known. One of these is BAS1 (PHYB ACTIVATION-TAGGED SUPPRESSOR1). BAS1 – a member of the P450 enzyme family is able to convert both CS and BL to their C-26 hydroxylated derivatives (Neff et al. 1999). Another example is an enzyme called UGT73C, which can catalyze the 23-O-glucosylation of CS and BL (Poppenberger et al. 2005). It is also known that enzyme, DRL1 (DWARF AND ROUND LEAF-1) acts like acyltransferase and probably promotes esterification of certain BRs (Zhu et al. 2013).

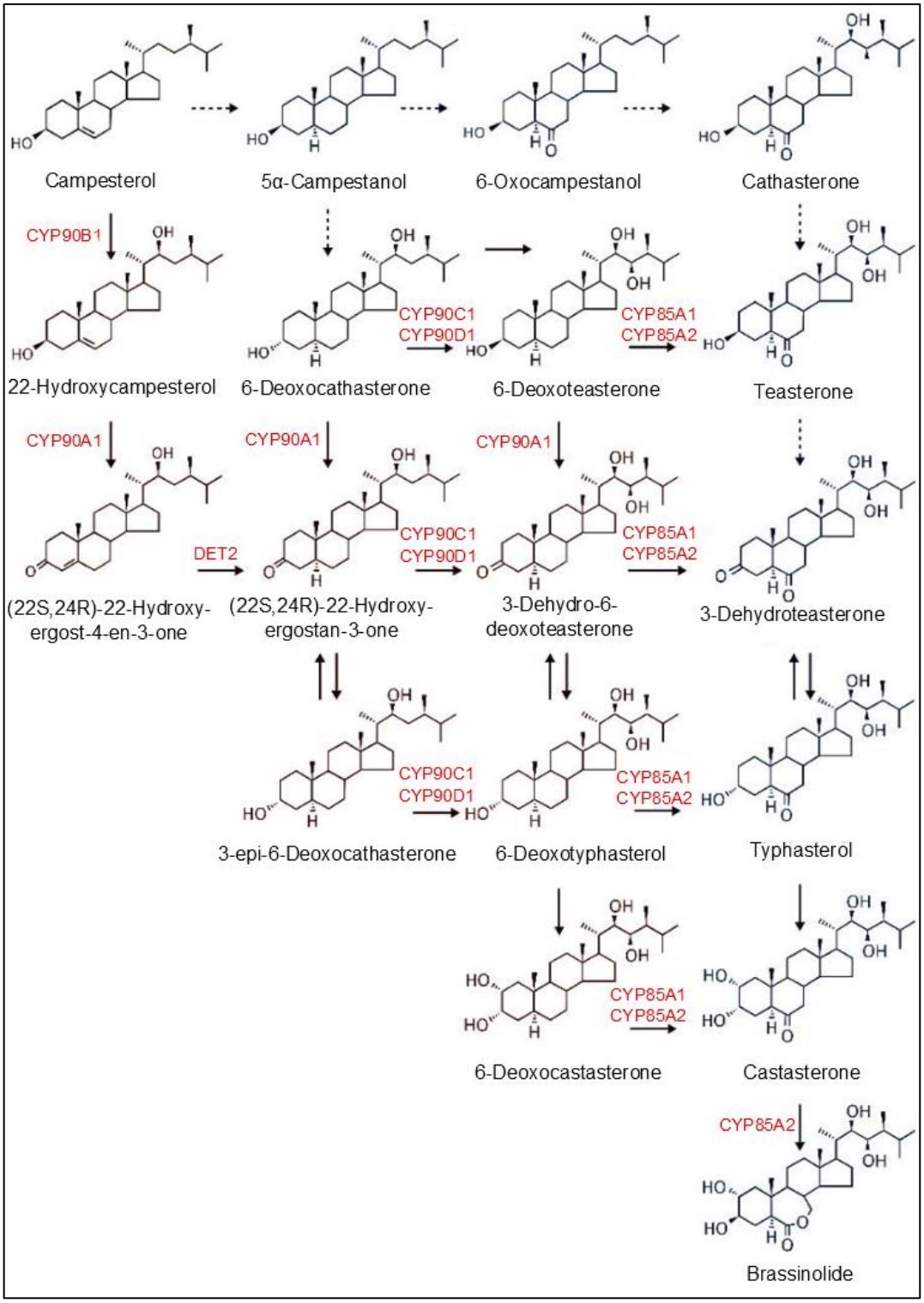

Figure 2. Proposed pathways and genes involved in brassinosteroid biosynthesis (adapted from Buchanan et al. 2015).

1.1.3 Signaling

In general, BR signaling pathway starts with perception of BRs by plasma membrane-localized receptors, after which the BR-mediated signals are transduced by downstream cystolic regulators to the nucleus, where the transcription of BR-responsive genes is activated (Zhu et al. 2013). BRs bind extracellularly to BRI1 (BRASSINOSTEROIDS INSENSITIVE 1) leucine-rich repeat receptor-like kinase (LRR-RLK) (Li and Chory 1997; Wang et al. 2001). Direct binding of BRs to the extracellular domain of BRI1, triggers dissociation of the inhibitory protein BKI1 (BRI1 kinase inhibitor-1) (Wang and Chory 2006) and formation of a heterodimer consisting of BRI1 and BAK1 (BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE 1, also known as SERK3 - SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3) and this complex initiates an intracellular phosphorylation cascade (Li and Nam 2002; Russinova et al. 2004). Activated BRI1 phosphorylates two plasma membrane-anchored cytoplasmic kinases BSK1 (BRASSINOSTEROID-SIGNALLING KINASE1) and CDG1 (CONSTITUTIVE DIFFERENTIAL GROWTH1) (Kim et al. 2011; Tang et al. 2008) which leads to phosphorylation and activation of BSU1 (BRI1-SUPPRESSOR1) (Kim et al. 2011). Subsequently, the activated BSU1 inhibits BIN2 (BRASSINOSTEROID INSENSITIVE2). The inhibition happens through dephosphorylation of conserved tyrosine residue (Kim and Wang 2010) and the inactivated BIN2 is degraded by the proteasome (Peng et al. 2008). In the case of no or low level of BRs, BIN2 remains in the active state and phosphorylates two homologous transcription factors BZR1 and BZR2 (BRASSINAZOLE RESISTANT1 and 2). BZR2 is also known as BES1 (BRI1-EMS-SUPPRESSOR1) which in the phosphorylated state cannot bind DNA and is retained in the cytoplasm, where both factors are degraded by proteasome (He et al. 2002; Yin et al. 2002). In the case of high BR level, BIN2 is inactivated, which means that transcription factors BZR1 and BZR2 are dephosphorylated by PP2A (PROTEIN PHOSPHATASE 2A) and they can move into the nucleus to bind to the promoters of their target genes which leads to regulation of the BR response (Tang et al. 2011; He et al. 2005; Yu et al. 2011, Planas-Riverola et al. 2019).

1.1.4 BR mutants

Both BR-deficient and BR-insensitive mutants, exhibit similar phenotypes with dramatic alterations, which are characteristic for lack of BRs. Light-grown BR mutant Arabidopsis plants have drastically reduced plant height and root system and, the rosette has typically compact structure with a small dark green leaves as a result of strong reduction of leaf expansion. Reduced growth of BR-deficient mutants is also often accompanied by the sterility of these plants. Unlike BR-insensitive mutants, the dwarf growth of BR-deficient mutants can be rescued by treatment with exogenously applied BRs (Müssig 2005).

BR mutants have been identified in other dicotyledons too. In pea plants, the *LK* gene encoding a *DET2* homologue and *LKB* gene as a *DWF1* homologue have been reported. Mutation in these genes results in BR biosynthesis pea mutant plants, which are also characterised by their dwarf growth. Since, the *LKA* gene encodes a homologue of the Arabidopsis BRI1 receptor in pea plants, the *lka* mutant is an example of pea mutant which is defective in BR perception. Similar homologues of genes encoding BR biosynthesis enzymes or BRI1 leucine-rich repeat receptor kinase, have been identified in tomato, rice and barley (Nomura et al. 1997, 1999, 2003, 2004).

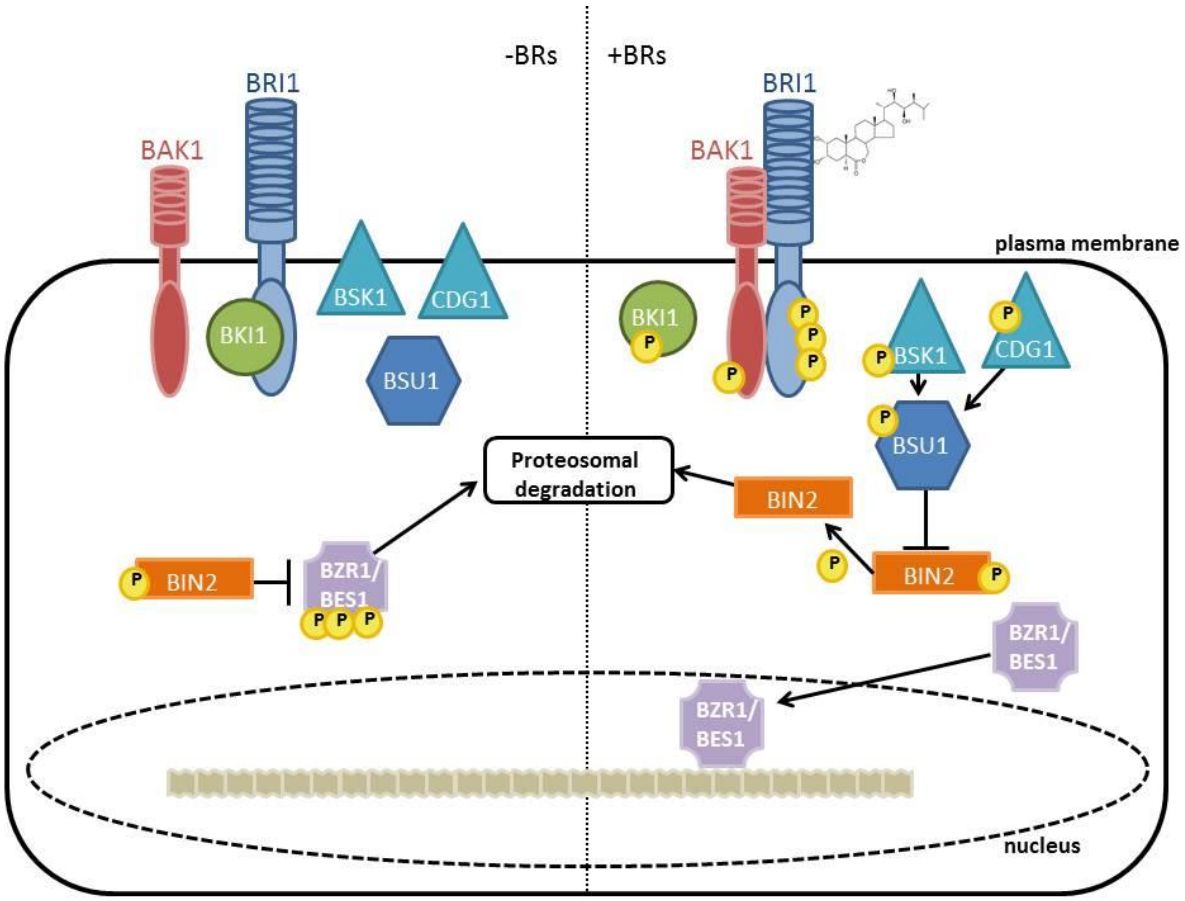

Figure 3. Proposed model for brassinosteroid (BR) signaling pathway in the absence and presence of BRs.

1.1.5 Physiological effects of BRs

The characteristic dwarf phenotype of both BR-insensitive and BR-deficient mutant provides clear evidence that BRs are essential for proper plant growth and development.

Cell elongation, cell division, cell differentiation

BRs are known for their promotion effect on cell expansion based on regulation of the expression of genes involved in cell wall modification, cytoskeleton rearrangements and cellulose biosynthesis (Clouse and Sasse 1998). It is assumed that besides changes in cell wall properties, BRs can affect water transport through aquaporins and the activity of a vacuolar H^+-ATPase which are both linked to cell elongation (Morillon et al. 2001; Schumacher et al. 1999). In addition to cell elongation, BRs also have a positive role in cell division. For example, treatment with 24-epibrassinolide causes increase in the mitotic index in onion root tip cells (Howell et al. 2007) and in tobacco BY-2 cell suspension cultures, brassinolide treatment also promotes cell proliferation (Miyazawa et al. 2003). Further experiments with Arabidopsis mutant plants *dwf7-1,* support the positive role of BRs in cell division, since these BR-deficient plants showed slower rate of cell division (Cheon et al. 2010). Several studies also show that BRs promote cell differentiation. Early studies using the two primary model systems for study of xylogenesis (*Heliantus tuberosus* and *Zinnia elegans*) showed that nanomolar levels of BL stimulate tracheid formation (Iwasaki and Shibaoka 1991). Further works on Arabidopsis supported the importance of BRs in the process of vascular differentiation. For example, BR-deficient mutant *dwf7,* had fewer vascular bundles and the spacing between bundles was irregular in the sense that two vascular bundles could be joined without a separating layer of parenchymas (Choe et al. 1999). Additional work on Arabidopsis confirmed that BRs modulate the number of vascular bundles which together with polar auxin transport determine the arrangement of vascular bundles in shoots (Ibanes et al. 2009).

Root growth

BRs affect root growth in a strong concentration dependent manner: treatment with low (nM) BR concentrations had a stimulative effect on primary root growth, while higher concertation (µM) causes inhibition (Haubrick and Assmann 2006). This is not only in conflict with the growth promoting effect in light-grown shoots but also with the reduced root length of both BR-deficient and BR-insensitive mutants. Given the dose response effects, the proper applied concentration is critical for growth promotion of roots and shoots. This threshold level tightly correlates with the biological activity of applied BRs and genotype of the plant. For example to exceed the critical concentration less 24-epibrassinolide than 24-epicastasterone is required (Müssig 2005). Since both loss-of-function (*bri1-116*) null mutants and gain-of-function (*bes1-D*) Arabidopsis mutant plants have reduced meristem size, it is assumed that not only proper concentration but also balanced BR signaling is required for optimal root growth (Gonzalez-Garcia et al. 2011). Additionally, BRs can also control the size of the root meristem through regulation of the PIN auxin efflux carriers' action. It has been shown that *bri1* mutation as well as treatment with brassinazole (BR biosynthesis inhibitor) leads to lower levels of PIN2 compered to wild type (Hacham et al. 2012).

Shoot growth

Very early reports of BR activity show that treatment with exogenously applied BRs in diverse plants had a dramatically positive effect on stem elongation. Probably because of these studies, BRs are widely known as a growth promoting substances (Mandava 1988). Several further studies have contributed to elucidating the mechanism of this action. For example, it has been shown that BRs play a role in carbohydrate allocation in tomato hypocotyls, a requisite for proper biosynthetic metabolism (Goetz et al. 2000). Further experiments on Arabidopsis using brassinazole suggested that BRs promote hypocotyl elongation more likely through cell expansion rather than cell divisions (Tanaka et al. 2003).

It has also been reported that likewise in the case of roots, the effect of BRs on shoot is integrated with other plant hormones. For example, the synergistic effect on stem

segment elongation has been known since 1985 when it was reported that the order of BRs-auxin co-treatment matters. BR treatment followed by IAA treatment resulted in synergistic enhancement of auxin-induced elongation, but in the case of the reverse order (auxins followed by BRs) BRs were inactive, suggesting that BRs adjust the response capacity to auxins (Katsumi 1985). Regarding another group of plant hormones, it has been noticed that BRs have a promoting effect on gibberellin biosynthesis which consequently leads to additive stimulation of plant elongation (Unterholzner et al. 2015). However, in comparison to light-grown plants the dark-grown plants show different growth response on BRs treatment. While the growth of Arabidopsis hypocotyls is stimulated by BRs treatment in the light, in the dark on the contrary, the hypocotyl growth is inhibited (Choe et al. 2001; Wang et al. 2002).

Reproductive organ and seed development

High content of BRs in flowers, pollen and seeds together with a plethora of studies related to this topic, demonstrate that BRs play a crucial role in regulation of reproductive development (Oklestkova et al. 2015). For example, BR signaling positively regulates ovule and seed number (Huang et al., 2013) and on the male side, lack of BRs in dwarf Arabidopsis mutant results in male sterility, due to reduction in pollen number and abnormal tapetum development (Ye et al. 2010) while exogenously applied BRs can stimulate pollen germination and pollen tube growth rates (Vogler et al. 2014). BRs play important role also in regulation of the size, mass, and shape of seeds via affecting the integument, endosperm, and embryo development (Jiang et al. 2013).

Stress tolerance

Besides direct effects on regulation of growth, BRs are also known for their ability to mediate biotic and abiotic stresses, including salt, drought, chilling or heat stress and pathogen attack (Krishna 2003; Oklestkova et al. 2015). For example, BRs through their crosstalk with the alternative oxidase (AOX) pathway improve plant tolerance to low temperature. In the case of chilling stress, photosystems are endangered by increased ROS production. To avoid damage to photosystems,

BRs induce AOX synthesis which can limit ROS synthesis (Deng et al. 2015). BRs are also able to influenced the fatty acid composition of membrane lipids and thereby reduce leakage of electrolyte which again leads to improving plant tolerance to temperature stress (Aghdam and Mohammadkhani 2014). BRs also play a crucial role in dealing with high-temperature stress, under this conditions BRs can stimulate initial Rubisco activity and thereby increase plant photosynthetic efficiency (Nawaz et al. 2017). Moreover exogenous plant treatment with BRs activates the antioxidant machinery which means that the activity of antixodant enzymes such as catalase (CAT) and ascorbate peroxidase (APX) is enhanced after the treatment (Xi et al. 2013). Increased production of ROS is also the result of salinity stress and it has been reported that in this case too, BRs increase the activity of antioxidant enzymes to alleviate the negative effects of salt stress and to strengthen oxidative stress tolerance (Anusha et al. 2016; Cheng et al. 2015). Reduction of ROS levels is also one way of decreasing the impact of drought stress on plants. This aside, under drought stress conditions, BRs increase relative water content, chlorophyll content and photosynthesis rates (Sairam 1994; Fialová 2014). They are also involved in plant defense mechanisms under biotic stress conditions. Besides well-known signal compounds such as jasmonic acid (JA), salycilic acid (SA) and ethylene (ET) also BRs are involved in plant-pathogen interaction (Ali et al 2013) mainly through positive regulation of metabolites and antioxidants to protect plant cells from a wide range of pathogens (Belkhadir et al. 2012). As in the case of BR-mediated regulation of growth, under stress conditions BRs cooperate at various levels with almost all groups of plant hormones to minimalize the damage and enhance the chance of stressed plants to survive and reproduce (Choudhary et al. 2012).

1.1.6 Perspectives

Combination of promotive effect of BRs on plant growth with positive effect on stress resistance and stress tolerance under various abiotic and biotic stresses, gives to BRs, great potential for application in agriculture to improve growth and yield. For this purpose, 24-epibrassinolide and 28-homobrassinolide have been already commercially synthesized and used in the field. Moreover, in Russia and Belarus 24-epibrassinolide

is used as an active ingredient of the commercial plant growth promoter Epin™ and it is recommended for the treatment of agricultural plants (Khripach et al. 2000).

1.2 ETHYLENE

1.2.1 Introduction

Owing to its simple structure and gaseous state, ethylene is unique among plant hormones and also one of the earliest discovered plant growth regulators. It was in 1901 that Dimitry Neljubov observed that illuminating gas affects the growth orientation of dark-grown pea seedlings, and subsequently he identified ethylene as the active component of the gas (Abeles et al. 1992). Since then, extensive studies have been performed to elucidate the ethylene biosynthetic pathway, mechanism of action and determine the most effective method for ethylene detection.

1.2.2 Biosynthesis

During the 20th century, the biochemistry of ethylene biosynthesis was intensively studied and this has resulted in a model of the ethylene biosynthesis pathway as we know it today. The major breakthrough in characterization of this pathway was identification of methionine (Met), S-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) as intermediates of ethylene biosynthesis (Lieberman et al. 1966; Adams and Yang 1977, 1979). The first step in the biosynthesis is activation of methionine by AdoMet synthetase yielding SAM which is a common precursor to many biosynthetic pathways. In the case of ethylene biosynthesis, SAM is converted to ACC by the enzyme ACC synthase (ACS) and ACC is finally oxidized by ACC oxidase (ACO) to form ethylene (Yang and Hoffman 1984). Since ethylene cannot be degraded or actively transported within plants the concentration of its immediate precursor – ACC has to be strictly controlled to maintain hormone homeostasis. One way of controlling the ethylene production is the formation of ACC derivatives like malonyl-ACC (MACC) and 1-(γ-L-glutamylamino) ACC (GACC) (Hoffman et al. 1982; Martin et al. 1995). ACC as the immediate precursor of ethylene biosynthesis can be transported within the plant, which can leads

to ethylene synthesis in specific tissue of stressed or senescent organs (Van de Poel and Van Der Straeten 2014). As mentioned above, the two key enzymes are part of the ethylene biosynthetic pathway – ACS and ACO. Unlike ACO which is constitutively present in most plant tissues, ACS occurs at very low levels, only in tissues that do not produce a large amount of ethylene and the activity of ACS is highly elevated under ethylene promoting conditions. Accordingly, ACS is considered the rate-limiting enzyme of ethylene biosynthesis (Yang and Hoffman 1984; Sato and Theologis 1989; Wang et al. 2002). Finally, targeting of these two key enzymes is another method for controlling ethylene production (Wang et al. 2002).

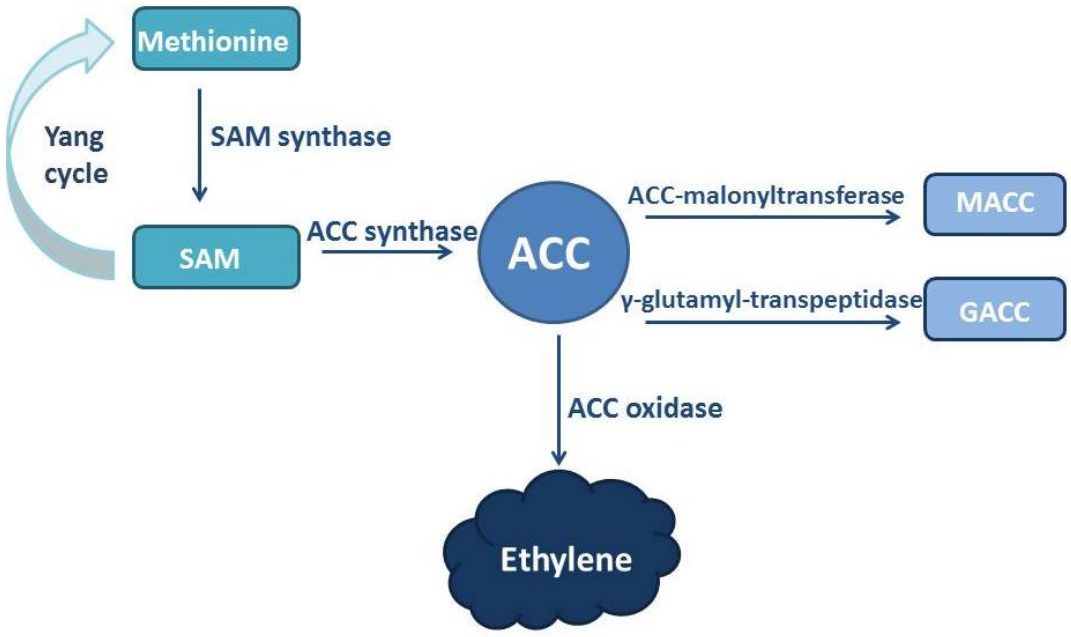

Figure 4. Scheme of the ethylene biosynthetic pathway, conjugation and catabolism.

1.2.3 Signaling

Even though ethylene as a plant growth regulator has been known for more than 100 years, the main components of its signaling pathway have only been identified in the last 25 years (Wen 2015). Since ethylene as a gas is freely diffusible into cells there is no requirement for its receptors on the plasma membrane. Therefore the ethylene receptor complex is localized in the endoplasmic reticulum (ER), the ethylene perception at the ER results in an energetically efficient and rapid response (Chen et al. 2002; Grefen et al. 2008). Nowadays, five ethylene receptors divided into two subfamilies are known. Subfamily I includes two receptors - Ethylene Response 1 (ETR1) and Ethylene Response Sensor 1 (ERS1) and subfamily II consists of three more receptors Ethylene Response 2 (ETR2) and Ethylene Response Sensor 2 (ERS2) and Ethylene Insensitive 4 (EIN4) (Lacey and Binder, 2014). Ethylene binds to the receptor via a copper factor and this results in the inactivation of the receptor (Hua and Meyerowitz 1998). When the receptor is in an inactive state (after ethylene binding) it cannot activate the Raf-like serine/threonine (Ser/Thr) kinase, CTR1, which is a negative regulator and downstream component of the signaling pathway (Kieber et al 1993). The active CTR1 physically interacts with another downstream component EIN2 (ethylene insensitive 2) and through phosphorylation directly regulates its activity (Ju et al. 2012). EIN2 is an integral membrane protein at ER but its C-terminus contains an NLS and in the presence of ethylene, the inactivation of the receptors and CTR1 results in cleavage of this EIN2 C-end. This cleaved carboxyl terminus then migrates into the nucleus, where it directly or indirectly regulates EIN3 and EIN3-LIKE1 (EIL1) transcription factors that subsequently regulate the expression of their immediate target genes such as the ETHYLENE RESPONSE FACTOR1 (ERF1) (Ju et al. 2012; Wen et al. 2012).

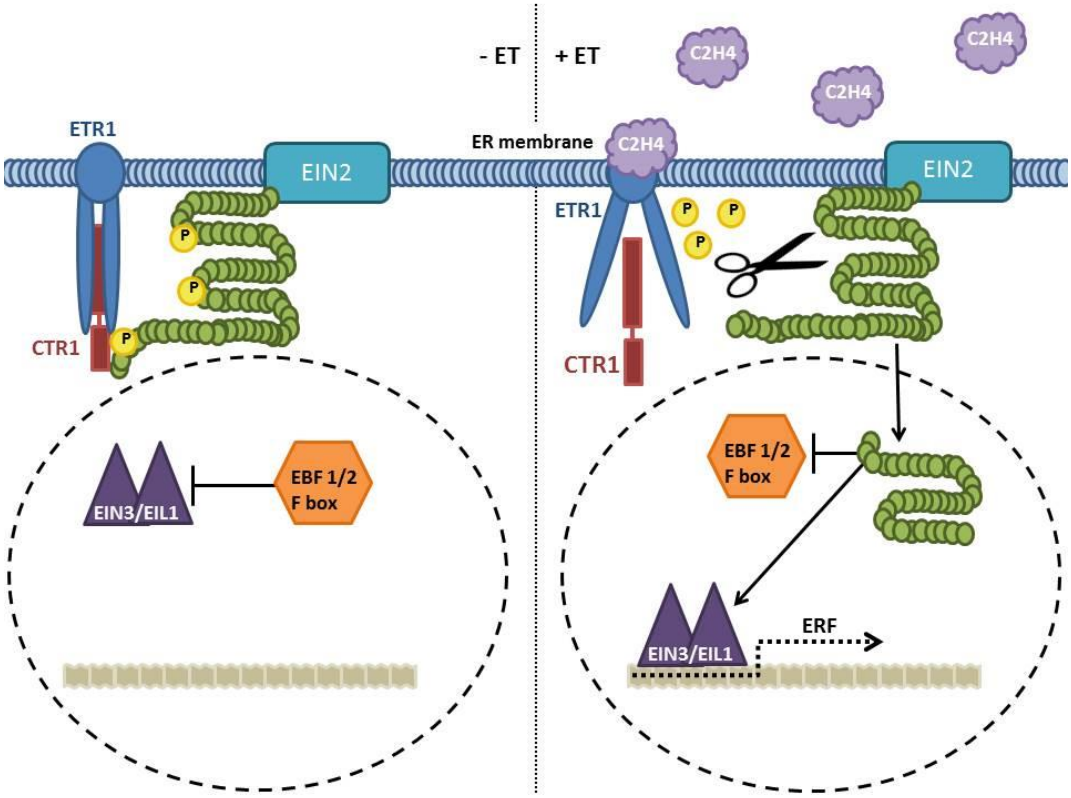

Figure 5. Proposed model for ethylene signaling pathway in the absence and in the presence of ethylene.

1.2.4 Physiological effects on plants

The plant hormone ethylene plays an important role during plant growth and development. Its role is also crucial in the regulation of plant response to stress and pathogen attack. The most widely documented ethylene response is the so-called triple response of etiolated seedlings. In dark-grown seedlings, the exogenous presence of ethylene causes inhibition of stem elongation, swelling of hypocotyls and exaggerated curvature of apical hook (Abeles et. al. 1992; Guo and Ecker 2003). Several components of the ethylene signal transduction pathway have been identified based on the molecular genetic screening of Arabidopsis mutant plants with an aberrant triple response. One class of mutants including *etr1, etr2, ein2, ein3, ein4, ein5* and *ein6* lacks ethylene responsiveness in the presence of exogenous ethylene or its metabolic precursor ACC. In contrast, the second class of mutants containing *eto1, eto2, eto3* and *ctr1* shows the triple response even in the absence of exogenous ethylene.

This effect is caused by endogenous ethylene overproduction (in the case of *eto* mutants) or by constitutive activation of the ethylene-signaling pathway (*ctr1*) (Guo and Ecker 2003).

Cell elongation

Ethylene is best known for its inhibitory effect on cell elongation of dark-grown plants (Guzmán and Ecker 1990). However several reports show the opposite effect - ethylene-stimulated cell elongation of the hypocotyls of light-grown seedlings (Smalle et al. 1997) and in the process of root hair growth (Pitts et al. 1998). The cell elongation is a result of combination of several processes like the cytoskeleton rearrangement, the cell wall relaxation and the water uptake and these processes are at least partially controlled by ethylene. The rearrangement of cortical microtubules (CMT) controls the direction of cell elongation (Bashline et al. 2014). It has been shown that ethylene can rapidly (within 10 minutes) affect microtubule reorientation in Arabidopsis roots and shoots and thereby change the growth pattern of seedlings by reducing the rate of elongation and increasing lateral expansion resulting in radial swelling (Le et al. 2004, 2005). However, the opposite effect was observed in upper hypocotyl cells in light-grown Arabidopsis (Le et al. 2005). Moreover, in Arabidopsis, expansins (cell wall-remodeling enzymes) have been shown to be regulated by ethylene during the process of root hair formation. During this process, At*EXP* expression is induced by ethylene and causes a local, (in many case) tissue specific, elongation response (Cho and Cosgrove 2002). Besides the promotive effect on fruit ripening, ethylene also positively affects flower senescence and leaf abscission (Bleecker and Kende 2000).

Hook formation

Apical hook formation is a crucial process during the early development of plants. The apical hook protects the delicate shoot apical meristem and cotyledons while the seedling grows through the soil (Guzmán and Ecker 1990). The formation of apical hook is initiated shortly after seed germination. Once the hook is formed, it remains completely closed until it is exposed to light after emergence from the soil, the hook then unfolds and the developmental processes can continue (Raz and Ecker 1999). In the presence of ethylene, dark-grown Arabidopsis seedlings show an exaggeration of the apical hook curvature (Guzmán and Ecker 1990), which is caused by the ability of ethylene to regulate the transition between hook maintenance and opening, by preventing hook opening (Gallego-Bartolomé et al. 2011). Experiments with ethylene mutant plants show that both ethylene synthesis and ethylene signaling are indispensable for proper development of the apical hook. For example, in dark-grown ethylene overproducer mutants - *eto1*, *eto2*, and *eto3* (displaying elevated levels of ethylene), an exaggerated hook was observed but reduced hook curvature was observed in mutant plants failing to increase ethylene biosynthesis like *cytokinin insensitive - cin1, cin2, cin3 and cin4*. Evidence of the importance of ethylene signaling in the process of apical hook formation has been reported where ethylene insensitive receptor mutants *etr1-1, ers1-1* show lack of apical hook, while ethylene hypersensitive mutants *etr1-7, etr2-3, ein4-7* display an exaggerated apical hook after ethylene treatment (Mazzella et al. 2014). Also an ethylene-responsive gene *HOOKLESS (HLS1)* has been identified (Lehman et al. 1996). The transcription of *HLS1* is activated through direct binding of the transcriptions factors EIN3/EIL1 to its promoter (An et al. 2012). *HLS1* encodes an *N*-acetyltransferase and it is indispensable for formation of apical hook (Lehman et al. 1996).

Fruit ripening

Fruit ripening as a developmental process is a series of biochemical events resulting in loss of chlorophyll, formation of flavors and aromas, flesh softening and abscission of the fruit. Because controlled ripening of edible fruits could have a potential in agriculture, the effect of ethylene on this process has been extensively investigated.

Based on the presence or absence of respiratory rise and increased ethylene production during the ripening process, fruits can be divided into climacteric and non-climacteric respectively. Ethylene has a great stimulating impact on ripening of climacteric fruits such as tomato, apple, peach, and banana, while ripening of non-climacteric fruits including citrus, grape, and strawberry is considered to be an ethylene-independent process (Lelievre et al. 1997; Barry and Giovannoni 2007). The mechanism of ethylene production differs in the case of normal growth and development including also stress responses and in the case of floral senescence and fruit ripening. Under normal conditions or during stress reaction, ethylene production is autoinhibitory, which means that exogenous ethylene inhibits further synthesis in plants. In contrast, during fruit ripening and floral senescence, ethylene production is stimulated by ethylene (Barry and Giovannoni 2007). The most genetically tractable plant system for studying fruit ripening is tomato and thus ample of reports have been published using the tomato plant as a model system. Extensive analysis has revealed differential expression of *ACO* and *ACS* genes during ripening in tomato fruit. This in combination with experiments using ethylene biosynthesis inhibitors and analysis of the *never-ripe* mutation in tomato (mutation in the ethylene receptor) provides clear proof of the crucial role of ethylene during the ripening of climacteric fruits (Barry and Giovannoni 2007).

Stress response

Plant hormones traditionally associated with plant defense against a wide variety of stress stimuli are salicylic acid (SA), JAs, ethylene and, indeed, abscisic acid (ABA) - the principal hormone regulating plant responses to abiotic stresses. Ethylene, in regard to positive effects on stress tolerance, is a well-established regulator of flooding tolerance. Experiments with rice show that submergence promotes ethylene accumulation resulting through antagonism with ABA, in GA-mediated stem elongation. Ethylene was also identified as a hormone improving plant survival rates under hypoxia (oxygen deficiency) caused by flooding. On the other hand, ET negatively affects stress tolerance in plants. For example, salt stress tolerance seems to be negatively affected by ethylene because in Arabidopsis, reduced salt tolerance correlates with increased ACC levels and Arabidopsis mutant (*acs7*) with reduced ethylene levels, exhibits

increased salt tolerance during germination. In contrast, other reports show that ET signaling positively regulates salt tolerance because ET receptor mutants such as *etr1* and *ein4* are more tolerant towards salt stress, and similarly, the *ctr1* mutant shows increased salt tolerance. Ethylene has a dual effect on stomatal closure as a reaction to drought stress. On the one hand, it inhibits ABA-induced stomatal closure and the *eto1* mutant (an ethylene overproducer) closes its stomata slower than wild type plants under conditions of drought stress. On the other hand, ethylene promotes stomatal closure by mediating ROS production in stomatal guard cells (Kazan 2015). However, from a large number of published studies, it is evident that plant responses to stress is a complex phenomenon. For this reason, it is important to consider various crosstalk and interactions between stress hormones (Munné-Bosch and Muller 2013).

1.2.5 Commercial use

Ethylene is one of the most widely used plant hormone in agriculture. Because of its gas state, ethylene is almost impossible to use in the field. This drawback, is overcome in practice using an ethylene-releasing compound such as Ethephon (2-chloroethylphosphonic acid) also known as Ethrel. Ethephon is the most widely used ethylene-releasing compound in agriculture and it is used to hasten the ripening of apples and tomatoes and accelerate abscission of flowers and fruits. In contrast, Ethylbloc® an ethylene binding inhibitor is used to extend the self-life of diverse climacteric fruits. Specific inhibitors of ethylene biosynthesis and action are also used to delay postharvest spoilage of cut flowers (Taiz and Zeiger 2010).

1.3 HORMONAL CROSSTALK

Plant hormones are structurally diverse compounds essential for the regulation of several signaling and metabolic systems with great importance for both plant development and plant responses to abiotic and biotic stresses. Unlike earlier works mainly focused on the developmental and/or growth effects of a single compound, nowadays it is more than evident that physiological processes are regulated in a complex way (Munné-Bosch and Muller 2013).

A large number of studies have shown that plant hormones act through multiple interactions at different levels rather than through isolated linear pathways. They play a key role in regulating plant growth and development as well as responses to environmental cues. Several hormones carry seemingly redundant information but in most cases they cannot be substituted as each hormone group is necessary for proper plant development. On the other hand, phytohormones often operate in a communication network to trigger specific outcomes. The term describing the phenomenon when one pathway influences another is commonly known as crosstalk but the term cross-regulation is broadly used in the literature for cases where multiple input signals influence a common biological outcome. Cross-regulation can be classified into three categories.

1. Primary cross-regulation – distinct signaling pathways regulate a shared transduction component (in both positive and negative manner)

2. Secondary cross-regulation – the output of one signaling pathway regulates the abundance or perception of a second signal

3. Tertiary cross-regulation – outputs of two distinct pathways influence the other (Kappusamy et al. 2009)

Even though the physiological evidence for hormone interactions are clear and widely described, many molecular mediators of crosstalk still await isolation. One issue complicating the identification of crosstalk components may be that the actual existence of crosstalk has sometimes less support than it appears (Depuydt and Hardtke 2011).

Regarding brassinosteroids and ethylene – the hormones of our interest, many studies indicate that both hormones interact at diverse levels with other phytohormones and thereby regulate various developmental processes in plants (Hu and Yu, 2014; Tong et al, 2014; Chaiwanon and Wang, 2015, Van de Poel et al., 2015). BRs have been described as having a positive influence on ET biosynthesis through post-transcriptional increase in ACS enzyme stability (Hansen et al. 2009).

Crosstalk between brassinosteroids and ethylene during plant growth and under abiotic stresses is reviewed in detail in Supplement III.

2. MATERIALS AND METHODS

2.1 Biological material

Pea seedlings - *Pisum sativum* var*. arvense* sort Arvica

Arabidopsis WT - *Arabidopsis thaliana* (Columbia ecotype, Col-0)

Arabidopsis ethylene signaling mutant lines - *Arabidopsis thaliana ein 2-5, etr 1-1, ein 3-1eil 1-1*

Arabidopsis BR mutant lines - *Arabidopsis thaliana dwf4, cpd, det2, rot3/cyp90d1, cyp85a1/cyp85a2, bri1/brl1/brl3*

2.2 Chemicals

All chromatographic solvents were of analytical grade or higher purity (Merck KGaA)

Chemical compounds - brassinolide, 24-epibrassinolide, indole-3-acetic acid, gibberellin GA3, trans-zeatin, thidiazuron and 1- aminocyclopropanecarboxylic acid, [D_4]ACC standard (Olchemim s.r.o. Czech Republic)

Brassinosteroid biosynthetic precursors were synthetized at the Laboratory of Growth Regulators Olomouc, Czech Republic)

2.3 Equipment

GC System gas chromatograph (Agilent Technologies, GC Systems) equipped with a flame ionic detector (FID) and HP-AL/S capillary column (50 m × 0.535 mm × 15 µm)

Laser-based photo-acoustic detector (ETD-300 ethylene detector, Sensor Sense, The Netherlands)

Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) system consisting of an ACQUITY UPLC® I-Class system (Waters, Milford, MA, USA) and a XevoTM TQ-S MS triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK).

2.4 Methods

2.4.1 The pea inhibition biotest

Pea seedlings (*Pisum sativum* var*. arvense* sort Arvica) germinating for 2 days were selected for uniformity from a large population and then transferred into pots containing perlite and 1/10 diluted Hoagland solution (half concentration, pH 5.7). After 24 h in a dark cultivation room (24 °C, humidity 75%) the seedlings were treated with different amounts of tested compounds in 5 µl fractionated lanolin. The substances were applied as microdrops to the scar left after the removal of the bract. The control plants were treated with lanolin alone. The inhibition of etiolated pea stems was measured after 4 days and the difference in length between the treated and control plants provided a measure of activity. The mean values were statistical analysed using the Student's t test (for more details see Supplement I, Supplement II, Supplement IV).

2.4.2 Determination of ethylene (The pea inhibition biotest)

For measurement of ethylene production, pea seedlings (8 plants/tested amount of substance) were placed in a 0.5 L glass container for 24 h in the dark. 1 ml of headspace gas was withdrawn from each container by syringe for each measurement and injected into a GC System gas chromatograph (Agilent Technologies, GC Systems) equipped with a flame ionic detector (FID) and HP-AL/S capillary column (50 m × 0.535 mm × 15 µm). The chromatographic analytical parameters were as follows: column temperature: 150 °C; detector temperature: 220 °C; and helium was used as a carrier gas. The area under the resultant peak (y-axis) versus sensitivity (x-axis; nl.ml-1) represented a quantitative measure of ethylene concentration; data were statistically analyzed using the Student's t test (for more details see Supplement I, Supplement II, Supplement IV).

2.4.3 Arabidopsis growth sensitivity assay

Arabidopsis thaliana (Columbia ecotype, Col-0; referred to as Arabidopsis) seedlings or Arabidopsis ethylene signaling mutant seedlings (*ein 2-5, etr 1-1, ein 3-1eil 1-1*) were stratified for 2 days at 4 °C and germinated on vertical half-strength

Murashige and Skoog (1% w/v sucrose) agar plates with different concentrations of tested compound at 22 °C in a 16 h/8 h light–dark cycle for 7 days. The plates were then scanned with an Epson high-resolution scanner and the entire root and hypocotyl length measured with ImageJ (http://rsbweb.nih.gov/ij/). P values were calculated with a two-tailed Student t-test using Excel software (see Supplement I, II).

2.4.4 Arabidopsis growth rescue assay

Arabidopsis *cyp85a1/cyp85a2* heterozygous mutant seedlings were stratified for 2 days at 4 °C and germinated on free vertical half-strength Murashige and Skoog (1% w/v sucrose) agar plates for 6 days. Then *cyp85a1/cyp85a2* homozygous plants were transferred to ½ Murashige and Skoog (1% w/v sucrose) agar plates containing DMSO or tested compound. 3 days after transfer, the plates were scanned with an Epson high-resolution scanner and the root length was measured with ImageJ (http://rsbweb.nih.gov/ij/).

2.4.5 Determination of ethylene production with photo-acoustic detector

Approximately 35 sterilized seeds of *Arabidopsis thaliana* (Columbia ecotype, Col-0; referred to as Arabidopsis) were placed in a 10 ml chromatography vials containing 5 ml of half-strength Murashige and Skoog (1% w/v sucrose) agar medium. Seeds in vials were vernalized for 2 days at 4°C in darkness and then vials were incubated at 22°C under long-day conditions (16 hours light/8 hours dark). After 4 days, vials were sealed and after another 24 hours, they were flushed at a flow rate of 2.5 Lh-1 and ethylene was measured with the laser-based photo-acoustic detector (ETD-300 ethylene detector, Sensor Sense, The Netherlands).

2.4.6 ACC determination

The tissue (50 mg of etiolated pea plants/50 pcs of 7day old light-grown Arabidopsis roots/hypocotyls) was homogenized in 1 ml of H_2O:methanol:chloroform (1:2:1), 50 pmol of internal standard ([D_4]ACC) was added to each sample, and after centrifugation (4 °C, 15 000 rpm) the supernatant was collected and evaporated todryness. The samples were derivatized using an AccQ-Tag Ultra kit (Waters)

and subsequently analyzed by an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) system consisting of an ACQUITY UPLC® I-Class system (Waters, Milford, MA, USA) and a XevoTM TQ-S MS triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK) (see Supplement IV).

3. SURVEY OF RESULTS

3.1 Design, synthesis and biological activities of new brassinosteroid analogues with a phenyl group in the side chain (Supplement I)

The aim of this study was to prepare a series of new brassinosteroid derivatives with a p-substituted phenyl group in the side chain. These novel brassinosteroid analogues were synthetized based on results from *in silico* molecular docking into the BRI1 receptor. To validate the docking method and to screen these new compounds in order to find compounds with strong brassinosteroid activity which could be potentially used in agriculture to improve growth and yield. Synthetized derivatives were tested in different brassinosteroid bioassays (the pea inhibition biotest, Arabidopsis growth bioassay, BES1 dephosphorylation assay) and the results were compared with naturally occurring brassinosteroids. Both types of brassinosteroids (6-ketones and B-lactones) were synthetized and 3 compounds (lactone 8f, 6-oxo derivatives 8c and 9c) with biological activity comparable with natural brassinosteroids were identified. It has been also shown that molecular docking into the BRI1 can be used as a powerful tool for prediction and design of new compounds with brassinosteroid activities, because analogues with similar or better binding energies than for brassinosteroids predicted by molecular docking also showed the strongest brassinosteroid activities. As a result of this work, new active compounds could be good candidates for application in agriculture. Progress in the chemical synthesis could help to overcome the economic restrictions which currently constrain the use of BRs in a large scale in agriculture. Finally *in silico* molecular docking appears to be a useful tool for predicting the brassinosteroid activity of new compounds.

Author contribution – co-author – screening of biological activities of new brassinosteroid analogues (performing the pea inhibition test, measurement of ethylene production in treated pea plants)

3.2 Synthesis of novel aryl brassinosteroids through alkene cross-metathesis and preliminary biological study (Supplement II)

The subsequent study is again aiming to synthesis and to test the biological activity of new phenyl analogue of brassinosteroids. In this study, a series of new aryl analogues of brassinosteroids was synthesized via alkene cross-metathesis which has been shown to be an efficient method for construction of the new side chains in the brassinosteroid structure. The biological activities of these newly prepared derivatives were established using different plant bioassays (the pea inhibition biotest, Arabidopsis growth bioassay). The ethylene production in pea seedlings treated with these compounds was also monitored and in order to compare the results with theoretical studies, *in silico* molecular docking into the BRI1 receptor was performed. Based on data from biological studies, 3 newly synthetized aryl analogues (10f, 10b, 10n) were identified as compounds with similar biological activity to 24-epiBL. Molecular docking into the BR receptor showed high binding affinity of these compounds which also predict good biological activity. In general, the results showed that the relationship between biological activity and substitution pattern in the phenyl group can be suggested as follows – no substitution or substitution with one small group like fluorine or chlorine leads to compounds with high brassinosteroid activity. In contrast, substitution with bulky groups (phenyl, methyl or others) or substitution with more than one group significantly reduces the biological activity of the berssinosteroid-like compound. Overall, alkene cross metathesis has been shown to be one prospective method for preparation of new brassinosteroid derivatives with potential use in agriculture to improve plant growth, enhance yield and/or increase the resistance of plants against various stresses.

Author contribution – first author - screening of biological activities of new brassinosteroid analogues (performing the pea inhibition test, Arabidopsis growth assays, measurement of ethylene production in treated pea plants)

3.3 Brassinosteroids induce strong, dose-dependent inhibition of etiolated pea seedling growth correlated with ethylene production (Supplement IV – submitted article)

The aim of this study was to examine the effect of brassinosteroids on the growth of etiolated pea seedlings. The results reported in this work, showed that BRs inhibit the etiolated growth of pea seedlings in a similar manner to the 'triple response' induced by ethylene. There was a correlation between growth inhibition and increase in ethylene/ACC levels which suggests that BRs' biological activities may be mediated by ethylene. And finally the data confirm the development of a new, highly sensitive and convenient bioassay for BR activity (in which ethylene production could also be monitored). This bioassay could be routinely used for evaluating the hormonal activities of new synthetic BR derivatives with potential agricultural uses.

Author contribution – first author - bioassay development, optimization, performing of the experiments, writing of manuscript

3.4 Unpublished data - biological activity of brassinolide biosynthetic precursors and crosstalk between BL and ethylene in plants

3.4.1 Biological activity of brassinolide biosynthetic precursors

The biosynthetic pathway as we know it today has been elucidated by a combination of genetic and analytical biochemistry approaches. The conversion of the membrane sterol – campesterol to final brassinosteroid (brassinolide) happens through a series of reductions, hydroxylations, epimerizations and oxidations – processes mediated by BR biosynthetic enzymes. Biochemical characterization of numerous mutants that are defective in these biosynthetic enzymes has helped to facilitate further validation of the biosynthetic pathway as well as to discover previously unknown steps (Choe 2006; Zhao and Li 2012).

It was found that lack of brassinosteroids in BR-deficient mutant plants results in characteristic phenotypes including extreme dwarfism often accompanied by male sterility. This characteristic BR deficient phenotype also occurs in case of BR signaling mutant plants. Despite their morphological similarity, BR-deficient and BR-insensitive mutants show different responses to exogenously-applied BL. In BR-deficient mutants, the dwarf growth can be rescued to a wild-type-like phenotype by treatment with exogenously applied brassinosteroids, whereas BR-insensitive mutants are generally insensitive to this treatment (Müssig 2005, Zhao and Li 2012).

Twenty one day old BR-deficient Arabidopsis plants (with mutation in various biosynthetic genes) are shown in Fig. 6, where phenotypes of the mutants can be compared with the phenotype of BR-insensitive mutant or wild type. Noticeably, two of the BR-deficient mutants exhibit bigger phenotype than others. These were *det2* and *cyp85a1/cyp85a2* mutants. Arabidopsis mutant *det2* was the first identified BR biosynthetic mutant with a de-etiolated phenotype when grown in the dark (Chory et al. 1991). Because of the significant sequence identity (38-42%) of DET2 and mammalian 5α-reductase, it has been proposed that the DET2 enzyme works

as 5α-reductase and converts campesterol to campestanol, (24R)-ergost-4-en-3-one to (24R)-5α-ergost-3-one and 22-OH-4-en-3-one to (22S, 24R)-22-hydroxy-5α-ergost-3-one (Zhao and Li 2012). In *det2* mutants, the campestanol level is reduced to about 10 % of the WT level and this residual campestanol may be the reason why the *det2* phenotype is not as severe as in cases of other BR mutants (Clouse 2011). DET2 is so far the only known catalytic enzyme of the BR-specific biosynthesis pathway which does not belong to the cytochrome P450 enzyme family. The second BR-deficient mutant with less severe phenotype is the *cyp85a1/cyp85a2* double mutant. Both enzymes CYP85A1 and CYP85A2 catalyze C-6 oxidase reactions –downstream in the BR biosynthetic pathway but only CYP85A2 is responsible for the ultimate oxidation step converting CS to BL (Zhao and Li 2012).

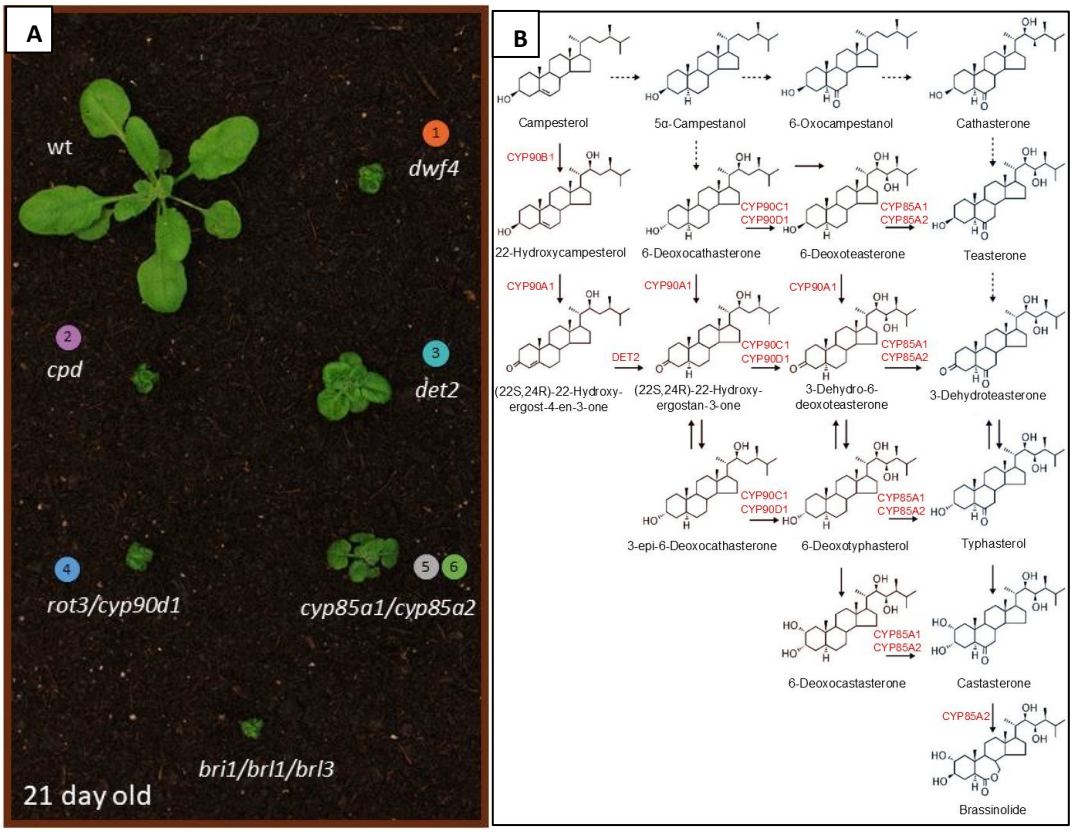

Figure 6. A comparison of Arabidopsis wild-type phenotype with other brassinosteroid mutants. A, Twenty-one-day-old normal-grown seedlings of wt and *dwf4, cpd, det2, rot3/cyp90d, cyp85a1/cyp85a2* mutant. Both Arabidopsis heterozygous mutant seedlings and wt seedlings were stratified for 2 days at4 °C and germinated on free vertical half-strength Murashige and Skoog (1% w/v sucrose) agar plates for 6 days. Then homozygous plants were transferred to soil, where they were grown for the next 15 days. B, Proposed pathways and genes involved in brassinosteroid biosynthesis (adapted from Buchanan et al. 2015).

In the past, several scientific groups invested much effort to elucidate the structure-activity relationship of brassinosteroids to determine which functional groups were essential for BRs´ activities. Given the high potential of BRs for application in agriculture to improve growth and yield is partially constrained by high cost of its synthesis, the identification of the active BR structure, which could be a template for new bioactive BR analogues, is still a relevant topic in the brassinosteroid field. For this purpose, we tested a huge library of BR biosynthetic precursors in two different biological assays (the pea inhibition test and Arabidopsis growth assay). In the pea inhibition assay, where biological activity is provided by inhibition of etiolated growth often accompanied by increase in ethylene production, we observed brassinosteroid biological activity in the case of typhasterol, 24-epiCS, 24-epiBL and brassinolide (Fig. 7) where the inhibition effect of brassinolide and 24-epiBL was accompanied by increased ethylene production and slight increase was observed after treatment with 24-epiCS in higher concentrations. No significant increase was found when typhasterol or other non-active precursors were applied (Fig. 8).

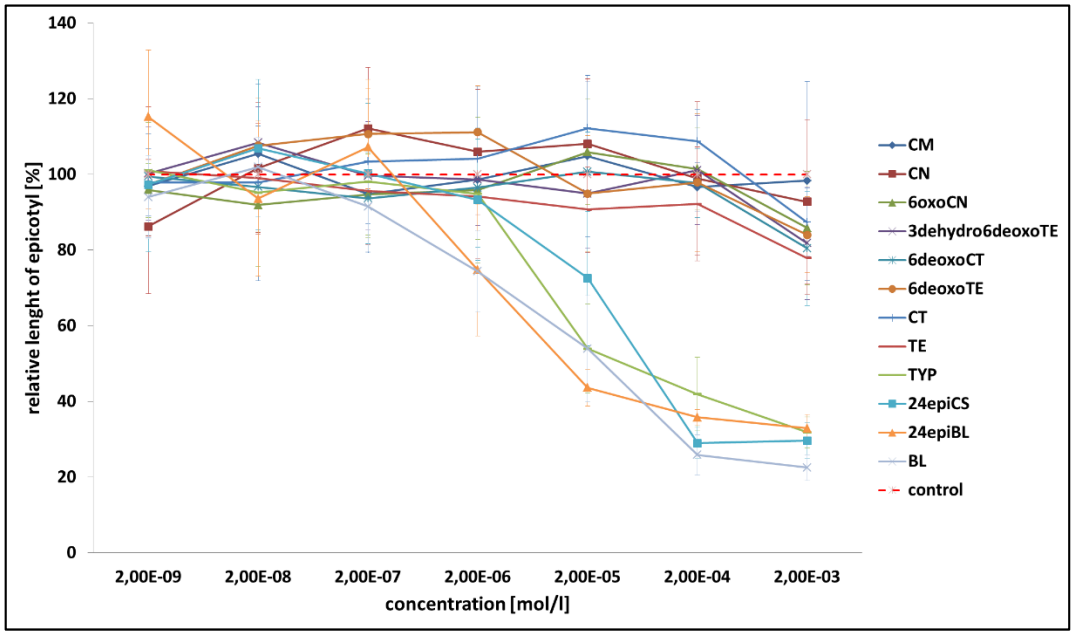

Figure 7. Effect of selected brassinolide biosynthetic precursors on inhibition of etiolated pea seedlings' growth. Pea seedlings (*Pisum sativum* var. *arvense* sort Arvica) germinating for 2 days were transferred into pots containing perlite and 1/10 diluted Hoagland solution. After 24 h in a dark the seedlings were treated with tested compounds in 5 µl fractionated lanolin. The substances were applied to the scar left after the removal of the bract. The control plants were treated with lanolin alone. The inhibition of etiolated pea stems was measured after 4 days. The mean values were subjected to statistical analysis using the Student's t-test. Error bars represent standard deviations (For statistical data see Table 1).

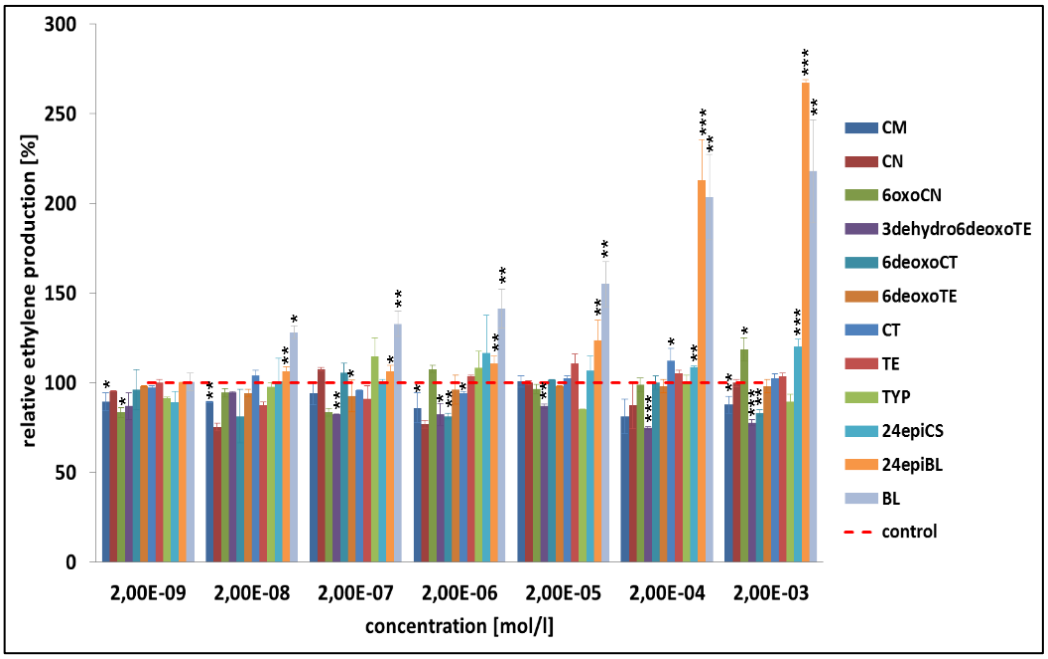

Figure 8. Effects of selected brassinolide biosynthetic precursors on ethylene production by etiolated pea seedlings determined by GC-FID. 5-day-old pea seedlings (8 plants/tested amount of substance) were closed in a 0.5 L glass container for 24 h in the dark, then 1 ml of headspace gas was withdrawn from each container by syringe for each measurement and injected into a GC System gas chromatograph (Agilent Technologies, GC Systems) equipped with a flame ionic detector (FID) and HP-AL/S capillary column (50 m × 0.535 mm × 15 µm). The area under the resultant peak (y-axis) versus sensitivity (x-axis; nl.ml-1) represented a quantitative measure of ethylene concentration; data were statistically analyzed using the Student's t test. Error bars represent standard deviations. Asterisks represent significant changes (t-test), *represents p value <0.05, **represent p value <0.01, ***represent p value <0.001.

Table 1. statistical data for Fig. 7 - Effect of selected brassinolide biosynthetic precursors on inhibition of etiolated pea seedlings' growth. Asterisks represent significant changes (t-test), *represents p value <0.05, **represent p value <0.01, ***represent p value <0.001.

campesterol [mol/l]	t test p values		cathasterone [mol/l]	t test p values	
2,00E-09	0,703676	-	2,00E-09	0,787968	-
2,00E-08	0,472697	-	2,00E-08	0,826469	-
2,00E-07	0,532782	-	2,00E-07	0,70138	-
2,00E-06	0,861406	-	2,00E-06	0,65211	-
2,00E-05	0,593448	-	2,00E-05	0,160784	-
2,00E-04	0,656579	-	2,00E-04	0,264383	-
2,00E-03	0,864597	-	2,00E-03	0,13575	-
campestanol [mol/l]	t test p values		teasterone [mol/l]	t test p values	
2,00E-09	0,045337	*	2,00E-09	0,901337	-
2,00E-08	0,605787	-	2,00E-08	0,880795	-
2,00E-07	0,529415	-	2,00E-07	0,499194	-
2,00E-06	0,956618	-	2,00E-06	0,391641	-
2,00E-05	0,862048	-	2,00E-05	0,139811	-
2,00E-04	0,456634	-	2,00E-04	0,244248	-
2,00E-03	0,200538	-	2,00E-03	0,001083	**
6-oxocampestanol [mol/l]	t test p values		typhasterol [mol/l]	t test p values	
2,00E-09	0,339281	-	2,00E-09	0,862909	-
2,00E-08	0,210602	-	2,00E-08	0,443491	-
2,00E-07	0,283786	-	2,00E-07	0,777734	-
2,00E-06	0,518751	-	2,00E-06	0,428619	-
2,00E-05	0,42857	-	2,00E-05	6,12E-07	***
2,00E-04	0,914638	-	2,00E-04	1,02E-08	***
2,00E-03	0,038706	*	2,00E-03	2,01E-10	***
3-dehydro-6deoxo-teasteron [mol/l]	t test p values		24epiCS [mol/l]	t test p values	
2,00E-09	0,972733	-	2,00E-09	0,74108	-
2,00E-08	0,193479	-	2,00E-08	0,430713	-
2,00E-07	0,956229	-	2,00E-07	0,975777	-
2,00E-06	0,846213	-	2,00E-06	0,408053	-
2,00E-05	0,4662	-	2,00E-05	0,002897	**
2,00E-04	0,869596	-	2,00E-04	8,65E-10	***
2,00E-03	0,016881	*	2,00E-03	1,08E-09	***
6-deoxocathasterone [mol/l]	t test p values		24epiBL [mol/l]	t test p values	
2,00E-09	0,918664	-	2,00E-09	0,061226	-
2,00E-08	0,498963	-	2,00E-08	0,47973	-
2,00E-07	0,192154	-	2,00E-07	0,340548	-
2,00E-06	0,514906	-	2,00E-06	0,003309	**
2,00E-05	0,876541	-	2,00E-05	1,5E-08	***
2,00E-04	0,602236	-	2,00E-04	1,17E-09	***
2,00E-03	0,001539	**	2,00E-03	5,85E-10	***
6-deoxoteasterone [mol/l]	t test p values		brassinolide [mol/l]	t test p values	
2,00E-09	0,494256	-	2,00E-09	0,51911	-
2,00E-08	0,125011	-	2,00E-08	0,522542	-
2,00E-07	0,049193	*	2,00E-07	0,325809	-
2,00E-06	0,027174	*	2,00E-06	0,002028	**
2,00E-05	0,34997	-	2,00E-05	2,54E-05	***
2,00E-04	0,718878	-	2,00E-04	1,64E-09	***
2,00E-03	0,001482	**	2,00E-03	4,96E-10	***

The characteristic effect of exogenously applied BRs on light-grown Arabidopsis was dual – BRs in higher concentrations caused inhibition of root growth and elongation of hypocotyls. The most active BRs – brassinolide, 24-epiBL and castasterone inhibited Arabidopsis root growth in 1 nM concentration, while isotyphasterol, typhasterol and 3-dehydroteasterone inhibited the root growth at 10 nM concentration. Additionaly treatment with 100 nM teasterone resulted in the inhibition of roots. (Fig. 9). The trend in elongation of hypocotyls was also dose dependent and like inhibition of roots – treatment with the most active BRs initiated elongation of hypocotyls in lower concentrations and in the highest tested concentration (1 µM), all compounds able to inhibit root growth (except teasterone) also caused the elongation of hypocotyls. A slight increase in elongation growth was also observed after treatment with 3-dehydro-6deoxoteasterone (Fig. 10).

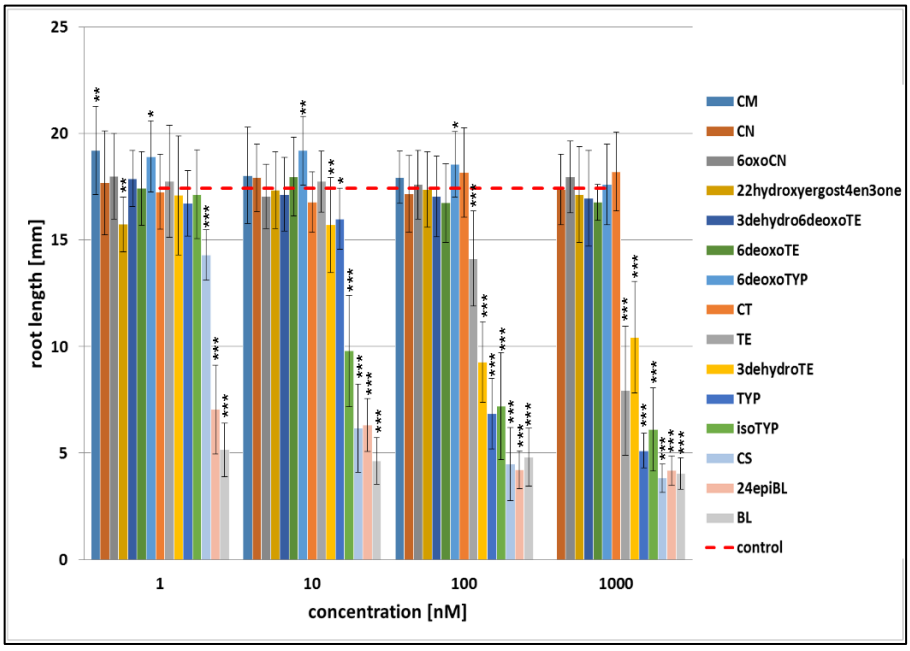

Figure 9. Effect of selected brassinolide biosynthetic precursors on Arabidopsis root growth. *Arabidopsis thaliana* (Columbia ecotype, Col-0) seedlings were stratified for 2 days at 4 °C and germinated on vertical half-strength Murashige and Skoog (1% w/v sucrose) agar plates with different concentrations of tested compound at 22 °C in a 16 h/8 h light–dark cycle for 7 days. Then the plates were scanned with an Epson high-resolution scanner and the entire root length was measured with ImageJ (http://rsbweb.nih.gov/ij/). Error bars represent standard deviations of the means. Asterisks represent significant changes (t-test), *represents p value <0.05, **represent p value <0.01, ***represent p value <0.001.

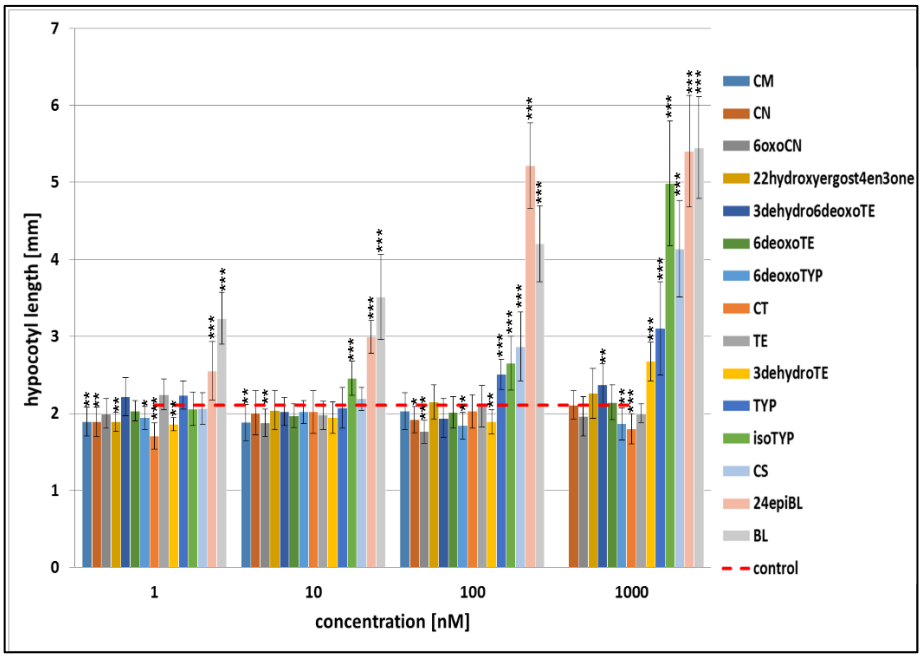

Figure 10. Effect of selected brassinolide biosynthetic precursors on Arabidopsis hypocotyl growth. *Arabidopsis thaliana* (Columbia ecotype, Col-0) seedlings were stratified for 2 days at 4 °C and germinated on vertical half-strength Murashige and Skoog (1% w/v sucrose) agar plates with different concentrations of tested compound at 22 °C in a 16 h/8 h light–dark cycle for 7 days. Then the plates were scanned with an Epson high-resolution scanner and the entire hypocotyl length was measured with ImageJ (http://rsbweb.nih.gov/ij/). Error bars represent standard deviations of the means. Asterisks represent significant changes (t-test), *represents p value <0.05, **represent p value <0.01, ***represent p value <0.001.

The limitation of this experimental setup is that the dose-dependent effect of selected precursors on a particular biological activity may be the effect of the compound downstream in the pathway. For this reason, we decided to use Arabidopsis biosynthetic double mutant *cyp85a1/cyp85a2* (enzymes encoding the last steps in the biosynthetic pathway) and rescue its short root phenotype by treatment with precursors located before and after this enzymatic step. The results from this experiment showed that typhasterol at 5nM concentration can rescue the mutant root growth to the level of wild type and even more, the root growth can be completely rescued by 1µM 3-dehydro-6-deoxoteasterone and also partially rescued by 10µM 6-deoxotyphasterol (Fig. 11). This suggests that these two intermediates have biological activity by themselves which could explain the larger phenotype of *cyp85a1/cyp85a2* mutant (Fig. 6) through accumulation of these bioactive compounds in this mutant.

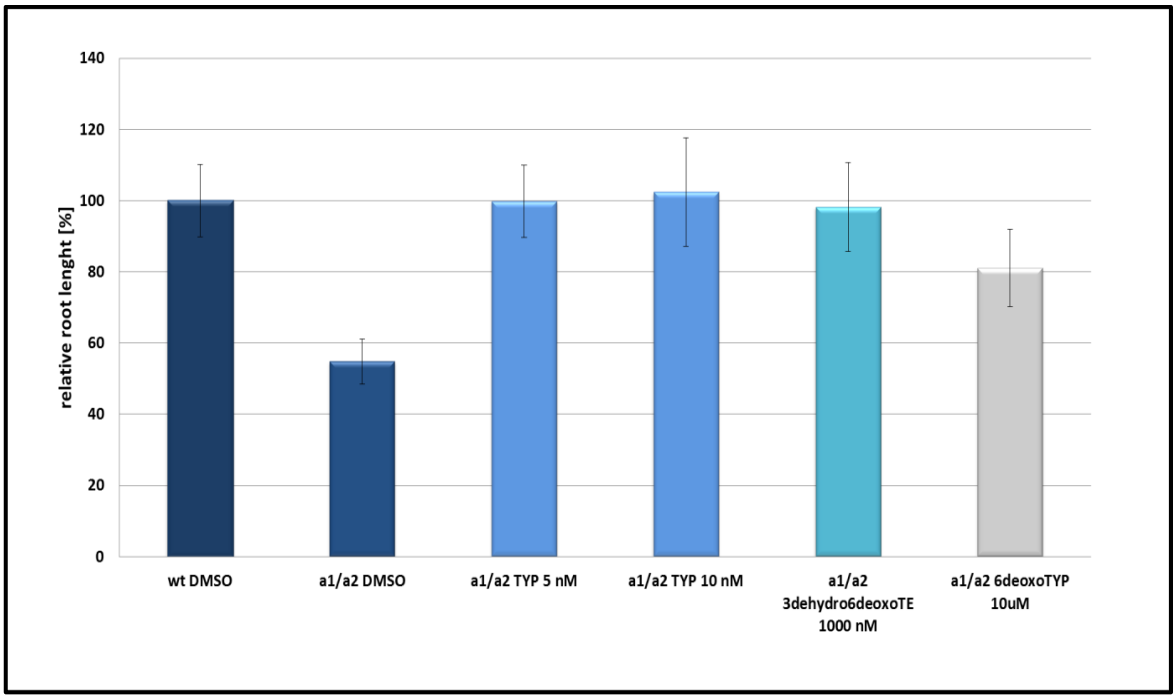

Figure 11. Effect of typhasterol, 3-dehydro-6-deoxoteasterone and 6-deoxotyphasterol on rescue of cyp85a1/cyp85a2 root growth. Arabidopsis cyp85a1/cyp85a2 heterozygous mutant seedlings were stratified for 2 days at 4 °C and germinated on free vertical half-strength Murashige and Skoog (1% w/v sucrose) agar plates for 6 days. Then cyp85a1/cyp85a2 homozygous plants were transferred to ½ MS plates containing DMSO or tested compound. 3 days after transfer, the plates were scanned with an Epson high-resolution scanner and the root length was measured with ImageJ. Error bars represent standard deviations of the means.

3.4.2 Brassinolide and its effect on ethylene production in Arabidopsis roots and shoots

As was mentioned earlier, in light-grown Arabidopsis, exogenously applied BRs caused inhibition of root growth and elongation of hypocotyls. In the case of brassinolide, the effective concentration for inhibition of roots was 1 nM whereas the elongation of hypocotyls is initiated by 10 nM BL and the significant elongation occurred after treatment with 100nM BL (Fig. 12). The same concentrations of BL were also required for induction of ethylene production in Arabidopsis of the same age grown under similar conditions (Fig. 13). Higher applied BL concentrations caused an increase in both the elongation of hypocotyls and ethylene production. Treatment with higher concentration of exogenously applied BL was also accompanied by further inhibition of roots.

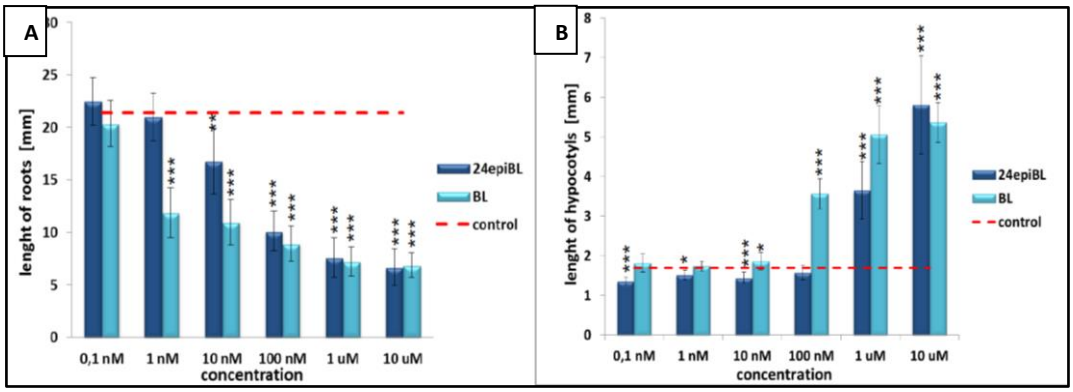

Figure 12. Effect of brassinolide and 24-epibrassinolide on Arabidopsis root (A) and hypocotyl (B) growth. *Arabidopsis thaliana* (Columbia ecotype, Col-0) seedlings were stratified for 2 days at 4 °C and germinated on vertical half-strength Murashige and Skoog (1% w/v sucrose) agar plates with different concentrations of tested compound at 22 °C in a 16 h/8 h light–dark cycle for 7 days. Then the plates were scanned with high-resolution scanner and the entire root length was measured with ImageJ software. Error bars represent standard deviations of the means. Asterisks represent significant changes (t-test), * p value <0.05, ** p value <0.01, *** p value <0.001.

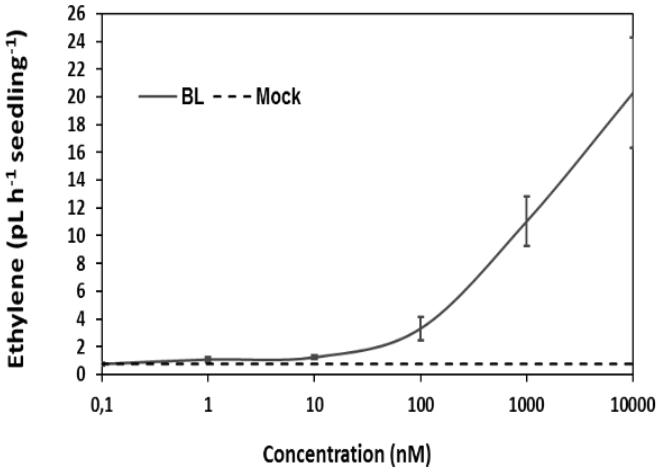

Figure 13. Effects of brassinolide on ethylene production by 7-day-old light-grown Arabidopsis determined by the laser-based photo-acoustic detector. Approximately 35 sterilized seeds of *Arabidopsis thaliana* (Col-0) were placed in a 10 ml chromatography vials containing 5 ml of half-strength Murashige and Skoog (1% w/v sucrose) agar medium. Seeds in vials were vernalized for 2 days at 4°C in darkness and then vials were incubated at 22°C under long-day conditions .After 4 days vials were sealed and after another 24 hours vials were flushed at flow rate of 2.5 Lh-1 and ethylene was measured with a laser-based photo-acoustic detector.

To investigate whether the root inhibition and hypocotyl elongation after treatment with BRs is mediated by produced ethylene, we treated ethylene signaling Arabidopsis mutant plants (*ein2-5; etr1-1; ein3-1 eil 1-1*) with brassinolide (100nM) and as a positive control we used treatment with ACC (the direct ethylene precursor). In the case of roots, BL significantly inhibited the growth of wild type roots as well as mutant roots. In contrast, the elongation of hypocotyls was at least partially inhibited in mutant plants after the treatment with both BL and ACC (Fig. 14). All together the data suggest that the effect on root inhibition was not ethylene dependent whereas the elongation of hypocotyls after BL treatment wass at least partially caused by increased ethylene production. Further quantification of ACC levels in wild-type Arabidopsis roots and shoots after BL treatment (10,100 and 1000

nM) showed no significant increase or decrease in ACC level in roots. In shoots significant accumulation of ACC was observed after treatment with BL at higher concentration (100nM and 1000 nM) in a dose dependent manner (Fig. 15). All data together suggest that the treatment with BRs (100nM and more) is dose-dependently accompanied by enhanced ethylene production and that BRs act differently in roots and shoots.

In shoots the elongation effect is ethylene dependent and probably mediated by enhanced levels of ACC in this tissue.

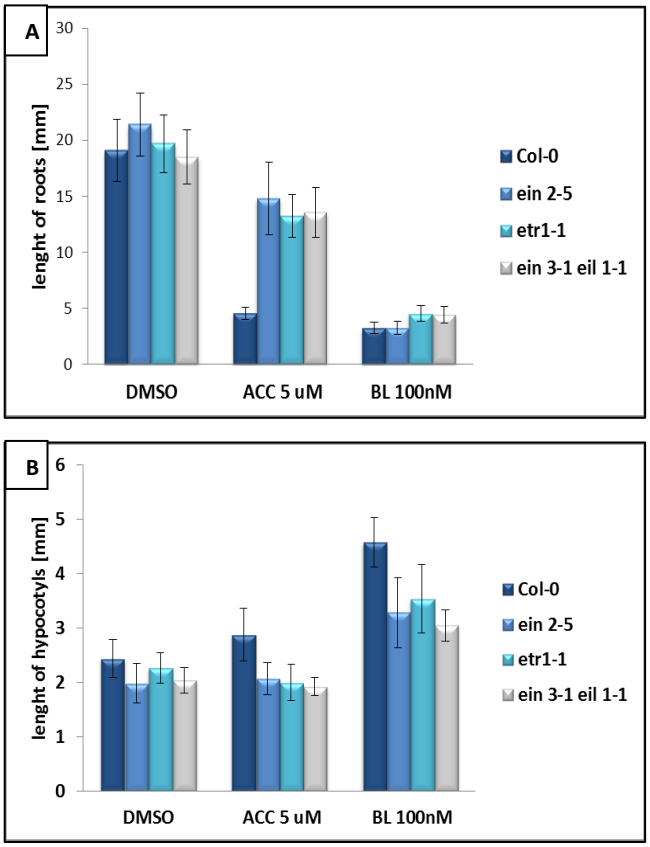

Figure 14. Effect of brassinolide on root (A) and hypocotyl (B) growth of Arabidopsis ethylene signaling mutant. Seedlings were stratified for 2 days at 4 °C and germinated on vertical half-strength Murashige and Skoog (1% w/v sucrose) agar plates with different concentrations of tested compound at 22 °C in a 16 h/8 h light–dark cycle for 7 days. Then the plates were scanned with an Epson high-resolution scanner and the entire root length was measured with ImageJ (http://rsbweb.nih.gov/ij/). Error bars represent standard deviations of the means.

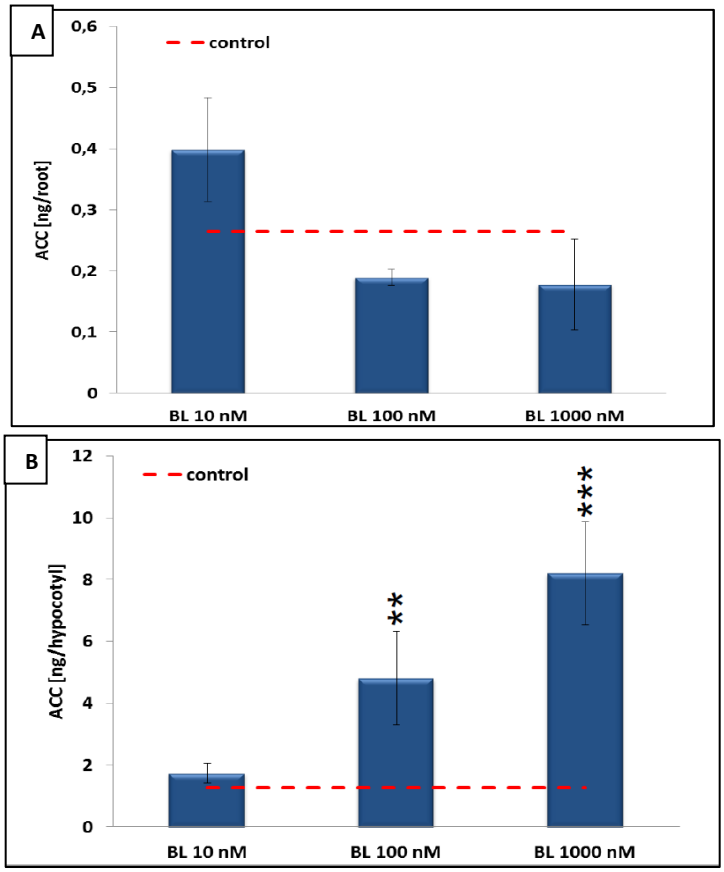

Figure 15. Effect of brassinolide on ACC level in Arabidopsis roots (A) and hypocotyls (B). WT seedlings of *Arabidopsis thaliana* (Col-0) were stratified for 2 days at 4 °C and germinated on vertical half-strength Murashige and Skoog (1% w/v sucrose) agar plates with different concentrations of tested compound at 22 °C in a 16 h/8 h light–dark cycle for 7 days. Then the tissue (50 pcs of roots/hypocotyls) was homogenized in H_2O:methanol:chloroform (1:2:1), 50 pmol of internal standard ([D_4]ACC) was added to each sample, and after centrifugation, the supernatant was collected and evaporated to dryness. The samples were derivatized and subsequently analyzed by an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) system consisting of an ACQUITY UPLC® I-Class system and a XevoTM TQ-S MS triple quadrupole mass spectrometer.

4. CONCLUSION AND PERSPECTIVES

This thesis focuses mainly on steroidal plant hormones – brassinosteroids, their effects on etiolated pea plants, the biological activity of BR precursors and novel synthetic analogues. The effects of BR described in this thesis are considered in relation to the interaction between BRs and the gaseous plant hormone, ethylene.

The most important outcomes of the work described are:

1. Treatment of etiolated pea plants with brassinosteroids induces all phenotypic elements of the ethylene 'triple response' to ethylene and the level of both ethylene and ACC increases in these plants in a dose-dependent manner. The results confirm that a highly sensitive bioassay for BRs using etiolated pea seedlings has been developed (Supplement IV).
2. Three compounds from the first series of brassinosteroid derivatives with p-substituted phenyl group in the side chain were identified as compounds with biological activity comparable with naturally occurring brassinosteroids (Supplement I).
3. It has been shown that molecular *in silico* docking into the BRI1 receptor can be used as a powerful tool for prediction and design of new compounds with brassinosteroid activities (Supplement I and II).
4. Through screening the second series of newly synthetized aryl BR analogues another 3 compounds with BR-like activity have been identified and the connection between the substitution pattern in the phenyl group and biological activity of the compound has been revealed (Supplement II).
5. Examination of BR biosynthetic precursors in the pea inhibition test and Arabidopsis growth test showed that BR biological activity occurs dose-dependently in downstream intermediates of the BR biosynthetic pathway

6. Arabidopsis growth rescue assay using *cyp85a1/cyp85a2* double mutant assigned the biological activity to intermediates located before the cyp85a1/cyp85a2 enzymatic step (3-dehydro-6-deoxoteasterone and 6-deoxotyphasterol)
7. The data suggests that in Arabidopsis, BRs act differently in roots and shoots. In shoots the elongation effect (observed after BR treatment) is ethylene dependent and probably mediated by enhanced ACC level in this tissue. The root inhibition occurring after the BR treatment is not dependent on ethylene signaling.
8. Comprehensive reviews on the interactions of brassinosteroids and ethylene during plant development and under various stress conditions have been published (Supplement III, Supplement V)

Plant hormone crosstalk is a complex topic of broad and current interest and the data presented in this thesis may form a basis for further research on a molecular level leading to elucidation of brassinosteroids-ethylene crosstalk. Newly synthetized BR analogues with high biological activity may have potential agriculture application by themselves or be used as a template for the synthesis of new compounds. *In silico* docking has also been shown to be a useful tool for the design of new bioactive compoundsand the newly developed sensitive bioassay for brassinosteroid is routinely used for examining BR biological activity.

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SUPPLEMENTS

SUPPLEMENT I

Kvasnica M., Oklestkova J., Bazgier V., Rárová L., **Korinkova P.,** Mikulík J., Budesinsky M., Béres T., Berka K., Lu Q., Russinova E., Strnad M. (2016). Design, synthesis and biological activities of new brassinosteroid analogues with a phenyl group in the side chain. Organic & Biomolecular Chemistry 14, 8691-8701.

- Co-author – screening of biological activities of new brassinosteroid analogues (performing the pea inhibition test, measurement of ethylene production in treated pea plants)

Supplementary information available https://doi.org/10.1039/C6OB01479H

SUPPLEMENT II

Korinkova P., Bazgier V., Oklestkova J., Rarova L., Strnad M., Kvasnica M. (2017). Synthesis of novel aryl brassinosteroids through alkene cross-metathesis and preliminary biological study. Steroids 127, 46-55.

- First author - screening of biological activities of new brassinosteroid analogues (performing the pea inhibition test, measurement of ethylene production in treated pea plants, Arabidopsis root and hypocotyls sensitivity assays)

Supplementary information available https://doi.org/10.1016/j.steroids.2017.08.010

SUPPLEMENT III

Jiroutová P., Oklestkova J., Strnad M., (2018) Crosstalk between Brassinosteroids and Ethylene during Plant Growth and under Abiotic Stress Conditions. Int. J. Mol. Sci 19 (10), 3283.

- First author – writing of manuscript

SUPPLEMENT IV – submitted article

Jiroutová P., Mikulík J., Novák O., Oklestkova J., Strnad M., (2019) Brassinosteroids induce strong, dose-dependent inhibition of etiolated pea seedling growth correlated with ethylene production. Biomoleculs

- First author - bioassay development, optimization, performing of the experiments, writing of manuscript

SUPPLEMENT V

J. Oklestkova, **P. Korinkova**, M. Strnad: The Crosstalk of Brassinosteroids and Other Hormones in Abiotic Stress Tolerance, in: Mechanisms Behind Phytohormonal Signalling and Crop Abiotic Stress Tolerance, NOVA SCIENCE PUBLISHERS, INC., NY 11788-3619, USA, 2017.

- Co-author – writing of manuscript

Organic & Biomolecular Chemistry

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Cite this: *Org. Biomol. Chem.*, 2016, **14**, 8691

Design, synthesis and biological activities of new brassinosteroid analogues with a phenyl group in the side chain†

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Received 12th July 2016,
Accepted 17th August 2016

DOI: 10.1039/c6ob01479h

www.rsc.org/obc

We have prepared and studied a series of new brassinosteroid derivatives with a *p*-substituted phenyl group in the side chain. To obtain the best comparison between molecular docking and biological activities both types of brassinosteroids were synthesized; 6-ketones, 10 examples, and B-lactones, 8 examples. The phenyl group was introduced into the steroid skeleton by Horner–Wadsworth–Emmons. The docking studies were carried out using AutoDock Vina 1.05. Plant biological activities were established using different brassinosteroid bioassays in comparison with natural brassinosteroids. Differences in the production of the plant hormone ethylene were also observed in etiolated pea seedlings after treatment with new brassinosteroids. The most active compounds were lactone **8f** and 6-oxo derivatives **8c** and **9c**, their biological activities were comparable or even better than naturally occurring brassinolide. Finally the cytotoxicity of the new derivatives was studied using human normal and cancer cell lines.

Introduction

Brassinosteroids (BRs, Fig. 1) are a class of plant steroid hormones, which are now known to be essential for many aspects of plant growth and development, such as cell division, elongation and differentiation, pollen tube growth, seed germination, regulation of gene expression, enzyme activation and photosynthesis.[1–3] They are also involved in defense against a wide range of biotic and abiotic stresses, such as water, temperature, oxidative stresses and high salinity.[4,5] Moreover, recent studies have shown that natural BRs have potential application in medicine due to their antiviral,[6,7] immunomodulatory and neuroprotective activities,[8,9] and anti-proliferative effects in animal cells *in vitro*.[10–15] In contrast to the mammalian nuclear steroid receptors, BRs are perceived at the cell surface by the transmembrane receptor complex formed by the receptor kinase BRI1 and its co-receptor BAK1.[16–19] The BRI1 receptor has a binding site for BRs located in the extracellular ectodomain. There, the nonpolar side of BRs fits into a highly

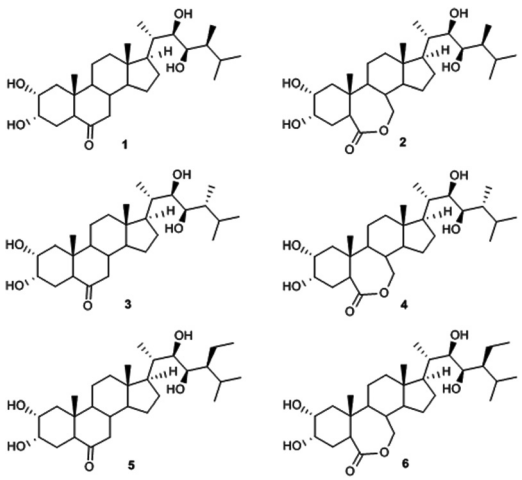

Fig. 1 Structures of the most common natural brassinosteroids; castasterone (**1**), brassinolide (**2**), 24-epicastasterone (**3**), 24-epibrassinolide (**4**), 28-homocastasterone (**5**), 28-homobrassinolide (**6**).

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†Electronic supplementary information (ESI) available. See DOI: 10.1039/c6ob01479h

nonpolar cavity of the receptor cleft, and the hydroxyl groups of BRs are exposed to the solvent or towards interactions with BAK1 or SERK1.[18,20] This structural knowledge, formed the basis of the idea of the brassinosteroid side chain modifications using a nonpolar group such as the phenyl group. Moreover, some analogues containing cycloalkyl substituents at C-24 (replacing the isopropyl group of brassinolide 1) exhibited significant activity in the rice lamina inclination biotest, where the compound with the cyclohexyl group, showed the lowest activity, whereas the cyclopentyl analogue was comparably active as brassinolide in this bioassay.[21,22]

The aim of this study was to synthesize new brassinosteroid derivatives with a *p*-substituted phenyl group in the side chain and study their biological properties. The phenyl group was chosen owing to its successful molecular docking into the active site of BRI1 using AutoDock Vina. Some compounds showed marked interactions with the BRI1 receptor. The biological activities of the newly prepared derivatives were confirmed by a plant bioassay (pea inhibition biotest, Arabidopsis root and hypocotyl sensitivity bioassay and BES-1 dephosphorylation assay). Their cytotoxic activities were studied using human normal and cancer cell lines.

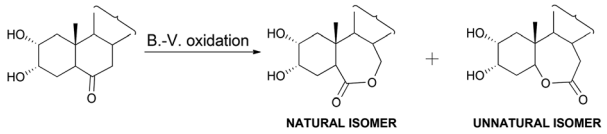
Results and discussion

Chemistry

For the preparation of the desired phenyl analogues of brassinosteroids, we set out from the known aldehyde **7** ((20*S*)-6,6-ethylenedioxy-5α-pregn-2-en-20-carbaldehyde) prepared according to a published procedure.[23] With this aldehyde Horner–Wadsworth–Emmons (HWE) reaction was then carried out with different commercially available *p*-substituted benzylphosphonates. Based on the character of the BRI1 non-polar part of the cavity for the side chain, we used these substituents in the *para* position: fluorine, chlorine, bromine, iodine, nitro, methyl, methoxy, nitrile, and isopropyl. Benzyl triphenylphosphonium chloride was used for preparation of non-substituted aryl analogues.

Despite the standard use of sodium hydride as a base in the HWE reaction, we observed isomerization of the methyl group on C-20 (see the ESI† for detailed reaction conditions and analysis). Using this base along with steric hindrance of the reaction site for bulky aryl groups, the reaction time increased and this led to enolization of the aldehyde. The aryl group of phosphonates used is crucial for this epimerization as it was not observed when smaller stabilizing groups were used (*e.g.* COOR, CN).[24] Using *n*-butyllithium instead solved the problem and only the desired aryl dienes **8a–17a** with 22*E* configuration were obtained. In almost all the cases, the reaction gave products in good yields (80–90%). Only compound **12a** was prepared in lower yield (65%) due to the presence of reactive iodine.

Further hydrolysis of the ketal group gave aryl dienons **8b–17b**, which were subjected to dihydroxylation in almost quantitative yield. Simultaneous Sharpless dihydroxylation of both



Scheme 1 Baeyer–Villiger oxidation of the B-ring showing formation of natural and unnatural isomeric lactones.

double bonds was used to minimize the formation of unnatural configurations of 22 and 23 hydroxy groups. As a chiral ligand, we used hydroquinidine 4-chlorobenzoate. The reaction rate was increased by addition of methanesulfonamide.[25] Without methanesulfonamide, the reaction took more than 48 hours. Such reaction conditions allowed us to isolate only the desired 22*R*,23*R*-isomers **8c–17c** in good yields (75–83%). The correct configuration was determined according to a published alternative preparation of **8c**.[26] The unnatural isomers (22*S*,23*S*) were formed only in trace amounts and therefore not isolated. Dihydroxylation of the double bond on the A-ring is stereocontrolled by A-ring conformation and the presence of a methyl group on C10. The attack of the reagent is always from the bottom side of the molecule and thus only 2α,3α-diol was detected.

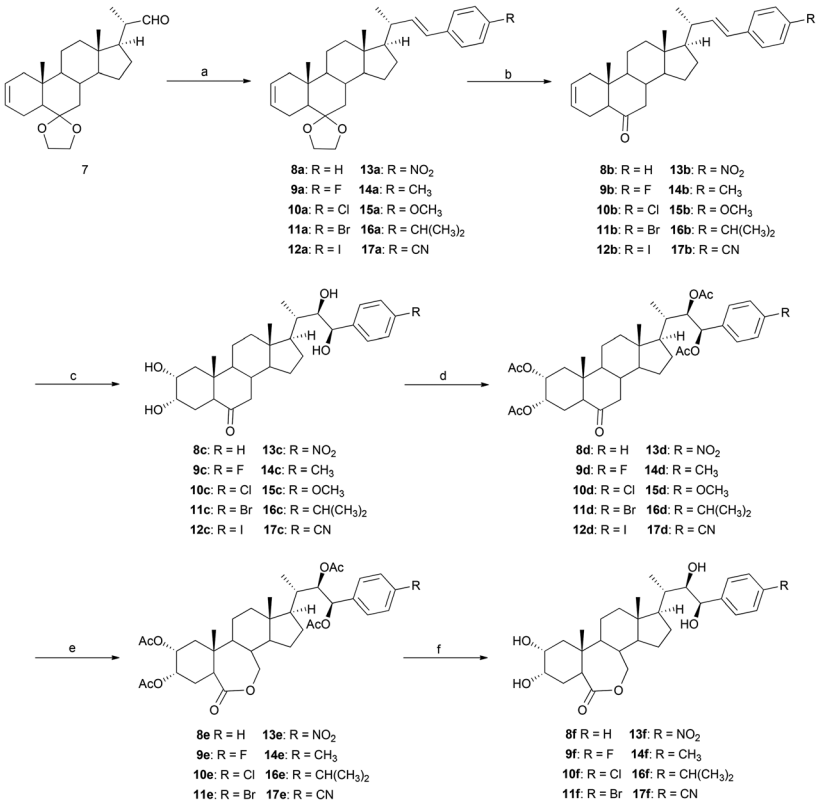
As brassinosteroids with lactone in the B-ring are known to show a higher biological activity than the corresponding 6-ketones, we decided to prepare them as well. The direct Baeyer–Villiger oxidation of tetrahydroxy-ketones with freshly prepared trifluoroperoxyacetic acid led to both isomers in a ratio of approx. 10–15 : 1 (with the natural isomer favoured). However, these mixtures were inseparable even by HPLC (Scheme 1).

For this reason, we had to prepare tetraacetates **8d–17d** first and carry out the oxidation reaction on these. This "detour" allowed us to prepare and easily separate the required lactones **8e–11e**, **13e**, **14e**, **16e**, and **17e** in good yields (79–88%). Unfortunately, two aryl-ketones **12d** and **15d** were unstable during the reaction and only a mixture of products was obtained in both cases. The instability of the iodophenyl derivative **12d** was predictable owing to the presence of easily oxidizable iodine. Due to the presence of the activating group, the methoxyphenyl derivative **15d** is more nucleophilic on the benzene ring than other derivatives and thus also undergoes the electrophilic aromatic substitution reaction (substitution with trifluoroacetate). These two reactions also failed using 3-chloroperoxybenzoic acid.

The last reaction step was basic hydrolysis of tetraacetoxy-lactones to the corresponding tetrahydroxy-lactones **8f–11f**, **13f**, **14f**, **16f**, and **17f** in almost quantitative yields. The reaction steps can be seen in Scheme 2. All compounds were characterized by NMR, IR and MS techniques. Compounds used for biological testing were also characterized for purity by elemental analysis and melting point.

Molecular docking

Molecular docking is a useful tool for understanding the pose and energetics of a protein–ligand complex. The binding site



Scheme 2 Synthesis of brassinosteroid phenyl analogues: (a) benzyltriphenylphosphonium bromide or diethyl arylphosphonates, *n*-BuLi/THF; (b) 5% HCl/THF; (c) OsO_4, $CH_3SO_2NH_2$, $K_3[Fe(CN)_6]$, K_3CO_2, hydroquinidine 4-chlorobenzoate/*t*-BuOH, H_2O; (d) Ac_2O/pyridine; (e) trifluoroperoxyacetic acid/CH_2Cl_2; (f) NaOH/THF, H_2O.

of BRI1 is located on the surface of the receptor ectodomain as a nonpolar cleft lined by nonpolar aromatic and aliphatic residues (I540, I563, W564, Y599, Y642, M657, F681, I682, I706), whereas hydroxyl groups form the cleft ridge (Y597, Y599, Y642, S647). Brassinolide fits into the cleft *via* its nonpolar side and displays its hydroxyl groups towards the solvent and protein partners (Fig. 2).

Since there is a space left in the cleft around the brassinolide chain, we were running molecular docking of BR derivatives with a phenyl ring on the tail replacing the 1′,2′-dimethylpropyl moiety of brassinolide. Molecular docking predicted similar or better binding energies than for brassinolide for compounds **8c**, **8f**, **9c**, **9f**, **10c**, **10f**, and **14f** (Fig. 3). This implies that derivatives with a phenyl ring on the tail or a phenyl ring with small groups such as fluorine, chlorine or methyl should be accommodated within the BRI1 cleft at least as easily as brassinolide itself and hence they were the best

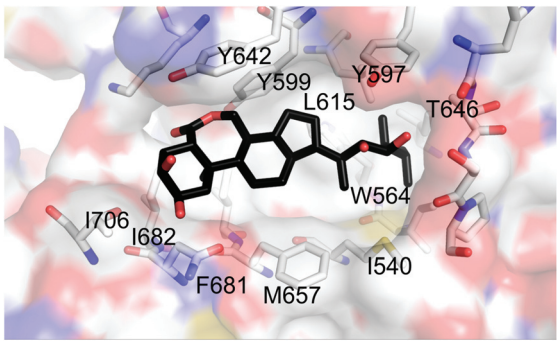


Fig. 2 3D view of brassinolide with amino acids around the active site.

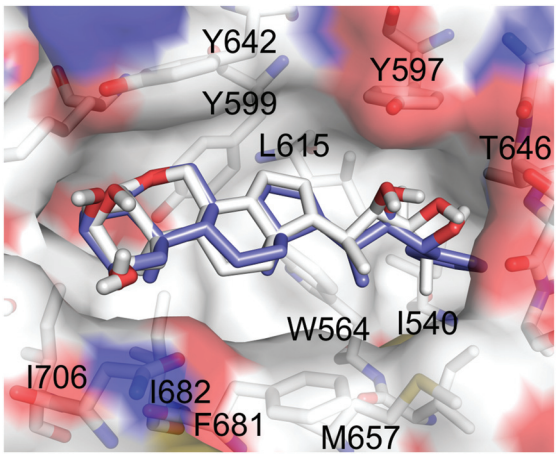


Fig. 3 Pose of **8f** within the BRI1 binding site. The structure of brassinolide and BRI1 binding sites are shown in white, and the structure of **8f** is shown in blue.

candidates for showing similar binding experimentally. On the other hand, derivatives with larger groups did not fit well into the cleft and the docked pose often revealed the tail out of the cleft completely (for the pose of all compounds see the ESI†). In all docking cases, lactones showed better binding energies than 6-ketones due to a better fit to cavity, close to Y599. The best compound to emerge from molecular docking was compound **8f**.

Biology

The response of plant tissues to applied BRs varies with BR concentration. In most cases, low concentration induces elongation and curvature as a result of cell division and cell elongation. Most BR bioassays are based on this effect.[27] However, there are other ways of regulating growth by BRs. For example, BRs inhibit the growth of etiolated pea seedlings at high concentration and this is probably caused by increased ethylene production. Ethylene effects alteration of the normal planes of cell growth. Radial swelling or abnormal radial expansion of the stem, such as that seen in the response of etiolated pea seedlings to ethylene application, results from inhibited elongation, increased radial expansion and probably also accounts for leaf epinasty.[28] These effects are known as the "triple response" of etiolated seedlings to ethylene.[29]

The IC_{50} values obtained from the pea inhibition biotest are summarized in Table 1. The most active lactones were **8f**, **9f**, **11f** and **14f** (IC_{50} 2.56×10^{-6}–1.4×10^{-6} mol L^{-1}) whereas brassinolide (BL), the most active natural BR used as a positive control, was about ten times less active (IC_{50} 2.2×10^{-5} mol L^{-1}). 6-Oxoderivatives **8c** and **9c** were also active in this bioassay. Their IC_{50} values were comparable with active lactones (Table 1). Compounds **15c**, **13f** and **16f** showed no inhibition of etiolated pea plants. Dose response curves for the most active BR derivatives are shown in Fig. 4.

The levels of ethylene were measured in cultivation vessels during the incubation of etiolated pea plants after treatment with different BR derivatives (Fig. 5). The highest concentration of ethylene (235 and 224 nL L^{-1}) was determined after 24 h treatment with **8c** and **14f** compared to 206 nL L^{-1} for BL treatment. However, the level of this gaseous plant hormone produced by untreated control pea plants was found to be significantly lower (about 80 nL L^{-1}, Fig. 5). Arteca *et al.*[30] also observed stimulated ethylene production in etiolated mung bean segments 4 hours after treatment with 1 μM BL and this increased production became greater over the following 20 h.

The most potent compounds (**8c**, **8f** and **9c**) were tested in Arabidopsis brassinosteroid sensitivity and BES-1 dephosphorylation bioassays. The potency of these compounds was as follows: **BL** ≥ **8f** > **9c** > **8c** in Arabidopsis sensitivity bioassays. The effects of the tested compounds on the Arabidopsis roots and hypocotyls are shown in the ESI (Fig. S3 and S4†). Compound **8f** significantly increased dephosphorylation of BES1 (Fig. 6), which is an important transcription factor in the BR signalling pathway. Altogether, these results confirm that the biological activity of compound **8f** is comparable with natural BR-brassinolide.

The antiproliferative activity of the prepared brassinosteroid derivatives was tested using several models of normal and

Table 1 IC_{50} (mol L^{-1}) values obtained from the pea inhibition biotest

Compound	IC_{50} (mol L^{-1})
Brassinolide	$2.2 \times 10^{-5} \pm 2 \times 10^{-6}$
8c	$2.5 \times 10^{-6} \pm 3 \times 10^{-7}$
8f	$1.8 \times 10^{-6} \pm 5 \times 10^{-8}$
9c	$2.0 \times 10^{-6} \pm 3 \times 10^{-7}$
9f	$2.6 \times 10^{-6} \pm 8 \times 10^{-8}$
10c	$1.8 \times 10^{-5} \pm 3 \times 10^{-6}$
10f	$2.3 \times 10^{-5} \pm 2 \times 10^{-6}$
11c	$2.7 \times 10^{-4} \pm 2 \times 10^{-5}$
11f	$1.7 \times 10^{-6} \pm 3 \times 10^{-7}$
12c	$2.1 \times 10^{-2} \pm 4 \times 10^{-3}$
13c	$4.0 \times 10^{-2} \pm 5 \times 10^{-4}$
13f	No inhibition
14c	$1.8 \times 10^{-4} \pm 7 \times 10^{-6}$
14f	$1.4 \times 10^{-6} \pm 2 \times 10^{-7}$
15c	No inhibition
16c	$2.1 \times 10^{-4} \pm 5 \times 10^{-6}$
16f	No inhibition
17c	$2.0 \times 10^{-3} \pm 6 \times 10^{-5}$
17f	$2.1 \times 10^{-4} \pm 5 \times 10^{-6}$

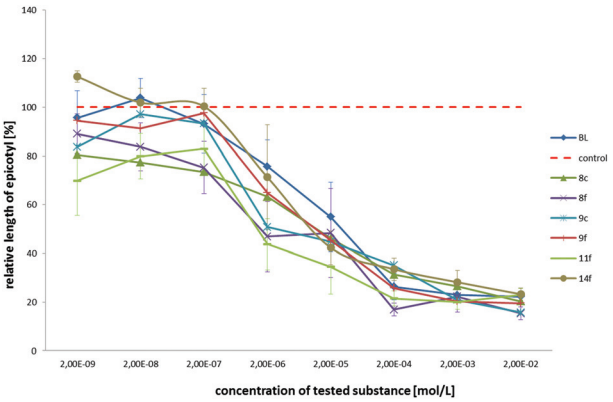


Fig. 4 Effect of selected brassinosteroid derivatives on the inhibition of etiolated pea seedlings. Error bars represent S.D.

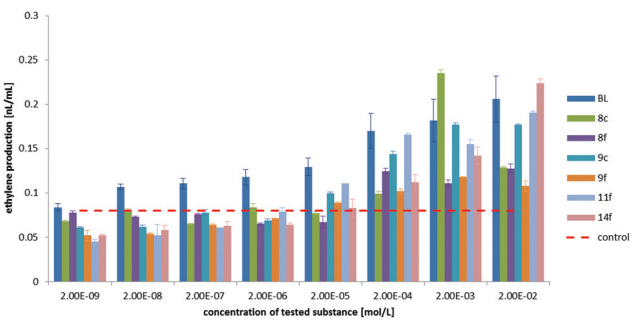


Fig. 5 Effect of selected brassinosteroid derivatives on ethylene production (nL mL^{-1}) in etiolated pea seedlings determined by GC-FID 24 h after ventilation. Error bars represent S.D.

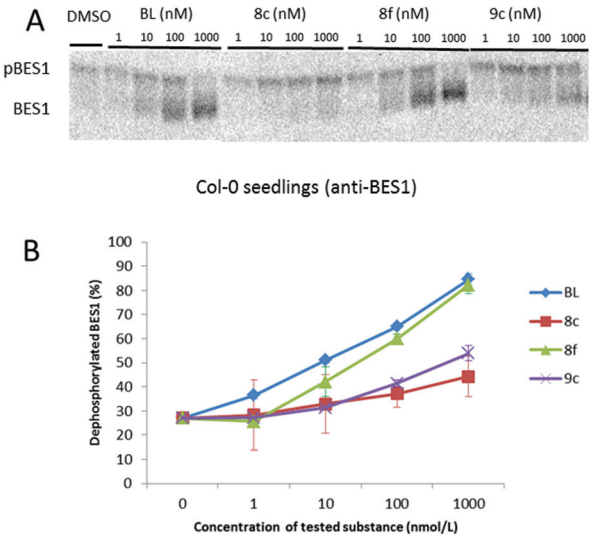


Fig. 6 (A) Immunoblot analysis of BES1 in *Arabidopsis thaliana* (Col-0) seedlings showing dephosphorylation of BES1 after BR treatment. (B) Graph shows the percentage of dephosphorylated BES1 relative to the total BES1 detected in Arabidopsis. The data are the average of two biological repeats. Error bars indicate S.E.M. of pBES1 and phosphorylated BES1.

cancer cell lines. We compared the *in vitro* cytotoxic activity of selected analogues against human foreskin fibroblasts (BJ) and cancer cell lines of various histopathological origins, including T-lymphoblastic leukemia CEM, breast carcinoma (MCF7) and cervical carcinoma (HeLa). Cells were exposed to six 3-fold dilutions of each drug for 72 h prior to determination of cell survival. The IC_{50} (concentration leading to 50% inhibition of viability) values obtained from the calcein AM cytotoxicity assay are presented in the ESI (Table S2†). Most tested BR analogues had no detectable cytotoxic activity, even when tested in concentrations of up to 50 μM. Only compounds **10f**, **11f** and **13f** showed moderate cytotoxic activity against CEM and HeLa cell lines (IC_{50} around 35 μM). No BR derivative mediated loss of viability was observed in the BJ fibroblasts. 24-Epibrassinolide was used as a control. It is a natural brassinosteroid with modest cytotoxicity against CEM cells (IC_{50} 44 μM).[15]

Experimental

General methods

The melting points were determined on a Stuart SMP30 instrument (Bibby Scientific Ltd, UK). Elemental analyses were performed using an EA 1108 elemental analyzer (Fison Instruments); the values (C, H, N) agreed with the calculated values within acceptable limits. The infrared spectra were recorded on a Thermo Scientific Nicolet spectrometer iZ10 using the ATR technique. The wave numbers are given in cm^{-1}. The NMR spectra were taken on a JEOL JNM-ECA 500 (JEOL, Tokyo, Japan; ^1H, 500 MHz; ^{13}C, 125 MHz) spectrometer equipped with a 5 mm JEOL Royal probe. ^1H NMR and ^{13}C NMR chemical shifts (δ) were calibrated using tetramethylsilane (TMS, ^1H δ = 0 ppm) or solvents: $CDCl_3$ (^1H δ = 7.26 ppm, ^{13}C δ = 77.00 ppm) or DMSO-d_6 (^1H δ = 2.46 ppm, ^{13}C δ = 40.00 ppm). Chemical shifts are given in ppm (δ-scale), coupling constants (J) in Hz. All values were obtained by first-order analysis. For API HRMS analysis, the samples were dissolved in chloroform (or chloroform : methanol; 1 : 1; v/v, in the case of hydroxylated compounds) to a concentration of 10 μg mL^{-1}. The ASAP (Atmospheric Solids Analysis Probe) was dipped into the sample solution, placed into the ion source and analysed in full scan mode. The source of the Synapt G2-Si mass spectrometer (Waters, Manchester, UK) was operated in positive ionisation mode (ASAP+), if not stated otherwise, at a source temperature of 120 °C. The corona needle current was kept at 5 μA and the collision energy at 4 V. The probe temperature was ramped up from 50 °C to 600 °C in 3 minutes. Data were acquired from 50 to 1000 Da with 1.0 s scan time in high resolution mode. The data were processed using the Masslynx 4.1 software (Waters). A mass accuracy of 1 ppm or less was achieved with the described instrumentation for all compounds. Merck silica gel Kieselgel 60 (230–400 mesh) was used for column chromatography. The HPLC system consisted of a Waters semi-preparative HPLC system including a quaternary pump, a liquid handler, and UV-VIS and ELSD detectors. The semi-preparative column was filled with silica gel. Reagents and solvents were purchased from Sigma–Aldrich and were not purified. For experimental procedures and data for compounds of series b, d, e, and f, see the ESI.†

(22*E*)-6,6-Ethylenedioxy-23-phenyl-24-nor-5α-chola-2,22-diene (8a). To a suspension of benzyl triphenylphosphonium chloride (0.81 mmol) in dried THF (10 mL) was added *n*-BuLi (1.6 M solution in *n*-hexane, 0.81 mmol) at 0 °C and stirred for 1 h. To the resultant red solution was added a solution of aldehyde 7 (200 mg, 0.54 mmol) in THF (10 mL) and stirred for 4 h at 25 °C. The reaction mixture was quenched with water and extracted with Et_2O (2 × 10 mL). The combined organic fractions were washed with brine and dried over anhydrous magnesium sulfate. Evaporation of the volatiles under reduced pressure followed by column chromatography on silica gel (Et_2O/cyclohexane – 1/19) gave 211 mg (88%) of the title compound **8a** as a colorless oil: IR ν (cm^{-1}) 2933, 1655, 1598. ^1H NMR ($CDCl_3$) δ 0.74, 0.89 (both s, 3H, CH_3), 1.11 (d, 3H, J = 6.7 Hz, CH_3), 1.68–1.74 (m, 2H), 1.78 (m, 2H), 1.93–2.03 (m, 3H), 2.09 (m, 1H), 2.26 (m, 1H, ΣJ = 37.6 Hz), 3.78 (m, 1H, ΣJ = 24.1 Hz, OCH), 3.88–3.99 (m, 3H, 3 × OCH), 5.54 (m, 1H, H-3), 5.66 (m, 1H, H-2), 6.06 (dd, 1H, J = 15.7, J' = 8.7 Hz, H-22), 6.29 (d, 1H, J = 15.7 Hz, H-23), 7.17 (m, 1H, Ar–H), 7.25–7.34 (m, 4H, 4 × Ar–H). ^{13}C NMR δ 12.25 (CH_3), 13.60 (CH_3), 20.41 (CH_3), 20.87 (CH_2), 21.43 (CH_2), 24.15 (CH_2), 28.36 (CH_2), 33.35 (CH), 35.91 (C), 39.68 (CH_2), 40.49 (CH), 41.20 (CH_2), 41.24 (CH_2), 42.65 (C), 48.08 (CH), 53.50 (CH), 55.93 (CH), 56.00 (CH), 64.08 (CH_2), 65.56 (CH_2), 110.02 (C), 124.80 (CH), 125.70 (CH), 125.90 (2 × CH), 126.64 (CH), 127.19 (CH), 128.43

(2 × CH), 137.30 (CH), 138.08 (C). HRMS: (API+) calculated for $C_{31}H_{43}O_2$ ($[M + H]^+$) 447.3263, Found 447.3266.

General procedure for the Wadsworth–Horner–Emmons (WHE) reaction

To a suspension of substituted diethyl phenylphosphonate (1.08 mmol) in dried THF (10 mL) was added *n*-BuLi (500 μL, 1.6 M solution in *n*-hexane, 0.81 mmol) at 0 °C and stirred for 1 h. To the resultant yellow solution was added a solution of aldehyde **7** (200 mg, 0.54 mmol) in THF (10 mL) and stirred for 4 h at room temperature. The reaction mixture was quenched with water and extracted with Et_2O (2 × 10 mL). The combined organic fractions were washed with brine and dried over anhydrous magnesium sulfate. Evaporation of the volatiles under reduced pressure followed by column chromatography on silica gel gave the desired product.

(22*E*)-6,6-Ethylenedioxy-23-(4-fluorophenyl)-24-nor-5α-chola-2,22-diene (9a). The general procedure for the WHE reaction with diethyl 4-fluorophenylphosphonate and chromatography on silica (Et_2O/cyclohexane – 1/19) yielded 221 mg (89%) of the title compound **9a** as a colorless oil: IR ν (cm^{-1}) 2933, 1654, 1599. ^{1}H NMR ($CDCl_3$) δ 0.74, 0.89 (both s, 3H, CH_3), 1.11 (d, 3H, J = 6.7 Hz, CH_3), 1.71 (m, 2H), 1.78 (m, 2H), 1.92–2.03 (m, 3H), 2.09 (m, 1H), 2.23 (m, 1H, ΣJ = 37.6 Hz), 3.77 (q, 1H, J = 6.8 Hz, OCH), 3.87–3.99 (m, 3H, 3 × OCH), 5.54 (m, 1H, H-3), 5.66 (m, 1H, H-2), 5.97 (dd, 1H, J = 15.6, J' = 8.9 Hz, H-22), 6.25 (d, 1H, J = 15.6 Hz, H-23), 6.94–6.98 (m, 2H, 2 × Ar–H), 7.25–7.28 (m, 2H, 2 × Ar–H). ^{13}C NMR δ 12.21 (CH_3), 13.57 (CH_3), 20.36 (CH_3), 20.86 (CH_2), 21.42 (CH_2), 24.12 (CH_2), 28.37 (CH_2), 33.34 (CH), 35.90 (C), 39.68 (CH_2), 40.45 (CH), 41.20 (CH_2), 41.24 (CH_2), 42.63 (C), 48.08 (CH), 53.50 (CH), 55.92 (CH), 56.00 (CH), 64.07 (CH_2), 65.55 (CH_2), 109.99 (C), 115.22 (d, J = 21.6 Hz, 2 × CH), 124.77 (CH), 125.69 (CH), 126.04 (CH), 127.25 (d, J = 7.2 Hz, 2 × CH), 134.18 (d, J = 3.6 Hz, C), 137.01 (d, J = 2.4 Hz, CH), 161.77 (d, J = 244.7 Hz, C). ^{19}F NMR $\{^1H\}$ δ −116.36 (s, 1F). HRMS: (API+) calculated for $C_{31}H_{42}FO_2$ ($[M + H]^+$) 465.3169, Found 465.3170.

(22*E*)-6,6-Ethylenedioxy-23-(4-chlorophenyl)-24-nor-5α-chola-2,22-diene (10a). The general procedure for the WHE reaction with diethyl 4-chlorophenylphosphonate and chromatography on silica (Et_2O/cyclohexane – 1/19) yielded 225 mg (87%) of the title compound **10a** as a colorless oil: IR ν (cm^{-1}) 2933, 1656, 1593. ^{1}H NMR ($CDCl_3$) δ 0.74, 0.89 (both s, 3H, CH_3), 1.11 (d, 3H, J = 6.7 Hz, CH_3), 1.71 (m, 2H), 1.78 (m, 2H), 1.92–2.03 (m, 3H), 2.09 (m, 1H), 2.25 (m, 1H, ΣJ = 37.6 Hz), 3.78 (q, 1H, J = 6.8 Hz, OCH), 3.88–4.00 (m, 3H, 3 × OCH), 5.54 (m, 1H, H-3), 5.66 (m, 1H, H-2), 6.04 (dd, 1H, J = 15.7, J' = 8.9 Hz, H-22), 6.25 (d, 1H, J = 15.7 Hz, H-23), 7.24 (m, 2H, 2 × Ar–H), 7.26 (m, 2H, 2 × Ar–H). ^{13}C NMR δ 12.24 (CH_3), 13.60 (CH_3), 20.30 (CH_3), 20.86 (CH_2), 21.43 (CH_2), 24.14 (CH_2), 28.36 (CH_2), 33.35 (CH), 35.91 (C), 39.68 (CH_2), 40.50 (CH), 41.21 (CH_2), 41.24 (CH_2), 42.67 (C), 48.08 (CH), 53.48 (CH), 55.85 (CH), 55.98 (CH), 64.09 (CH_2), 65.58 (CH_2), 110.01 (C), 124.79 (CH), 125.70 (CH), 126.06 (CH), 127.11 (2 × CH), 128.53 (2 × CH), 132.13 (C), 136.56 (C), 138.02 (CH). HRMS: (API+) calculated for $C_{31}H_{42}ClO_2$ ($[M + H]^+$) 481.2873, Found 481.2878.

(22*E*)-6,6-Ethylenedioxy-23-(4-bromophenyl)-24-nor-5α-chola-2,22-diene (11a). The general procedure for the WHE reaction with diethyl 4-bromophenylphosphonate and chromatography on silica (Et_2O/cyclohexane – 1/19) afforded 231 mg (82%) of the title compound **11a** as a colorless oil: IR ν (cm^{-1}) 2933, 1656, 1595. ^{1}H NMR ($CDCl_3$) δ 0.74, 0.89 (both s, 3H, CH_3), 1.11 (d, 3H, J = 6.7 Hz, CH_3), 1.71 (m, 2H), 1.78 (m, 2H), 1.92–2.03 (m, 3H), 2.09 (m, 1H), 2.25 (m, 1H, ΣJ = 37.6 Hz), 3.78 (q, 1H, J = 6.8 Hz, OCH), 3.88–4.00 (m, 3H, 3 × OCH), 5.54 (m, 1H, H-3), 5.66 (m, 1H, H-2), 6.05 (dd, 1H, J = 15.7, J' = 8.7 Hz, H-22), 6.23 (d, 1H, J = 15.7 Hz, H-23), 7.18 (m, 2H, 2 × Ar–H), 7.39 (m, 2H, 2 × Ar–H). ^{13}C NMR δ 12.23 (CH_3), 13.59 (CH_3), 20.25 (CH_3), 20.85 (CH_2), 21.42 (CH_2), 24.13 (CH_2), 28.35 (CH_2), 33.34 (CH), 35.90 (C), 39.68 (CH_2), 40.51 (CH), 41.20 (CH_2), 41.23 (CH_2), 42.67 (C), 48.07 (CH), 53.47 (CH), 55.82 (CH), 55.97 (CH), 64.08 (CH_2), 65.57 (CH_2), 110.00 (C), 120.21 (C), 124.78 (CH), 125.69 (CH), 126.10 (CH), 127.46 (2 × CH), 131.46 (2 × CH), 137.01 (C), 138.15 (CH). HRMS: (API+) calculated for $C_{31}H_{42}{}^{79}BrO_2$ ($[M + H]^+$) 525.2368, Found 525.2370.

(22*E*)-6,6-Ethylenedioxy-23-(4-iodophenyl)-24-nor-5α-chola-2,22-diene (12a). The general procedure for the WHE reaction with diethyl 4-iodophenylphosphonate and chromatography on silica (Et_2O/cyclohexane – 1/19) afforded 200 mg (65%) of the title compound **12a** as a colorless oil: IR ν (cm^{-1}) 2933, 1656, 1595. ^{1}H NMR ($CDCl_3$) δ 0.74, 0.89 (both s, 3H, CH_3), 1.11 (d, 3H, J = 6.7 Hz, CH_3), 1.71 (m, 2H), 1.78 (m, 2H), 1.92–2.03 (m, 3H), 2.09 (m, 1H), 2.24 (m, 1H, ΣJ = 37.1 Hz), 3.78 (q, 1H, J = 6.8 Hz, OCH), 3.88–4.00 (m, 3H, 3 × OCH), 5.54 (m, 1H, H-3), 5.66 (m, 1H, H-2), 6.06 (dd, 1H, J = 15.7, J' = 8.7 Hz, H-22), 6.21 (d, 1H, J = 15.7 Hz, H-23), 7.06 (m, 2H, 2 × Ar–H), 7.59 (m, 2H, 2 × Ar–H). ^{13}C NMR δ 12.23 (CH_3), 13.59 (CH_3), 20.24 (CH_3), 20.85 (CH_2), 21.42 (CH_2), 24.13 (CH_2), 28.33 (CH_2), 33.34 (CH), 35.90 (C), 39.67 (CH_2), 40.50 (CH), 41.20 (CH_2), 41.23 (CH_2), 42.67 (C), 48.07 (CH), 53.47 (CH), 55.81 (CH), 55.96 (CH), 64.08 (CH_2), 65.57 (CH_2), 91.52 (C), 110.00 (C), 124.78 (CH), 125.70 (CH), 126.20 (CH), 127.75 (2 × CH), 137.43 (2 × CH), 137.60 (C), 138.29 (CH). HRMS: (API+) calculated for $C_{31}H_{42}IO_2$ ($[M + H]^+$) 573.2229, Found 573.2230.

(22*E*)-6,6-Ethylenedioxy-23-(4-nitrophenyl)-24-nor-5α-chola-2,22-diene (13a). The general procedure for the WHE reaction with diethyl 4-nitrophenylphosphonate and chromatography on silica (Et_2O/cyclohexane – 1/19) afforded 230 mg (87%) of the title compound **13a** as a colorless oil: IR ν (cm^{-1}) 2930, 1646, 1594, 1510, 1346. ^{1}H NMR ($CDCl_3$) δ 0.76, 0.89 (both s, 3H, CH_3), 1.15 (d, 3H, J = 6.5 Hz, CH_3), 1.72 (m, 2H), 1.78 (m, 2H), 1.92–2.04 (m, 3H), 2.09 (m, 1H), 2.32 (m, 1H, ΣJ = 37.0 Hz), 3.78 (q, 1H, J = 6.8 Hz, OCH), 3.88–4.00 (m, 3H, 3 × OCH), 5.54 (m, 1H, H-3), 5.66 (m, 1H, H-2), 6.28 (dd, 1H, J = 15.7, J' = 8.6 Hz, H-22), 6.37 (d, 1H, J = 15.7 Hz, H-23), 7.43 (m, 2H, 2 × Ar–H), 8.15 (m, 2H, 2 × Ar–H). ^{13}C NMR δ 12.26 (CH_3), 13.59 (CH_3), 20.02 (CH_3), 20.85 (CH_2), 21.42 (CH_2), 24.14 (CH_2), 28.32 (CH_2), 33.34 (CH), 35.90 (C), 39.68 (CH_2), 40.75 (CH), 41.21 (CH_2), 41.23 (CH_2), 42.79 (C), 48.08 (CH), 53.46 (CH), 55.63 (CH), 55.92 (CH), 64.09 (CH_2), 65.58 (CH_2),

109.97 (C), 123.94 (2 × CH), 124.74 (CH), 125.70 (2 × CH), 126.31 (2 × CH), 142.50 (CH), 144.67 (C), 146.31 (C). HRMS: (API+) calculated for $C_{31}H_{42}NO_4$ ($[M + H]^+$) 492.3114, Found 492.3118.

(22*E*)-6,6-Ethylenedioxy-23-(4-methylphenyl)-24-nor-5α-chola-2,22-diene (14a). The general procedure for the WHE reaction with diethyl 4-methylphenylphosphonate and chromatography on silica (Et_2O/cyclohexane – 1/19) afforded 200 mg (81%) of the title compound **14a** as a colorless oil: IR ν (cm^{-1}) 2934, 1656, 1595. ^1H NMR ($CDCl_3$) δ 0.74, 0.89 (both s, 3H, CH_3), 1.11 (d, 3H, J = 6.7 Hz, CH_3), 1.71 (m, 2H), 1.77 (m, 2H), 1.92–2.03 (m, 3H), 2.09 (m, 1H), 2.23 (m, 1H, ΣJ = 37.2 Hz), 2.31 (s, 3H, CH_3), 3.77 (q, 1H, J = 6.5 Hz, OCH), 3.87–3.98 (m, 3H, 3 × OCH), 5.54 (m, 1H, H-3), 5.66 (m, 1H, H-2), 6.00 (dd, 1H, J = 15.6, J' = 8.9 Hz, H-22), 6.26 (d, 1H, J = 15.6 Hz, H-23), 7.08 (d, 2H, J = 7.9 Hz, 2 × Ar–H), 7.21 (d, 2H, J = 7.9 Hz, 2 × Ar–H). ^{13}C NMR δ 12.20 (CH_3), 13.56 (CH_3), 20.42 (CH_3), 20.84 (CH_2), 21.07 (CH_3), 21.40 (CH_2), 24.11 (CH_2), 28.32 (CH_2), 33.32 (CH), 35.87 (C), 39.66 (CH_2), 40.42 (CH), 41.16 (CH_2), 41.21 (CH_2), 42.58 (C), 48.04 (CH), 53.47 (CH), 55.96 (2 × CH), 64.03 (CH_2), 65.53 (CH_2), 109.97 (C), 124.77 (CH), 125.67 (CH), 125.76 (2 × CH), 126.96 (CH), 129.08 (2 × CH), 135.24 (C), 136.25 (CH), one aromatic C not detected. HRMS: (API+) calculated for $C_{32}H_{45}O_2$ ($[M + H]^+$) 461.3420, Found 461.3422.

(22*E*)-6,6-Ethylenedioxy-23-(4-methoxyphenyl)-24-nor-5α-chola-2,22-diene (15a). The general procedure for the WHE reaction with diethyl 4-methoxyphenylphosphonate and chromatography on silica (Et_2O/cyclohexane – 1/19) afforded 203 mg (80%) of the title compound **15a** as a colorless oil: IR ν (cm^{-1}) 2934, 1656, 1594. ^1H NMR ($CDCl_3$) δ 0.74, 0.89 (both s, 3H, CH_3), 1.11 (d, 3H, J = 6.7 Hz, CH_3), 1.71 (m, 2H), 1.78 (m, 2H), 1.92–2.03 (m, 3H), 2.09 (m, 1H), 2.22 (m, 1H, ΣJ = 37.4 Hz), 3.77 (q, 1H, J = 6.7 Hz, OCH), 3.79 (s, 3H, OCH_3), 3.87–3.99 (m, 3H, 3 × OCH), 5.54 (m, 1H, H-3), 5.66 (m, 1H, H-2), 5.91 (dd, 1H, J = 15.7, J' = 8.7 Hz, H-22), 6.24 (d, 1H, J = 15.7 Hz, H-23), 6.82 (m, 2H, 2 × Ar–H), 7.25 (m, 2H, 2 × Ar–H). ^{13}C NMR δ 12.21 (CH_3), 13.58 (CH_3), 20.50 (CH_3), 20.85 (CH_2), 21.41 (CH_2), 24.12 (CH_2), 28.38 (CH_2), 33.33 (CH), 35.88 (C), 39.67 (CH_2), 40.43 (CH), 41.18 (CH_2), 41.22 (CH_2), 42.58 (C), 48.06 (CH), 53.48 (CH), 55.24 (CH_3), 55.99 (CH), 56.02 (CH), 64.05 (CH_2), 65.54 (CH_2), 109.99 (C), 113.83 (2 × CH), 124.78 (CH), 125.68 (CH), 126.48 (CH), 126.92 (2 × CH), 130.87 (C), 135.23 (CH), 158.48 (C). HRMS: (API+) calculated for $C_{32}H_{45}O_3$ ($[M + H]^+$) 477.3369, Found 477.3371.

(22*E*)-6,6-Ethylenedioxy-23-(4-isopropylphenyl)-24-nor-5α-chola-2,22-diene (16a). The general procedure for the WHE reaction with diethyl 4-isopropylphenylphosphonate and chromatography on silica (Et_2O/cyclohexane – 1/19) afforded 231 mg (88%) of the title compound **16a** as a colorless oil: IR ν (cm^{-1}) 2935, 1656, 1593. ^1H NMR ($CDCl_3$) δ 0.74, 0.89 (both s, 3H, CH_3), 1.11 (d, 3H, J = 6.7 Hz, CH_3), 1.229, 1.231 (both d, 3H, J = 7.0 Hz, CH_3), 1.71 (m, 2H), 1.78 (m, 2H), 1.93–2.03 (m, 3H), 2.09 (m, 1H), 2.24 (m, 1H, ΣJ = 37.6 Hz), 2.87 (septet, 1H, J = 7.0 Hz, C*H*(CH_3)$_2$), 3.78 (q, 1H, J = 6.8 Hz, OCH), 3.88–4.00 (m, 3H, 3 × OCH), 5.54 (m, 1H, H-3), 5.66 (m, 1H, H-2), 6.01 (dd, 1H, J = 15.8, J' = 8.6 Hz, H-22), 6.27 (d, 1H, J = 15.8 Hz, H-23), 7.15 (d, 2H, J = 8.3 Hz, 2 × Ar–H), 7.26 (d, 2H, J = 8.3 Hz, 2 × Ar–H). ^{13}C NMR δ 12.24 (CH_3), 13.60 (CH_3), 20.49 (CH_3), 20.86 (CH_2), 21.43 (CH_2), 23.97 (2 × CH_3), 24.14 (CH_2), 28.35 (CH_2), 33.35 (CH), 33.79 (CH), 35.91 (C), 39.68 (CH_2), 40.50 (CH), 41.19 (CH_2), 41.24 (CH_2), 42.61 (C), 48.07 (CH), 53.49 (CH), 55.97 (CH), 56.00 (CH), 64.07 (CH_2), 65.56 (CH_2), 110.02 (C), 124.81 (CH), 125.69 (CH), 125.86 (2 × CH), 126.50 (2 × CH), 126.97 (CH), 135.69 (C), 136.44 (CH), 147.43 (C). HRMS: (API+) calculated for $C_{34}H_{49}O_2$ ($[M + H]^+$) 489.3733, Found 489.3736.

(22*E*)-6,6-Ethylenedioxy-23-(4-cyanophenyl)-24-nor-5α-chola-2,22-diene (17a). The general procedure for the WHE reaction with diethyl 4-cyanophenylphosphonate and chromatography on silica (Et_2O/cyclohexane – 1/19) afforded 220 mg (87%) of the title compound **17a** as an amorphous solid: IR ν (cm^{-1}) 2933, 2224, 1646, 1604. ^1H NMR ($CDCl_3$) δ 0.75, 0.89 (both s, 3H, CH_3), 1.13 (d, 3H, J = 6.7 Hz, CH_3), 1.71 (m, 2H), 1.78 (m, 2H), 1.93–2.03 (m, 3H), 2.09 (m, 1H), 2.30 (m, 1H, ΣJ = 37.2 Hz), 3.78 (q, 1H, J = 6.8 Hz, OCH), 3.88–4.00 (m, 3H, 3 × OCH), 5.54 (m, 1H, H-3), 5.66 (m, 1H, H-2), 6.21 (dd, 1H, J = 15.7, J' = 8.6 Hz, H-22), 6.31 (d, 1H, J = 15.7 Hz, H-23), 7.39 (m, 2H, 2 × Ar–H), 7.56 (m, 2H, 2 × Ar–H). ^{13}C NMR δ 12.25 (CH_3), 13.59 (CH_3), 20.07 (CH_3), 20.84 (CH_2), 21.42 (CH_2), 24.13 (CH_2), 28.32 (CH_2), 33.34 (CH), 35.90 (C), 39.67 (CH_2), 40.66 (CH), 41.20 (CH_2), 41.22 (CH_2), 42.76 (C), 48.08 (CH), 53.45 (CH), 55.65 (CH), 55.92 (CH), 64.09 (CH_2), 65.58 (CH_2), 109.77 (C), 109.97 (C), 119.21 (C), 124.75 (CH), 125.70 (CH), 125.99 (CH), 126.36 (2 × CH), 132.30 (2 × CH), 141.46 (CH), 142.62 (C). HRMS: (API+) calculated for $C_{32}H_{42}NO_2$ ($[M + H]^+$) 472.3216, Found 472.3218.

General procedure for dihydroxylation of dienes

To a solution of diene (160 mg), hydroquinidine 4-chlorobenzoate (45 mg; 0.097 mmol), methanesulfonamide (65 mg; 0.68 mmol), potassium carbonate (280 mg; 2.03 mmol), and potassium ferricyanide (700 mg; 2.13 mmol) in the mixture of *t*-butanol and water (15 mL; 1 : 1 v/v) was added 0.2 mL of osmium tetroxide in *t*-butanol (1 g per 20 mL; 0.039 mmol). The reaction mixture was stirred at room temperature for 24 h. A saturated solution of sodium sulfite (3 mL) was then added. After an additional 30 minutes of stirring, the reaction mixture was diluted with ethyl acetate (30 mL) and extracted with water (2 × 20 mL). The combined organic fractions were dried over anhydrous magnesium sulfate and evaporated under reduced pressure. Column chromatography on silica gel gave the desired product.

(22*R*,23*R*)-2α,3α,22,23-Tetrahydroxy-23-phenyl-24-nor-5α-cholan-6-one (8c). The general procedure for dihydroxylation of **8b** and chromatography on silica ($MeOH/CHCl_3$ – 1/16) afforded 144 mg (77%) of the title compound **8c** as a white solid: m. p. 268–270 °C (EtOH), IR ν (cm^{-1}) 3344, 2940, 1708, 1496. ^1H NMR (DMSO-d_6) δ 0.30, 0.59 (both s, 3H, CH_3), 0.86 (d, 3H, J = 6.4 Hz, CH_3), 1.80 (m, 1H), 1.86 (m, 1H), 1.97 (dd, 1H, J = 13.1, J' = 4.6 Hz), 2.07 (t, 1H, J = 12.6 Hz), 2.58 (dd, 1H, J = 12.1, J' = 3.2 Hz), 3.44 (m, 1H), 3.48 (dd, 1H, J = 8.4, J' =

4.4 Hz), 3.74 (m, 1H), 4.19 (d, 1H, J = 2.8 Hz, OH), 4.32 (d, 1H, J = 6.1 Hz, OH), 4.35 (dd, 1H, J = 8.6, J' = 3.9 Hz), 4.51 (d, 1H, J = 4.3 Hz, OH), 5.14 (d, 1H, J = 3.9 Hz, OH), 7.22–7.27 (m, 3H), 7.31 (m, 2H). ^{13}C NMR δ 11.39 (CH_3), 12.47 (CH_3), 13.36 (CH_3), 20.79 (CH_2), 23.28 (CH_2), 26.82 (CH_2), 27.22 (CH_2), 36.29 (CH), 37.02 (CH), 39.14 (C), 41.84 (C), 42.03 (CH_2), 45.95 (CH_2), 50.28 (CH), 51.88 (CH), 52.85 (CH), 55.95 (CH), 67.10 (CH), 67.49 (CH), 75.16 (CH), 76.30 (CH), 127.01 (2 × CH), 127.21 (CH), 128.07 (2 × CH), 143.28 (C), 211.57 (C). One CH_2 covered by a DMSO multiplet. HRMS: (API+) calculated for $C_{29}H_{43}O_5$ ($[M + H]^+$) 471.3110, Found 471.3108. Anal. Calcd for $C_{29}H_{42}O_5$: C, 74.01; H, 8.99. Found: C, 73.95; H, 9.06%.

(22*R*,23*R*)-2α,3α,22,23-Tetrahydroxy-23-(4-fluorophenyl)-24-nor-5α-cholan-6-one (9c). The general procedure for dihydroxylation of **9b** and chromatography on silica (MeOH/$CHCl_3$ – 1/16) afforded 149 mg (80%) of the title compound **9c** as a white solid: m. p. 277–279 °C (EtOH), IR ν (cm^{-1}) 3251, 2937, 1709, 1607, 1513. ^{1}H NMR (DMSO-d_6) δ 0.33, 0.60 (both s, 3H, CH_3), 0.84 (d, 3H, J = 6.7 Hz, CH_3), 1.80 (m, 1H), 1.85 (m, 1H), 1.98 (dd, 1H, J = 13.0, J' = 4.8 Hz), 2.07 (t, 1H, J = 12.6 Hz), 2.58 (dd, 1H, J = 12.2, J' = 3.1 Hz), 3.42–3.47 (m, 2H), 3.74 (m, 1H), 4.19 (d, 1H, J = 2.4 Hz, OH), 4.32 (d, 1H, J = 6.1 Hz, OH), 4.37 (dd, 1H, J = 8.7, J' = 3.5 Hz), 4.54 (d, 1H, J = 4.3 Hz, OH), 5.14 (d, 1H, J = 4.0 Hz, OH), 7.14 (m, 2H), 7.30 (m, 2H). ^{13}C NMR δ 11.42 (CH_3), 12.44 (CH_3), 13.38 (CH_3), 20.80 (CH_2), 23.30 (CH_2), 26.84 (CH_2), 27.28 (CH_2), 36.35 (CH), 37.04 (CH), 39.15 (C), 41.86 (C), 42.06 (CH_2), 45.97 (CH_2), 50.31 (CH), 51.89 (CH), 52.88 (CH), 55.97 (CH), 67.11 (CH), 67.51 (CH), 74.40 (CH), 76.34 (CH), 114.84 (d, J = 21.6 Hz, 2 × CH), 128.86 (d, J = 8.4 Hz, 2 × CH), 139.58 (d, J = 2.4 Hz, C), 161.26 (d, J = 242.3 Hz, C), 211.62 (C). One CH_2 covered by a DMSO multiplet. ^{19}F NMR $\{^1H\}$ δ −115.37 (s, 1F). HRMS: (API+) calculated for $C_{29}H_{42}FO_5$ ($[M + H]^+$) 489.3016, Found 489.3017. Anal. Calcd for $C_{29}H_{41}FO_5$: C, 71.28; H, 8.46. Found: C, 71.88; H, 8.55%.

(22*R*,23*R*)-2α,3α,22,23-Tetrahydroxy-23-(4-chlorophenyl)-24-nor-5α-cholan-6-one (10c). The general procedure for dihydroxylation of **10b** and chromatography on silica (MeOH/$CHCl_3$ – 1/16) afforded 152 mg (82%) of the title compound **10c** as a white solid: m. p. 251–253 °C (EtOH), IR ν (cm^{-1}) 3220, 2940, 1712, 1598, 1494. ^{1}H NMR (DMSO-d_6) δ 0.34, 0.60 (both s, 3H, CH_3), 0.85 (d, 3H, J = 6.7 Hz, CH_3), 1.80 (m, 1H), 1.85 (m, 1H), 1.98 (dd, 1H, J = 13.0, J' = 4.8 Hz), 2.07 (t, 1H, J = 12.6 Hz), 2.58 (dd, 1H, J = 12.2, J' = 3.4 Hz), 3.42–3.48 (m, 2H), 3.74 (m, 1H), 4.19 (d, 1H, J = 2.4 Hz, OH), 4.33 (d, 1H, J = 6.1 Hz, OH), 4.37 (dd, 1H, J = 8.4, J' = 3.8 Hz), 4.58 (d, 1H, J = 4.6 Hz, OH), 5.20 (d, 1H, J = 3.8 Hz, OH), 7.29 (m, 2H), 7.37 (m, 2H). ^{13}C NMR δ 11.46 (CH_3), 12.49 (CH_3), 13.38 (CH_3), 20.80 (CH_2), 23.30 (CH_2), 26.84 (CH_2), 27.29 (CH_2), 36.47 (CH), 37.05 (CH), 39.15 (C), 41.87 (C), 42.07 (CH_2), 45.98 (CH_2), 50.31 (CH), 51.89 (CH), 52.89 (CH), 55.97 (CH), 67.12 (CH), 67.51 (CH), 74.44 (CH), 76.21 (CH), 128.10 (2 × CH), 128.87 (2 × CH), 131.52 (C), 142.41 (C), 211.62 (C). One CH_2 covered by a DMSO multiplet. HRMS: (API+) calculated for $C_{29}H_{42}ClO_5$ ($[M + H]^+$) 505.2721, Found 505.2723. Anal. Calcd for $C_{29}H_{41}ClO_5$: C, 68.96; H, 8.18. Found: C, 68.90; H, 8.28%.

(22*R*,23*R*)-2α,3α,22,23-Tetrahydroxy-23-(4-bromophenyl)-24-nor-5α-cholan-6-one (11c). The general procedure for dihydroxylation of **11b** and chromatography on silica (MeOH/$CHCl_3$ – 1/16) afforded 146 mg (80%) of the title compound **11c** as a white solid: m. p. 247–249 °C (i-PrOH), IR ν (cm^{-1}) 3237, 2941, 1709, 1600, 1490. ^{1}H NMR (DMSO-d_6) δ 0.34, 0.60 (both s, 3H, CH_3), 0.84 (d, 3H, J = 6.7 Hz, CH_3), 1.79 (m, 1H), 1.87 (m, 1H), 1.98 (dd, 1H, J = 13.0, J' = 4.6 Hz), 2.07 (t, 1H, J = 12.5 Hz), 2.58 (dd, 1H, J = 12.2, J' = 3.1 Hz), 3.42–3.47 (m, 2H), 3.74 (m, 1H), 4.19 (d, 1H, J = 2.8 Hz, OH), 4.33 (d, 1H, J = 6.1 Hz, OH), 4.36 (dd, 1H, J = 8.6, J' = 3.8 Hz), 4.58 (d, 1H, J = 4.3 Hz, OH), 5.20 (d, 1H, J = 3.8 Hz, OH), 7.23 (d, 2H, J = 8.6 Hz, 2 × Ar–H), 7.51 (d, 2H, J = 8.6 Hz, 2 × Ar–H). ^{13}C NMR δ 11.49 (CH_3), 12.50 (CH_3), 13.38 (CH_3), 20.81 (CH_2), 23.31 (CH_2), 26.84 (CH_2), 27.30 (CH_2), 36.50 (CH), 37.06 (CH), 39.15 (C), 41.87 (C), 42.08 (CH_2), 45.99 (CH_2), 50.32 (CH), 51.89 (CH), 52.89 (CH), 55.98 (CH), 67.13 (CH), 67.52 (CH), 74.51 (CH), 76.16 (CH), 120.08 (C), 129.26 (2 × CH), 131.01 (2 × CH), 142.83 (C), 211.62 (C). One CH_2 covered by a DMSO multiplet. HRMS: (API+) calculated for $C_{29}H_{42}{}^{79}BrO_5$ ($[M + H]^+$) 549.2216, Found 549.2216. Anal. Calcd for $C_{29}H_{41}BrO_5$: C, 63.38; H, 7.52. Found: C, 63.29; H, 7.55%.

(22*R*,23*R*)-2α,3α,22,23-Tetrahydroxy-23-(4-iodophenyl)-24-nor-5α-cholan-6-one (12c). The general procedure for dihydroxylation of **12b** and chromatography on silica (MeOH/$CHCl_3$ – 1/16) afforded 150 mg (83%) of the title compound **12c** as a white solid: m. p. 253–255 °C (EtOH), IR ν (cm^{-1}) 3193, 2943, 1710, 1590. ^{1}H NMR (DMSO-d_6) δ 0.35, 0.60 (both s, 3H, CH_3), 0.84 (d, 3H, J = 6.7 Hz, CH_3), 1.79 (m, 1H), 1.86 (m, 1H), 1.98 (dd, 1H, J = 13.2, J' = 4.9 Hz), 2.07 (t, 1H, J = 12.5 Hz), 2.58 (dd, 1H, J = 12.2, J' = 3.4 Hz), 3.41–3.48 (m, 2H), 3.74 (m, 1H), 4.19 (d, 1H, J = 2.8 Hz, OH), 4.32 (d, 1H, J = 6.1 Hz, OH), 4.34 (dd, 1H, J = 8.6, J' = 3.7 Hz), 4.56 (d, 1H, J = 4.3 Hz, OH), 5.18 (d, 1H, J = 3.8 Hz, OH), 7.09 (m, 2H, 2 × Ar–H), 7.67 (m, 2H, 2 × Ar–H). ^{13}C NMR δ 11.49 (CH_3), 12.48 (CH_3), 13.36 (CH_3), 20.80 (CH_2), 23.29 (CH_2), 26.83 (CH_2), 27.28 (CH_2), 36.51 (CH), 37.03 (CH), 39.14 (C), 41.85 (C), 42.05 (CH_2), 45.96 (CH_2), 50.30 (CH), 51.87 (CH), 52.86 (CH), 55.95 (CH), 67.11 (CH), 67.49 (CH), 74.60 (CH), 76.07 (CH), 92.95 (C), 129.40 (2 × CH), 136.83 (2 × CH), 143.19 (C), 211.57 (C). One CH_2 covered by a DMSO multiplet. HRMS: (API+) calculated for $C_{29}H_{42}IO_5$ ($[M + H]^+$) 597.2077, Found 597.2076. Anal. Calcd for $C_{29}H_{41}IO_5$: C, 58.39; H, 6.93. Found: C, 58.32; H, 7.01%.

(22*R*,23*R*)-2α,3α,22,23-Tetrahydroxy-23-(4-nitrophenyl)-24-nor-5α-cholan-6-one (13c). The general procedure for dihydroxylation of **13b** and chromatography on silica (MeOH/$CHCl_3$ – 1/16) afforded 144 mg (78%) of the title compound **13c** as a white solid: m. p. 244–246 °C (EtOH), IR ν (cm^{-1}) 3180, 2940, 1711, 1605, 1523, 1349. ^{1}H NMR (DMSO-d_6) δ 0.34, 0.59 (both s, 3H, CH_3), 0.87 (d, 3H, J = 6.7 Hz, CH_3), 1.80 (m, 1H), 1.86 (m, 1H), 1.97 (dd, 1H, J = 13.2, J' = 4.6 Hz), 2.06 (t, 1H, J = 12.5 Hz), 2.58 (dd, 1H, J = 12.2, J' = 3.4 Hz), 3.45 (m, 1H), 3.50 (m, 1H), 3.74 (m, 1H), 4.19 (d, 1H, J = 2.8 Hz, OH), 4.33 (d, 1H, J = 6.1 Hz, OH), 4.53 (dd, 1H, J = 7.9, J' = 3.5 Hz), 4.74 (d, 1H, J = 4.6 Hz, OH), 5.47 (d, 1H, J = 3.5 Hz, OH), 7.57 (m, 2H), 8.19 (m, 2H). ^{13}C NMR δ 11.46 (CH_3), 12.64 (CH_3), 13.37 (CH_3),

20.80 (CH_2), 23.30 (CH_2), 26.83 (CH_2), 27.32 (CH_2), 36.77 (CH), 37.03 (CH), 39.14 (C), 41.86 (C), 42.09 (CH_2), 45.97 (CH_2), 50.31 (CH), 51.92 (CH), 52.87 (CH), 55.95 (CH), 67.11 (CH), 67.51 (CH), 74.46 (CH), 76.09 (CH), 123.31 (2 × CH), 128.25 (2 × CH), 146.62 (C), 151.53 (C), 211.61 (C). One CH_2 covered by a DMSO multiplet. HRMS: (ESI-) calculated for $C_{29}H_{41}NO_7$ ($[M^{\bullet}]^-$) 515.2883, Found 515.2888. Anal. Calcd for $C_{29}H_{41}NO_7$: C, 67.55; H, 8.01. Found: C, 67.45; H, 8.09%.

(22*R*,23*R*)-2α,3α,22,23-Tetrahydroxy-23-(4-methylphenyl)-24-nor-5α-cholan-6-one (14c). The general procedure for dihydroxylation of **14b** and chromatography on silica (MeOH/$CHCl_3$ – 1/16) afforded 145 mg (78%) of the title compound **14c** as a white solid: m. p. 271–272 °C (EtOH), IR ν (cm^{-1}) 3215, 2937, 1710, 1610, 1516. ^{1}H NMR (DMSO-d_6) δ 0.32, 0.59 (both s, 3H, CH_3), 0.84 (d, 3H, J = 6.7 Hz, CH_3), 1.80 (m, 1H), 1.86 (m, 1H), 1.98 (dd, 1H, J = 13.2, J' = 4.6 Hz), 2.07 (t, 1H, J = 12.5 Hz), 2.27 (s, 3H, CH_3), 2.58 (dd, 1H, J = 12.2, J' = 3.4 Hz), 3.42–3.48 (m, 2H), 3.74 (m, 1H), 4.19 (d, 1H, J = 2.8 Hz, OH), 4.31 (dd, 1H, J = 8.6, J' = 3.8 Hz), 4.34 (d, 1H, J = 6.1 Hz, OH), 4.46 (d, 1H, J = 4.4 Hz, OH), 5.00 (d, 1H, J = 3.8 Hz, OH), 7.10–7.15 (m, 4H). ^{13}C NMR δ 11.50 (CH_3), 12.44 (CH_3), 13.40 (CH_3), 20.83 (CH_2, CH_3), 23.32 (CH_2), 26.85 (CH_2), 27.28 (CH_2), 36.38 (CH), 37.07 (CH), 39.15 (C), 41.89 (C), 42.07 (CH_2), 46.00 (CH_2), 50.33 (CH), 51.90 (CH), 52.91 (CH), 56.00 (CH), 67.14 (CH), 67.52 (CH), 74.96 (CH), 76.36 (CH), 126.99 (2 × CH), 128.71 (2 × CH), 136.15 (C), 140.20(C), 211.68 (C). One CH_2 covered by a DMSO multiplet. HRMS: (API+) calculated for $C_{30}H_{45}O_5$ ($[M + H]^+$) 485.3267, Found 485.3270. Anal. Calcd for $C_{30}H_{44}O_5$: C, 74.34; H, 9.15. Found: C, 74.30; H, 9.20%.

(22*R*,23*R*)-2α,3α,22,23-Tetrahydroxy-23-(4-methoxyphenyl)-24-nor-5α-cholan-6-one (15c). The general procedure for dihydroxylation of **15b** and chromatography on silica (MeOH/$CHCl_3$ – 1/16) afforded 150 mg (81%) of the title compound **15c** as a white solid: m. p. 242–244 °C (EtOH), IR ν (cm^{-1}) 3197, 2934, 1710, 1616, 1513. ^{1}H NMR (DMSO-d_6) δ 0.33, 0.60 (both s, 3H, CH_3), 0.84 (d, 3H, J = 6.7 Hz, CH_3), 1.80 (m, 1H), 1.86 (m, 1H), 1.98 (dd, 1H, J = 13.2, J' = 4.6 Hz), 2.07 (t, 1H, J = 12.7 Hz), 2.58 (dd, 1H, J = 12.2, J' = 3.4 Hz), 3.42–3.49 (m, 2H), 3.72 (s, 3H, CH_3), 3.74 (m, 1H), 4.18 (d, 1H, J = 2.5 Hz, OH), 4.30 (dd, 1H, J = 8.6, J' = 3.8 Hz), 4.32 (d, 1H, J = 6.4 Hz, OH), 4.44 (d, 1H, J = 4.1 Hz, OH), 4.96 (d, 1H, J = 3.8 Hz, OH), 6.87 (m, 2H), 7.17 (m, 2H). ^{13}C NMR δ 11.48 (CH_3), 12.38 (CH_3), 13.37 (CH_3), 20.80 (CH_2), 23.30 (CH_2), 26.83 (CH_2), 27.26 (CH_2), 36.31 (CH), 37.04 (CH), 39.14 (C), 41.86 (C), 42.04 (CH_2), 45.97 (CH_2), 50.30 (CH), 51.89 (CH), 52.88 (CH), 54.97 (CH_3), 55.98 (CH), 67.11 (CH), 67.50 (CH), 74.59 (CH), 76.29 (CH), 113.43 (2 × CH), 128.12 (2 × CH), 135.17 (C), 158.29 (C), 211.59 (C). One CH_2 covered by a DMSO multiplet. HRMS: (API+) calculated for $C_{30}H_{45}O_6$ ($[M + H]^+$) 501.3218, Found 501.3216. Anal. Calcd for $C_{30}H_{44}O_6$: C, 71.97; H, 8.86. Found: C, 71.91; H, 8.97%.

(22*R*,23*R*)-2α,3α,22,23-Tetrahydroxy-23-(4-isopropylphenyl)-24-nor-5α-cholan-6-one (16c). The general procedure for dihydroxylation of **16b** and chromatography on silica (MeOH/$CHCl_3$ – 1/16) afforded 146 mg (79%) of the title compound **16c** as a white solid: m. p. 247–249 °C (EtOH), IR ν (cm^{-1}) 3215, 2938, 1710, 1617, 1513. ^{1}H NMR (DMSO-d_6) δ 0.31, 0.59 (both s, 3H, CH_3), 0.84 (d, 3H, J = 6.7 Hz, CH_3), 1.17 (d, 6H, J = 6.9 Hz, 2 × CH_3), 1.77–1.86 (m, 2H), 1.98 (dd, 1H, J = 13.2, J' = 4.6 Hz), 2.07 (t, 1H, J = 12.5 Hz), 2.58 (dd, 1H, J = 12.1, J' = 3.2 Hz), 2.86 (septet, 1H, J = 6.9 Hz), 3.44 (m, 1H), 3.50 (dt, 1H, J = 8.3, J' = 4.1 Hz), 3.74 (m, 1H), 4.19 (d, 1H, J = 2.8 Hz, OH), 4.31 (dd, 1H, J = 8.6, J' = 3.8 Hz), 4.33 (d, 1H, J = 6.1 Hz, OH), 4.44 (d, 1H, J = 4.0 Hz, OH), 5.00 (d, 1H, J = 3.8 Hz, OH), 7.17 (s, 4H). ^{13}C NMR δ 11.46 (CH_3), 12.50 (CH_3), 13.37 (CH_3), 20.80 (CH_2), 23.32 (CH_2), 23.92 (CH_3), 24.02 (CH_3), 26.85 (CH_2), 27.20 (CH_2), 33.11 (CH), 36.33 (CH), 37.05 (CH), 39.14 (C), 41.87 (C), 42.07 (CH_2), 45.98 (CH_2), 50.32 (CH), 51.97 (CH), 52.89 (CH), 55.96 (CH), 67.12 (CH), 67.52 (CH), 75.05 (CH), 76.18 (CH), 125.97 (2 × CH), 127.04 (2 × CH), 140.66 (C), 147.21 (C), 211.62 (C). One CH_2 covered by a DMSO multiplet. HRMS: (API+) calculated for $C_{32}H_{49}O_5$ ($[M + H]^+$) 513.3580, Found 513.3585. Anal. Calcd for $C_{32}H_{48}O_5$: C, 74.96; H, 9.44. Found: C, 74.89; H, 9.51%.

(22*R*,23*R*)-2α,3α,22,23-Tetrahydroxy-23-(4-cyanophenyl)-24-nor-5α-cholan-6-one (17c). The general procedure for dihydroxylation of **17b** and chromatography on silica (MeOH/$CHCl_3$ – 1/16) afforded 139 mg (75%) of the title compound **17c** as a white solid: m. p. 260–262 °C (EtOH), IR ν (cm^{-1}) 3198, 2936, 2230, 1706, 1611, 1510. ^{1}H NMR (DMSO-d_6) δ 0.33, 0.59 (both s, 3H, CH_3), 0.85 (d, 3H, J = 6.7 Hz, CH_3), 1.74–1.88 (m, 2H), 1.98 (dd, 1H, J = 13.2, J' = 4.8 Hz), 2.06 (t, 1H, J = 12.5 Hz), 2.57 (dd, 1H, J = 12.2, J' = 3.4 Hz), 3.46–3.50 (m, 2H), 3.74 (m, 1H), 4.20 (d, 1H, J = 2.4 Hz, OH), 4.34 (d, 1H, J = 6.1 Hz, OH), 4.46 (dd, 1H, J = 8.1, J' = 4.3 Hz), 4.69 (d, 1H, J = 4.3 Hz, OH), 5.04 (d, 1H, J = 4.0 Hz, OH), 7.48 (d, 2H, J = 8.3 Hz, 2 × Ar–H), 7.79 (d, 2H, J = 8.3 Hz, 2 × Ar–H). ^{13}C NMR δ 11.47 (CH_3), 12.64 (CH_3), 13.41 (CH_3), 20.83 (CH_2), 23.33 (CH_2), 26.87 (CH_2), 27.33 (CH_2), 36.71 (CH), 37.08 (CH), 39.14 (C), 41.89 (C), 42.11 (CH_2), 46.00 (CH_2), 50.35 (CH), 51.94 (CH), 52.91 (CH), 55.98 (CH), 67.15 (CH), 67.54 (CH), 74.74 (CH), 76.12 (CH), 109.86 (C), 119.03 (C), 128.05 (2 × CH), 132.14 (2 × CH), 149.34 (C), 211.68 (C). One CH_2 covered by a DMSO multiplet. HRMS: (API+) calculated for $C_{30}H_{42}NO_5$ ($[M + H]^+$) 496.3063, Found 496.3064. Anal. Calcd for $C_{30}H_{41}NO_5$: C, 72.70; H, 8.34. Found: C, 72.69; H, 8.39%.

Molecular docking

Docking was performed to predict the conformation and energy ranking between the BRI1 receptor (PDB ID: 3RGZ) and the steroid molecule. The docking studies were carried out using AutoDock Vina 1.05.[31] All 3D structures of BRI1 ligands were obtained with Marvin 5.10.3 [32] software, which can be used for drawing, displaying and characterization of chemical structures, substructures and reactions. Ligands were prepared as derivatives of the natural ligand brassinolide (BLD). Polar hydrogens were added to all ligands and proteins with the AutoDock Tools (ADT)[16] program prior to docking with the Autodock Vina program. A grid box with a size of 40 Å was centered on the active site of the protein. The exhaustiveness parameter was set to 20 (default 8). After docking, we compared the docked ligand with brassinolide crystal-like poses and the best crystal-like poses of each ligand were analyzed.

The pea inhibition biotest

Pea seedlings (*Pisum arvense* L. sort Arvica) germinating for 2 days were selected for uniformity from a large population and then transferred into pots containing perlite and 1/10 diluted Hoagland solution (half concentration, pH 5.7). After 24 h in a dark cultivation room (24 °C, humidity 75%) the seedlings were treated with different amounts of tested compounds in 5 μl fractionated lanolin. The substances were applied as microdrops to the scar left after the removal of the bract. The control plants were treated with lanolin alone. At least seven plants were used for each experiment and the assays were repeated at least three times. The inhibition of etiolated pea stems was measured after 4 days and the difference in length between the treated and control plants provided a measure of activity. For each treatment, 8 seedlings were analyzed in two biological replicates. The mean values were subjected to the statistical analysis using the Student's *t* test.

Determination of ethylene production

For measurement of ethylene production, pea seedlings (8 plants/tested amount of substance) were placed in a 0.5 L glass container for 24 h in the dark. One milliliter of headspace gas from the chamber was withdrawn for each measurement and injected into a gas chromatograph (Agilent Technologies, GC Systems) equipped with a flame ionic detector (FID) and a capillary column (HP-AL/S stationary phase, 15 μm, i.d. = 0.535). The chromatographic analytical parameters were as follows: column temperature: 150 °C; detector temperature: 220 °C; and helium was used as a carrier gas. The area under the resultant peak (*y*-axis) *versus* sensitivity (*x*-axis; nL mL^{-1}) represented a quantitative measure of ethylene concentration. The measurements were done in triplicate and data were statistically analyzed using the Student's *t* test.

Arabidopsis brassinosteroid sensitivity assays

Arabidopsis thaliana L. (Heyhn.) (Columbia ecotype, Col-0; referred to as Arabidopsis) seedlings were stratified for 2 d at 4 °C and germinated on vertical half-strength Murashige and Skoog (1% w/v sucrose) agar plates with different concentrations of BL (Fuji Chemical Industries) and BR derivatives at 22 °C in a 16 h/8 h light–dark cycle for 5 d. For the hypocotyl assay, after stratification, the plants were exposed to light for 6 h and grown in the dark for 5 days. Roots and hypocotyls were then straightened on solid media plates, scanned with an Epson high-resolution scanner and the entire root and hypocotyl length measured with ImageJ (http://rsbweb.nih.gov/ij/). For each treatment, more than 25 seedlings were analyzed in two biological repeats. *P* values were calculated with a two-tailed Student *t*-test using Excel software.

BES-1 dephosphorylation assay

For BES1 dephosphorylation studies, thirty to sixty 5-day-old Arabidopsis seedlings grown on BL and new BR derivatives in continuous light were used. DMSO was used as the control solvent. The protein extraction and western blot analysis were carried out as previously described.[33] Endogenous BES1 was detected using rabbit polyclonal anti-BES1 antibodies (1 : 1000)[34] and HRP-conjugated anti-rabbit antibodies (1 : 10 000; NA934, GE Healthcare). Signals were detected using ECL (ECL plus, GE Healthcare).

Cell cultures

The screening cell lines: T-lymphoblastic leukemia CEM; breast carcinoma MCF7 (estrogen-sensitive); cervical carcinoma cell line HeLa; and human foreskin fibroblasts BJ were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium, Sigma, MO, USA). Media used were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1% penicillin–streptomycin. The cell lines were maintained under standard cell culture conditions at 37 °C and 5% CO_2 in a humid environment. The cells were subcultured twice or thrice a week using the standard trypsinization procedure.

Calcein AM cytotoxicity assay

Suspensions with approximately 1.0×10^5 cells per mL were distributed in 96-well microtiter plates and after 24 h of stabilization the BR analogues tested were added at the desired concentrations in DMSO. Control cultures were treated with DMSO alone, and the final concentration of DMSO in the reaction mixture never exceeded 0.6%. In most cases, six serial 3-fold dilutions of the test substances were added at time zero in 20 μl aliquots to the microtiter plate wells and the highest final concentration in the wells was 50 μM. After incubation for 72 h, calcein AM solution (2 μM, Molecular Probes) was added and the cells were incubated for an additional hour. The fluorescence of the viable cells was then quantified using a Fluoroskan Ascent instrument (Labsystems, Finland). The percentage of the surviving cells in each well was calculated by dividing the intensity of the fluorescence signals from the exposed wells by the intensity of signals from the control wells and multiplying by 100. These ratios were then used to construct dose–response curves from which IC_{50} values, the concentrations of the respective compounds that were lethal to 50% of the tumor cells, were calculated.

Conclusions

Several novel brassinosteroid 23-phenyl analogues were synthesized based on molecular docking into the BRI1 receptor. The introduction of a phenyl group with no or small non-polar substituents (fluorine, chlorine, methyl) resulted in new compounds with plant growth promoting activities comparable with natural brassinosteroids. The results of biological screenings showed that molecular docking into BRI1 is a powerful tool for prediction and design of new compounds with strong brassinosteroid activities. New active compounds might be good candidates for potential application in agriculture to improve growth and yield or to increase the resistance of plants against various biotic and abiotic stresses. Recent

progress in the chemical synthesis also leads to overcoming economic restrictions, which are currently major constraints for using BRs at a large scale in the field.

Author contribution

M. Kvasnica – chemical synthesis; J. Oklestkova, P. Kořínková, J. Mikulik, M. Strnad, Q. Lu, E. Russinova – screening of biological activities on plants; L. Rárová – screening of cytotoxicity; M. Budesinsky, T. Beres – analysis of compounds prepared; V. Bazgier, K. Berka – molecular docking.

Acknowledgements

We thank Yanhai Yin for sharing BES1 antibodies. This work was supported by projects of the Ministry of Education, Youth and Sports CR NPUI LO1204, by grants GJ15-08202Y and 14-27669P of the Grant Agency of the Czech Republic. This work was also supported by student projects IGA_PrF_2016_018 and IGA_PrF_2016_028 of the Palacky University in Olomouc. This work was supported by the ELIXIR CZ research infrastructure project (MEYS Grant No: LM2015047) including access to computing and storage facilities. Qing Lu is indebted to the Chinese Scholarship Council (CSC) for his predoctoral fellowship.

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Steroids 127 (2017) 46–55

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Synthesis of novel aryl brassinosteroids through alkene cross-metathesis and preliminary biological study

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ARTICLE INFO

Keywords:
Brassinosteroids
Cross-metathesis
Organic synthesis
Molecular docking
BRI1 receptor kinase
Plant bioassays

ABSTRACT

A series of phenyl analogues of brassinosteroids was prepared via alkene cross-metathesis using commercially available styrenes and 24-nor-5α-chola-2,22-dien-6-one. All derivatives were successfully docked into the active site of BRI1 using AutoDock Vina. Plant growth promoting activity was measured using the pea inhibition biotest and Arabidopsis root sensitivity assay and then was compared with naturally occuring brassinosteroids. Differences in the production of plant hormone ethylene were also observed in etiolated pea seedlings after treatment with the new and also five known brassinosteroid phenyl analogues. Antiproliferative activity was also studied using normal human fibroblast and human cancer cell lines.

1. Introduction

Brassinosteroids (BRs, Fig. 1) represent a large group of plant steroids with more than 70 structurally and functionally related compounds [1]. BRs have been found at low concentrations throughout the plant kingdom, widely distributed in higher and lower plants, and have been detected in various plant parts such as pollen, seeds, leaves, stems, roots, and flowers. They are essential for many aspects of plant growth and development, such as cell division, elongation and differentiation, pollen tube growth, seed germination, regulation of gene expression, enzyme activation and photosynthesis [2–5]. At the molecular level, BRs change the gene expression and the metabolism of nucleic acids and proteins. BRs have structures similar to those of animal steroid hormone. Unlike animals, plants perceive steroids at cell membrane, using the membrane-integral receptor kinase brassinosteroid insensitive 1 (BRI1) [6–8]. The encoded protein, BRI1, belongs to a large family of plant LRR (leucine-rich repeat) receptor-like kinases, characterized by an extracellular LRR domain, a single-pass transmembrane segment and a cytoplasmic kinase domain. BRI1 has been established as an authentic brassinosteroid receptor by genetic and biochemical investigations [9]. Crystal structures of BRI1 in both free (PDB ID: 3RIZ, 3RGX), and brassinolide-bound (PDB ID: 3RJ0, 3BRZ), forms are available, following independent X-ray diffraction structural determinations by two groups [9,10]. The structure of the ligand-binding domain resembles a superhelix of 25 twisted LRRs. A 70-amino acid island domain between LRRs 21 and 22 folds back into the interior of the superhelix, creating a surface pocket where the brassinosteroids bind. These recently published structures of *Arabidopsis thaliana* BRI1 enable the rational design of brassinosteroid-like antagonists and agonists. Recent studies [11,12] have indicated that molecular docking is a powerful tool to predict how effective incorporation of different functional groups into brassinosteroid skeleton is and to design new types of BRs with biological activities comparable to natural BRs [11].

The aim of our study is related to the synthesis of new aryl analogues of BRs by alkene cross-metathesis and to study of their biological properties. Alkene cross-metathesis was chosen for preparation of all aryl analogues as an efficient method for construction of the new side chains using different commercially available substituted styrenes. The biological activities of newly prepared derivatives were evaluated using plant bioassays (pea inhibition biotest and Arabidopsis root sensitivity bioassay) and Calcein AM cytotoxicity assay. All derivative structures were subjected to docking studies using AutoDock Vina [13] in order to analyze the results with theoretical studies.

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http://dx.doi.org/10.1016/j.steroids.2017.08.010
Received 14 June 2017; Received in revised form 18 July 2017; Accepted 9 August 2017
Available online 31 August 2017
0039-128X/

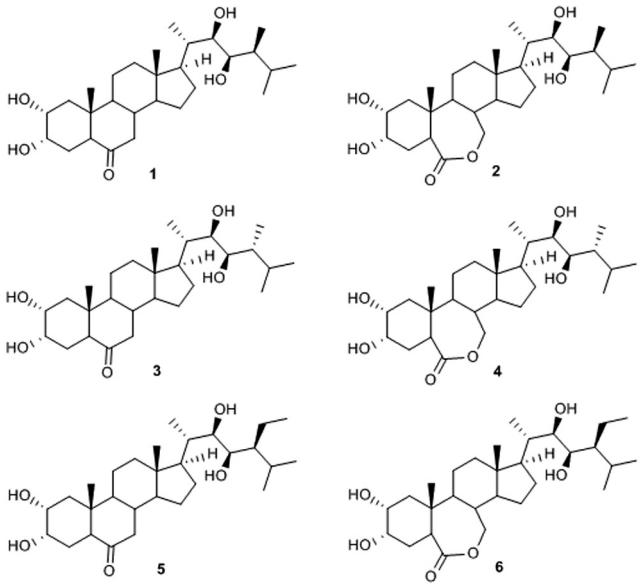


Fig. 1. Structures of most common natural brassinosteroids; castasterone (1), brassinolide (2), 24-epicastasterone (3), 24-epibrassinolide (4), 28-homocastasterone (5), 28-homobrassinolide (6).

2. Experimental

2.1. General methods

The melting points were determined on a Stuart SMP30 instrument (Bibby Scientific Ltd., UK). Elemental analyses were performed using an EA 1108 elemental analyzer (Fison Instruments); the values (C, H, N) agreed with the calculated values within acceptable limits. The infrared spectra were recorded on a Thermo Scientific Nicolet spectrometer iZ10 using the ATR technique. The wave numbers are given in cm^{-1}. The NMR spectra were taken on a JEOL JNM-ECA 500 (JEOL, Tokyo, Japan; 1H, 500 MHz; 13C, 125 MHz) spectrometer equipped with a 5 mm JEOL Royal probe. 1H NMR and 13C NMR chemical shifts (δ) were calibrated using tetramethylsilane (TMS, 1H δ = 0 ppm) or solvents: CDCl3 (1H δ = 7.26 ppm, 13C δ = 77.00 ppm) or DMSO-d_6 (1H δ = 2.46 ppm, 13C δ = 40.00 ppm). Chemical shifts are given in ppm (δ-scale), coupling constants (J) in Hz. All values were obtained by first-order analysis. For API HRMS analysis, the samples were dissolved in chloroform (or chloroform: methanol; 1:1; v/v, in case of hydroxylated compounds) to a concentration 10 μg.mL^{-1}. The ASAP (Atmospheric Solids Analysis Probe) was dipped into the sample solution, placed into the ion source and analysed in fullscan mode. The source of the Synapt G2-Si Mass Spectrometer (Waters, Manchester, UK) was operated in positive ionisation mode (ASAP+), if not stated otherwise, at source temperature of 120 °C. The Corona needle current was kept at 5 μA and the collision energy at value 4. The probe temperature was ramped up from 50 °C to 600 °C in 3 min. Data were acquired from 50 to 1000 Da with 1.0 s scan time in High Resolution Mode. The data were processed using the Masslynx 4.1 software (Waters). Mass accuracy of 1 ppm or less was achieved with the described instrumentation for all compounds. Merck silica gel Kieselgel 60 (230–400 mesh) was used for column chromatography. The HPLC system consisted of a Waters semi-preparative HPLC system including quaternary pump, liquid handler, UV–VIS and ELSD detectors. The semi preparative column was filled with silica gel. Reagents and solvents were purchased from Sigma–Aldrich and were not purified.

2.1.1. General procedure for cross metathesis

Hoveyda-Grubbs 2nd generation catalyst (19 mg; 0.03 mmol) was added to a solution of dien **7** (100 mg; 0.31 mmol) and styrene (2.48 mmol) in dichloroethane (5 mL). The reaction mixture was heated at 80 °C for 5 h. Then, another portion of H-G catalyst (19 mg; 0.03 mmol) was added and the reaction mixture was heated at 80 °C for additional 5 h. Then, the solvent was evaporated and crude solid was purified by column chromatography on silica gel (mobile phase – 3% ethyl acetate in cyclohexane, R_f of products 0.18–0.25). In some cases, stated in each experiment, HPLC had to be used due to very close retention time of product and starting material (mobile phase – 0.5% ethyl acetate in cyclohexane).

2.1.2. (22E)-23-phenyl-24-nor-5α-chola-2,22-dien-6-one (8a)

The general procedure with styrene afforded 120 mg (81%) of the title compound **8** as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.72, 0.74 (both s, 3H, CH_3), 1.14 (d, 3H, J = 6.4 Hz, CH_3), 1.69–1.80 (m, 2H), 1.96–2.04 (m, 4H), 2.07 (dt, 1H, J = 12.6, J' = 3.3 Hz), 2.23–2.31 (m, 2H), 2.34–2.37, (m, 2H), 5.57 (m, 1H, H-3), 5.69 (m, 1H, H-2), 6.06 (dd, 1H, J = 15.9, J' = 8.9 Hz, H-22), 6.30 (d, 1H, J = 15.9 Hz, H-23), 7.19 (m, 1H, Ar-H), 7.27–7.35 (m, 4H, 4 × Ar-H). ^{13}C NMR δ 12.19 (C-18), 13.50 (C-19), 20.38 (C-21), 21.10, 21.70, 23.92, 28.21, 37.68, 39.35, 39.37, 40.04, 40.39, 42.92, 46.96, 53.40, 53.83, 55.83, 56.75, 124.49 (C-3), 124.95 (C-2), 125.92 (2 × C), 126.72, 127.42 (C-23), 128.45 (2 × C), 136.94 (C-22), 137.98, 211.98 (C-6). Spectral data in agreement with literature [11].

2.1.3. (22E)-23-(2-fluorophenyl)-24-nor-5α-chola-2,22-dien-6-one (8b)

The general procedure with *o*-fluorostyrene afforded 84 mg (65%) of the title compound **9a** as a colorless oil: IR ν (cm^{-1}) 2930, 1702, 1655, 1593, 1560, 965. ^{1}H NMR ($CDCl_3$) δ 0.73, 0.75 (both s, 3H, CH_3); 1.15 (d, 3H, J = 6.7 Hz, CH_3); 1.72–1.81 (m, 2H); 1.97–2.06 (m, 4H); 2.08 (dt, 1H, J = 12.6, J' = 3.5 Hz); 2.24–2.33 (m, 2H); 2.34–2.38 (m, 2H); 5.58 (m, 1H, H-3); 5.70 (m, 1H, H-2); 6.14 (dd, 1H, J = 15.9, J' = 8.6 Hz, H-22); 6.47 (d, 1H, J = 15.9 Hz, H-23); 7.01 (ddd, J = 10.9, J' = 7.8, J'' = 0.9 Hz, Ar-H); 7.07 (td, 1H, J = 7.8, J' = 1.2 Hz, Ar-H); 7.16 (m, 1H, Ar-H); 7.41 (td, 1H, J = 7.8, J' = 1.8 Hz, Ar-H). ^{13}C NMR δ 12.19 (C-18), 13.48 (C-19), 20.27 (C-21), 21.08, 21.70, 23.91, 28.17, 37.65, 39.32, 39.35, 40.01, 40.76, 42.92, 46.93, 53.37, 53.80, 55.68, 56.71, 115.56 (d, J = 22.8 Hz), 119.79 (d, J = 3.6 Hz), 123.91 (d, J = 3.6 Hz), 124.48 (C-3), 124.94 (C-2), 125.62 (d, J = 13.2 Hz), 126.92 (d, J = 3.6 Hz), 127.86 (d, J = 8.4 Hz), 139.50 (d, J = 3.6 Hz), 159.94 (d, J = 248.3 Hz, C-F), 211.90 (C-6). ^{19}F NMR {^{1}H} δ −118.83 (s, 1F). HRMS: (API+) calculated for $C_{29}H_{38}FO$ ($[M+H]^+$) 421.2907, Found 421.2910.

2.1.4. (22E)-23-(3-fluorophenyl)-24-nor-5α-chola-2,22-dien-6-one (8c)

The general procedure with *m*-fluorostyrene afforded 94 mg (73%) of the title compound **10a** as a colorless oil: IR ν (cm^{-1}) 2933, 1705, 1656, 1593, 1560, 966. ^{1}H NMR ($CDCl_3$) δ 0.73, 0.74 (both s, 3H, CH_3); 1.14 (d, 3H, J = 6.7 Hz, CH_3); 1.71–1.80 (m, 2H); 1.97–2.04 (m, 4H); 2.07 (dt, 1H, J = 12.5, J' = 3.2 Hz); 2.23–2.31 (m, 2H); 2.34–2.38 (m, 2H); 5.58 (m, 1H, H-3); 5.70 (m, 1H, H-2); 6.08 (dd, 1H, J = 15.7, J' = 8.7 Hz, H-22); 6.28 (d, 1H, J = 15.7 Hz, H-23); 6.88 (td, 1H, J = 8.3, J' = 2.6 Hz, Ar-H); 7.03 (m, 1H, Ar-H); 7.08 (b d, 1H, J = 8.3 Hz, Ar-H); 7.24 (td, 1H, J = 7.8, J' = 6.1 Hz, Ar-H). ^{13}C NMR δ 12.16 (C-18), 13.46 (C-19), 20.22 (C-21), 21.06, 21.68, 23.87, 28.16, 37.62, 39.30, 39.32, 39.98, 40.30, 42.90, 46.90, 53.33, 53.77, 55.67, 56.66, 112.25 (d, J = 21.6 Hz), 113.44 (d, J = 21.6 Hz), 121.81 (d, J = 2.4 Hz), 124.45 (C-3), 124.92 (C-2), 126.46 (d, J = 2.4 Hz), 129.79 (d, J = 8.4 Hz), 138.28, 140.35 (d, J = 7.2 Hz), 163.08 (d, J = 244.7 Hz, C-F), 211.82 (C-6). ^{19}F NMR {^{1}H} δ −113.78 (s, 1F). HRMS: (API+) calculated for $C_{29}H_{38}FO$ ($[M+H]^+$) 421.2907, Found 421.2910.

2.1.5. (22E)-23-(4-fluorophenyl)-24-nor-5α-chola-2,22-dien-6-one (8d)

The general procedure with *p*-fluorostyrene afforded 96 mg (75%) of the title compound **8** as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.72, 0.73 (both s, 3H, CH_3), 1.13 (d, 3H, J = 6.7 Hz, CH_3), 1.71–1.80 (m, 2H),

1.96–2.04 (m, 4H), 2.08 (dt, 1H, J = 12.5, J' = 3.2 Hz), 2.22–2.31 (m, 2H), 2.33–2.37, (m, 2H), 5.57 (m, 1H, H-3), 5.69 (m, 1H, H-2), 5.97 (dd, 1H, J = 15.7, J' = 8.7 Hz, H-22), 6.26 (d, 1H, J = 15.7 Hz, H-23), 6.97 (m, 2H, 2 × Ar-H), 7.27 (m, 2H, 2 × Ar-H). ^{13}C NMR δ 12.17 (C-18), 13.48 (C-19), 20.35 (C-21), 21.08, 21.69, 23.88, 28.21, 37.65, 39.32, 39.34, 40.01, 40.33, 42.89, 46.92, 53.36, 53.80, 55.79, 56.71, 115.25 (d, J = 20.4 Hz, 2 × C), 124.47 (C-3), 124.93 (C-2), 126.25 (C-23), 127.28 (d, J = 8.4 Hz, 2 × C), 134.07 (d, J = 3.6 Hz), 136.65 (d, J = 2.4 Hz, C-22), 161.79 (d, J = 244.7 Hz, C-F), 211.93 (C-6). Spectral data in agreement with literature [11].

*2.1.6. (22E)-23-(2-chlorophenyl)-24-nor-5α-chola-2,22-dien-6-one **(8e)***

The general procedure with *o*-chlorostyrene afforded 86 mg (64%) of the title compound **11a** as a colorless oil: IR ν (cm^{-1}) 2941, 1701, 1655, 1593, 1563, 964. ^{1}H NMR ($CDCl_3$) δ 0.73, 0.75 (both s, 3H, CH_3); 1.16 (d, 3H, J = 6.7 Hz, CH_3); 1.71–1.81 (m, 2H); 1.96–2.04 (m, 4H); 2.08 (dt, 1H, J = 12.5, J' = 3.2 Hz); 2.22–2.33 (m, 2H); 2.34–2.38 (m, 2H); 5.58 (m, 1H, H-3); 5.70 (m, 1H, H-2); 6.04 (dd, 1H, J = 15.6, J' = 8.9 Hz, H-22); 6.69 (d, 1H, J = 15.9 Hz, H-23); 7.13 (m, 1H, ΣJ = 16.8 Hz, Ar-H); 7.19 (m, 1H, ΣJ = 15.9 Hz, Ar-H); 7.33 (dd, 1H, J = 7.9, J' = 1.2 Hz, Ar-H); 7.47 (dd, 1H, J = 7.9, J' = 1.5 Hz, Ar-H). ^{13}C NMR δ 12.19 (C-18), 13.46 (C-19), 20.26 (C-21), 21.07, 21.68, 23.91, 28.17, 37.62, 39.30, 39.32, 39.98, 40.51, 42.93, 46.91, 53.34, 53.77, 55.63, 56.68, 123.80, 124.46 (C-3), 124.91 (C-2), 126.53, 126.64, 127.73, 129.52, 132.58, 136.00, 139.72, 211.87 (C-6). HRMS: (API+) calculated for $C_{29}H_{38}ClO$ ([M+H]$^+$) 437.2611, Found 437.2614.

*2.1.7. (22E)-23-(3-chlorophenyl)-24-nor-5α-chola-2,22-dien-6-one **(8f)***

The general procedure with *m*-chlorostyrene afforded 94 mg (70%) of the title compound **12a** as a colorless oil: IR ν (cm^{-1}) 2944, 1701, 1654, 1593, 1562, 964. ^{1}H NMR ($CDCl_3$) δ 0.72, 0.73 (both s, 3H, CH_3); 1.13 (d, 3H, J = 6.7 Hz, CH_3); 1.70–1.80 (m, 2H); 1.96–2.05 (m, 4H); 2.07 (dt, 1H, J = 12.5, J' = 3.2 Hz); 2.22–2.31 (m, 2H); 2.33–2.37 (m, 2H); 5.58 (m, 1H, H-3); 5.69 (m, 1H, H-2); 6.07 (dd, 1H, J = 15.9, J' = 8.9 Hz, H-22); 6.25 (d, 1H, J = 15.9 Hz, H-23); 7.14–7.22 (m, 3H, 3 × Ar-H); 7.31 (t, 1H, J = 1.7 Hz, Ar-H). ^{13}C NMR δ 12.18 (C-18), 13.48 (C-19), 20.24 (C-21), 21.08, 21.69, 23.89, 28.19, 37.64, 39.31, 39.33, 40.01, 40.39, 42.92, 46.92, 53.34, 53.79, 55.66, 56.68, 124.23, 124.47 (C-3), 124.94 (C-2), 126.22, 126.63, 127.74, 129.63, 134.36, 138.47, 139.85, 211.92 (C-6). HRMS: (API+) calculated for $C_{29}H_{38}ClO$ ([M+H]$^+$) 437.2611, Found 437.2615.

*2.1.8. (22E)-23-(4-chlorophenyl)-24-nor-5α-chola-2,22-dien-6-one **(8g)***

The general procedure with *p*-chlorostyrene afforded 100 mg (75%) of the title compound **8** as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.72, 0.73 (both s, 3H, CH_3), 1.13 (d, 3H, J = 6.7 Hz, CH_3), 1.70–1.80 (m, 2H), 1.96–2.04 (m, 4H), 2.07 (dt, 1H, J = 12.5, J' = 3.4 Hz), 2.22–2.30 (m, 2H), 2.34–2.37, (m, 2H), 5.57 (m, 1H, H-3), 5.69 (m, 1H, H-2), 6.03 (dd, 1H, J = 15.9, J' = 8.9 Hz, H-22), 6.27 (d, 1H, J = 15.9 Hz, H-23), 7.24 (s, 4H, 4 × Ar-H). ^{13}C NMR δ 12.19 (C-18), 13.50 (C-19), 20.27 (C-21), 21.09, 21.70, 23.91, 28.21, 37.66, 39.33, 39.36, 40.04, 40.39, 42.93, 46.94, 53.37, 53.82, 55.75, 56.72, 124.49 (C-3), 124.96 (C-2), 126.30 (C-23), 127.12 (2 × C), 128.55 (2 × C), 132.22, 136.45, 137.64 (C-22), 211.96 (C-6). Spectral data in agreement with literature [11].

*2.1.9. (22E)-23-(2-bromophenyl)-24-nor-5α-chola-2,22-dien-6-one **(8h)***

The general procedure with *o*-bromostyrene afforded 87 mg (59%) of the title compound **13a** as a colorless oil: IR ν (cm^{-1}) 2944, 1699, 1654, 1593, 1564, 964. ^{1}H NMR ($CDCl_3$) δ 0.72, 0.75 (both s, 3H, CH_3); 1.15 (d, 3H, J = 6.7 Hz, CH_3); 1.71–1.81 (m, 2H); 1.96–2.04 (m, 4H); 2.08 (dt, 1H, J = 12.5, J' = 3.1 Hz); 2.22–2.38 (m, 4H); 5.57 (m, 1H, H-3); 5.69 (m, 1H, H-2); 5.99 (dd, 1H, J = 15.6, J' = 8.9 Hz, H-22); 6.63 (d, 1H, J = 15.6 Hz, H-23); 7.05 (m, 1H, ΣJ = 16.8 Hz, Ar-H); 7.23 (m, 1H, ΣJ = 16.2 Hz, Ar-H); 7.45 (dd, 1H, J = 7.6, J' = 1.5 Hz, Ar-H); 7.52 (m, 1H, ΣJ = 9.2 Hz, Ar-H). ^{13}C NMR δ 12.24 (C-18), 13.51 (C-19), 20.28 (C-21), 21.10, 21.70, 23.96, 28.21, 37.66, 39.34, 39.36, 40.03, 40.47, 42.98, 46.95, 53.39, 53.83, 55.66, 56.72, 123.26, 124.50 (C-3), 124.96 (C-2), 126.50, 126.81, 127.33, 128.06, 132.77, 137.80, 139.93, 211.97 (C-6). HRMS: (API+) calculated for $C_{29}H_{38}{}^{79}BrO$ ([M+H]$^+$) 481.2106, Found 481.2107.

*2.1.10. (22E)-23-(3-bromophenyl)-24-nor-5α-chola-2,22-dien-6-one **(8i)***

The general procedure with *m*-bromostyrene afforded 97 mg (66%) of the title compound **14a** as a colorless oil: IR ν (cm^{-1}) 2944, 1700, 1655, 1588, 1560, 964. ^{1}H NMR ($CDCl_3$) δ 0.72, 0.73 (both s, 3H, CH_3); 1.13 (d, 3H, J = 6.7 Hz, CH_3); 1.71–1.80 (m, 2H); 1.96–2.04 (m, 4H); 2.06 (dt, 1H, J = 12.5, J' = 3.4 Hz); 2.22–2.31 (m, 2H); 2.34–2.37, (m, 2H); 5.58 (m, 1H, H-3); 5.69 (m, 1H, H-2); 6.06 (dd, 1H, J = 15.7, J' = 8.7 Hz, H-22); 6.23 (d, 1H, J = 15.7 Hz, H-23); 7.14 (t, 1H, J = 7.8 Hz, Ar-H); 7.22 (m, 1H, Ar-H); 7.30 (m, 1H, Ar-H); 7.47 (t, 1H, J = 1.7 Hz, Ar-H). ^{13}C NMR δ 12.19 (C-18), 13.50 (C-19), 20.25 (C-21), 21.09, 21.70, 23.91, 28.20, 37.66, 39.34, 39.36, 40.03, 40.41, 42.95, 46.94, 53.37, 53.82, 55.69, 56.71, 122.69, 124.48 (C-3), 124.69, 124.96 (C-2), 126.15, 128.70, 129.56, 129.95, 138.55, 140.17, 211.92 (C-6). HRMS: (API+) calculated for $C_{29}H_{38}{}^{79}BrO$ ([M+H]$^+$) 481.2106, Found 481.2108.

*2.1.11. (22E)-23-(4-bromophenyl)-24-nor-5α-chola-2,22-dien-6-one **(8j)***

The general procedure with *p*-bromostyrene afforded 107 mg (73%) of the title compound **8** as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.72, 0.73 (both s, 3H, CH_3), 1.13 (d, 3H, J = 6.7 Hz, CH_3), 1.70–1.79 (m, 2H), 1.96–2.04 (m, 4H), 2.07 (dt, 1H, J = 12.5, J' = 3.3 Hz), 2.22–2.30 (m, 2H), 2.33–2.37, (m, 2H), 5.57 (m, 1H, H-3), 5.69 (m, 1H, H-2), 6.05 (dd, 1H, J = 15.7, J' = 8.7 Hz, H-22), 6.24 (d, 1H, J = 15.7 Hz, H-23), 7.18 (m, 2H, 2 × Ar-H), 7.39 (m, 2H, 2 × Ar-H). ^{13}C NMR δ 12.19 (C-18), 13.49 (C-19), 20.24 (C21), 21.08, 21.70, 23.90, 28.19, 37.65, 39.32, 39.35, 40.02, 40.40, 42.92, 46.93, 53.36, 53.81, 55.71, 56.70, 120.30, 124.47 (C-3), 124.95 (C-2), 126.33 (C-23), 127.47 (2 × C), 131.48 (2 × C), 136.89, 137.77 (C-22), 211.92 (C-6). Spectral data in agreement with literature [11].

*2.1.12. (22E)-23-(2-methylphenyl)-24-nor-5α-chola-2,22-dien-6-one **(8k)***

The general procedure with *o*-methylstyrene afforded 83 mg (65%) of the title compound **15a** as a colorless oil: IR ν (cm^{-1}) 2932, 1701, 1653, 1593, 1563, 964. ^{1}H NMR ($CDCl_3$) δ 0.72, 0.74 (both s, 3H, CH_3); 1.14 (d, 3H, J = 6.7 Hz, CH_3); 1.71–1.80 (m, 2H); 1.96–2.04 (m, 4H); 2.07 (dt, 1H, J = 12.6, J' = 3.6 Hz); 2.22–2.31 (m, 2H); 2.32 (s, 3H, Ar-**CH_3**); 2.33–2.37 (m, 2H); 5.57 (m, 1H, H-3); 5.69 (m, 1H, J = 15.6 Hz, H-2); 5.89 (dd, 1H, J = 15.6, J' = 8.9 Hz, H-22); 6.49 (d, 1H, J = 15.6 Hz, H-23); 7.10–7.15 (m, 3H, 3 × Ar-H); 7.37 (d, 1H, J = 7.3 Hz, Ar-H). ^{13}C NMR δ 12.20 (C-18), 13.48 (C-19), 19.58 (Ar-**CH_3**), 20.53 (C-21), 21.08, 21.69, 23.92, 28.30, 37.65, 39.31, 39.34, 40.01, 40.72, 42.87, 46.93, 53.35, 53.78, 55.71, 56.73, 124.48 (C-3), 124.93 (C-2), 125.28, 125.45, 125.92, 126.67, 130.06, 134.94, 137.15, 138.43, 211.98 (C-6). HRMS: (API+) calculated for $C_{30}H_{41}O$ ([M+H]$^+$) 417.3157, Found 417.3160.

*2.1.13. (22E)-23-(3-methylphenyl)-24-nor-5α-chola-2,22-dien-6-one **(8l)***

The general procedure with *m*-methylstyrene afforded 88 mg (69%) of the title compound **16a** as a colorless oil: IR ν (cm^{-1}) 2933, 1700, 1653, 1593, 1562, 964. ^{1}H NMR ($CDCl_3$) δ 0.71, 0.73 (both s, 3H, CH_3); 1.12 (d, 3H, J = 6.7 Hz, CH_3); 1.71–1.79 (m, 2H); 1.95–2.04 (m, 4H); 2.06 (dt, 1H, J = 12.6, J' = 3.5 Hz); 2.20–2.30 (m, 2H); 2.32 (s, 3H, Ar-**CH_3**); 2.33–2.37 (m, 2H); 5.57 (m, 1H, H-3); 5.68 (m, 1H, H-2); 6.04 (dd, 1H, J = 15.9, J' = 8.9 Hz, H-22); 6.27 (d, 1H, J = 15.9 Hz, H-23); 7.00 (d, v, J = 7.3 Hz, Ar-H); 7.11–7.19 (m, 3H, 3 × Ar-H). ^{13}C NMR δ 12.19 (C-18), 13.49 (C-19), 20.40 (C-21), 21.09, 21.40 (Ar-**CH_3**), 21.70, 23.90, 28.20, 37.66, 39.32, 39.35, 40.03, 40.40, 42.88, 46.94, 53.37, 53.80, 55.82, 56.73, 123.09, 124.49 (C-3), 124.94 (C-2), 126.60, 127.45, 127.51, 128.36, 136.73, 137.89, 137.95, 211.99 (C-6). HRMS: (API+) calculated for $C_{30}H_{41}O$ ([M+H]$^+$) 417.3157, Found 417.3161.

*2.1.14. (22E)-23-(4-methylphenyl)-24-nor-5α-chola-2,22-dien-6-one **(8m)***

The general procedure with *p*-methylstyrene afforded 91 mg (71%) of the title compound **8** as a colorless oil. ^{1}H NMR ($CDCl_3$) δ ^{1}H NMR ($CDCl_3$) δ 0.72, 0.73 (both s, 3H, CH_3), 1.12 (d, 3H, J = 6.7 Hz, CH_3), 1.70–1.79 (m, 2H), 1.96–2.04 (m, 4H), 2.07 (dt, 1H, J = 12.5, J' = 3.4 Hz), 2.22–2.30 (m, 2H), 2.32 (s, 3H, CH_3), 2.33–2.37, (m, 2H), 5.57 (m, 1H, H-3), 5.69 (m, 1H, H-2), 6.00 (dd, 1H, J = 15.9, J' = 8.9 Hz, H-22), 6.27 (d, 1H, J = 15.9 Hz, H-23), 7.09 (d, 2H, J = 7.9 Hz, 2×Ar-H), 7.22 (d, 2H, J = 7.9 Hz, 2×Ar-H). ^{13}C NMR δ 12.19 (C-18), 13.49 (C-19), 20.42 (C-21), 21.09 (CH_2, Ar-**CH_3**), 21.70, 23.90, 28.19, 37.68, 39.34, 39.36, 40.04, 40.34, 42.88, 46.95, 53.39, 53.81, 55.89, 56.75, 124.50 (C-3), 124.95 (C-2), 125.80 (2×C), 127.21 (C-23), 129.13 (2×C), 135.18, 135.94 (C-22), 136.40, 212.00 (C-6). Spectral data in agreement with literature [11].

*2.1.15. (22E)-23-(2-trifluoromethylphenyl)-24-nor-5α-chola-2,22-dien-6-one **(8n)***

The general procedure with *o*-trifluoromethylstyrene afforded 82 mg (57%) of the title compound **17a** as a colorless oil: IR ν (cm^{-1}) 2942, 1701, 1653, 1593, 1560, 1128, 964. ^{1}H NMR ($CDCl_3$) δ 0.73, 0.76 (both s, 3H, CH_3); 1.15 (d, 3H, J = 6.7 Hz, CH_3); 1.71–1.81 (m, 2H); 1.96–2.04 (m, 4H); 2.07 (dt, 1H, J = 12.6, J' = 3.6 Hz); 2.23–2.31 (m, 2H); 2.31–2.38 (m, 2H); 5.58 (m, 1H, H-3); 5.69 (m, 1H, H-2); 6.00 (dd, 1H, J = 15.6, J' = 8.9 Hz, H-22); 6.68 (qd, 1H, J = 15.6, J' = 2.1 Hz, H-23); 7.29 (t, 1H, J = 7.7 Hz, Ar-H); 7.46 (t, 1H, J = 7.7 Hz, Ar-H); 7.55 (d, 1H, J = 7.7 Hz, Ar-H); 7.60 (d, 1H, J = 7.7 Hz, Ar-H). ^{13}C NMR δ 12.19 (C-18), 13.48 (C-19), 20.17 (C-21), 21.08, 21.70, 23.94, 28.02, 37.63, 39.32 (2×C), 40.00, 40.59, 42.95, 46.92, 53.37, 53.80, 55.68, 56.69, 123.72, 124.38 (q, J = 273.5 Hz), 124.47 (C-3), 124.93 (C-2), 125.58 (q, J = 6.0 Hz), 126.44, 126.92 (q, J = 30.0 Hz), 127.28, 131.65, 137.26, 141.19, 211.90 (C-6). ^{19}F NMR {^{1}H} δ -59.56 (s, 1F). HRMS: (API+) calculated for $C_{30}H_{38}F_3O$ ([M+H]$^{+}$) 471.2875, Found 471.2876.

*2.1.16. (22E)-23-(3-trifluoromethylphenyl)-24-nor-5α-chola-2,22-dien-6-one **(8o)***

The general procedure with *m*-trifluoromethylstyrene afforded 95 mg (66%) of the title compound **18** as a colorless oil: IR ν (cm^{-1}) 2940, 1701, 1655, 1593, 1561, 1124, 964. ^{1}H NMR ($CDCl_3$) δ 0.72, 0.74 (both s, 3H, CH_3); 1.14 (d, 3H, J = 6.7 Hz, CH_3); 1.71–1.80 (m, 2H); 1.97–2.05 (m, 4H); 2.07 (dt, 1H, J = 12.6, J' = 3.3 Hz); 2.22–2.32 (m, 2H); 2.34–2.37 (m, 2H); 5.58 (m, 1H, H-3); 5.69 (m, 1H, H-2); 6.14 (dd, 1H, J = 15.7, J' = 8.7 Hz, H-22); 6.34 (d, 1H, J = 15.7 Hz, H-23); 7.37–7.44 (m, 2H, 2×Ar-H); 7.48 (d, 1H, J = 7.3 Hz, Ar-H), 7.56 (b s, 1H, Ar-H). ^{13}C NMR δ 12.19 (C-18), 13.50 (C-19), 20.24 (C-21), 21.09, 21.70, 23.91, 28.23, 37.66, 39.36, 39.37, 40.03, 40.48, 42.96, 46.93, 53.37, 53.82, 55.64, 56.71, 122.52 (q, J = 3.6 Hz), 123.26 (q, J = 3.6 Hz), 124.19 (q, J = 272.3 Hz), 124.48 (C-3), 124.96 (C-2), 126.27, 128.85, 129.14, 130.81 (q, J = 32.4 Hz), 138.71, 138.90, 211.92 (C-6). ^{19}F NMR {^{1}H} δ -62.59 (s, 1F). HRMS: (API+) calculated for $C_{30}H_{38}F_3O$ ([M+H]$^{+}$) 471.2875, Found 471.2879.

*2.1.17. (22E)-23-(4-trifluoromethylphenyl)-24-nor-5α-chola-2,22-dien-6-one **(8p)***

The general procedure with *p*-trifluoromethylstyrene afforded 102 mg (71%) of the title compound **19a** as a colorless oil: IR ν (cm^{-1}) 2940, 1702, 1653, 1593, 1562, 1127, 963. ^{1}H NMR ($CDCl_3$) δ 0.72, 0.74 (both s, 3H, CH_3); 1.15 (d, 3H, J = 6.7 Hz, CH_3); 1.71–1.81 (m, 2H); 1.96–2.05 (m, 4H); 2.07 (dt, 1H, J = 12.5, J' = 3.4 Hz); 2.21–2.31 (m, 2H); 2.34–2.37 (m, 2H); 5.58 (m, 1H, H-3); 5.69 (m, 1H, H-2); 6.17 (dd, 1H, J = 15.7, J' = 8.7 Hz, H-22); 6.34 (d, 1H, J = 15.7 Hz, H-23); 7.41 (d, 2H, J = 8.4 Hz, 2×Ar-H); 7.53 (d, 2H, J = 8.4 Hz, 2×Ar-H). ^{13}C NMR δ 12.21 (C-18), 13.51 (C-19), 20.19 (C-21), 21.10, 21.70, 23.92, 28.19, 37.67, 39.34, 39.37, 40.04, 40.49, 42.99, 46.94, 53.38, 53.83, 55.85, 56.70, 124.28 (q, J = 271.5 Hz), 124.48 (C-3), 124.97 (C-2), 125.38, 125.40 (q, J = 3.6 Hz, 2×C), 126.04 (2×C), 126.35, 128.56 (q, J = 32.4 Hz), 139.66, 211.91 (C-6). ^{19}F NMR {^{1}H} δ -62.25 (s, 1F). HRMS: (API+) calculated for $C_{30}H_{38}F_3O$ ([M+H]$^{+}$) 471.2875, Found 471.2879.

*2.1.18. (22E)-23-(2,4-dimethylphenyl)-24-nor-5α-chola-2,22-dien-6-one **(8q)***

The general procedure with 2,4-dimethylstyrene and purification on HPLC afforded 81 mg (61%) of the title compound **20a** as a colorless oil: IR ν (cm^{-1}) 2935, 1701, 1652, 1593, 1560, 964. ^{1}H NMR ($CDCl_3$) δ 0.72, 0.74 (both s, 3H, CH_3); 1.13 (d, 3H, J = 6.7 Hz, CH_3); 1.71–1.80 (m, 2H); 1.96–2.04 (m, 4H); 2.07 (dt, 1H, J = 12.5, J' = 3.4 Hz); 2.22–2.37 (m, 4H); 2.28 (s, 6 H, 2×Ar-**CH_3**); 5.57 (m, 1H, H-3); 5.69 (m, 1H, H-2); 5.85 (dd, 1H, J = 15.6, J' = 8.9 Hz, H-22); 6.45 (d, 1H, J = 15.6 Hz, H-23); 6.94 (s, 1H, Ar-H); 6.95 (d, 1H, J = 8.3 Hz, Ar-H); 7.27 (d, 1H, J = 8.3 Hz, Ar-H). ^{13}C NMR δ 12.20 (C-18), 13.48 (C-19), 19.76 (Ar-**CH_3**), 20.57 (C-21), 20.97 (Ar-**CH_3**), 21.08, 21.70, 23.92, 28.28, 37.66, 39.32, 39.35, 40.02, 40.68, 42.85, 46.93, 53.37, 53.79, 55.78, 56.75, 124.48 (C-3), 124.93 (C-2), 125.09, 125.38, 126.64, 130.84, 134.29, 134.77, 136.28, 137.58, 211.98 (C-6). HRMS: (API+) calculated for $C_{31}H_{43}O$ ([M+H]$^{+}$) 431.3314, Found 431.3318.

*2.1.19. (22E)-23-(2,5-dimethylphenyl)-24-nor-5α-chola-2,22-dien-6-one **(8r)***

The general procedure with 2,5-dimethylstyrene and purification on HPLC afforded 77 mg (58%) of the title compound **21a** as a colorless oil: IR ν (cm^{-1}) 2931, 1699, 1653, 1593, 1560, 963. ^{1}H NMR ($CDCl_3$) δ 0.72, 0.74 (both s, 3H, CH_3); 1.13 (d, 3H, J = 6.7 Hz, CH_3); 1.71–1.80 (m, 2H); 1.96–2.04 (m, 4H); 2.08 (dt, 1H, J = 12.5, J' = 3.3 Hz); 2.22–2.31 (m, 2H); 2.27, 2.30 (both s, 3H, Ar-**CH_3**); 2.32–2.37 (m, 2H); 5.57 (m, 1H, H-3); 5.69 (m, 1H, H-2); 5.88 (dd, 1H, J = 15.6, J' = 8.9 Hz, H-22); 6.46 (d, 1H, J = 15.6 Hz, H-23); 6.92 (dd, 1H, J = 7.6, J' = 1.2 Hz, Ar-H); 7.00 (d, 1H, J = 7.6 Hz, Ar-H); 7.18 (b s, 1H, Ar-H). ^{13}C NMR δ 12.20 (C-18), 13.48 (C-19), 19.36 (Ar-**CH_3**), 20.57 (C-21), 21.00 (Ar-**CH_3**), 21.09, 21.70, 23.92, 28.31, 37.66, 39.33, 39.37, 40.02, 40.73, 42.87, 46.93, 53.37, 53.80, 55.74, 56.77, 124.48 (C-3), 124.94 (C-2), 125.37, 126.07, 127.43, 130.01, 131.89, 135.23, 136.88, 138.12, 211.95 (C-6). HRMS: (API+) calculated for $C_{31}H_{43}O$ ([M+H]$^{+}$) 431.3314, Found 431.3318.

*2.1.20. (22E)-23-(4-t-butylphenyl)-24-nor-5α-chola-2,22-dien-6-one **(8s)***

The general procedure with *p-t*-butylstyrene and purification on HPLC afforded 84 mg (60%) of the title compound **22a** as a colorless oil: IR ν (cm^{-1}) 2939, 1702, 1651, 1593, 1564, 1372, 963. ^{1}H NMR ($CDCl_3$) δ 0.73, 0.74 (both s, 3H, CH_3); 1.13 (d, 3H, J = 6.7 Hz, CH_3); 1.32 (s, 9 H, *t*-Bu); 1.71–1.80 (m, 2H); 1.97–2.05 (m, 4H); 2.08 (dt, 1H, J = 12.7, J' = 3.4 Hz); 2.23–2.32 (m, 2H); 2.35–2.38 (m, 2H); 5.59 (m, 1H, H-3); 5.70 (m, 1H, H-2); 6.03 (dd, 1H, J = 15.7, J' = 8.7 Hz, H-22); 6.30 (d, 1H, J = 15.7 Hz, H-23); 7.27–7.29 (m, 2H, 2×Ar-H); 7.32–7.34 (m, 2H, 2×Ar-H). ^{13}C NMR δ 12.18 (C-18), 13.48 (C-19), 20.46 (C-21), 21.08, 21.70, 23.90, 28.17, 31.28 (3×C, *t*-Bu), 37.66, 39.32, 39.35, 40.02, 40.39, 42.86, 46.94, 53.38, 53.80, 55.84, 56.74, 124.48 (C-3), 124.94 (C-2), 125.35 (2×C), 125.60 (2×C), 127.09, 135.17, 136.17, 149.72, 211.97 (C-6). HRMS: (API+) calculated for $C_{33}H_{47}O$ ([M+H]$^{+}$) 459.3627, Found 459.3629.

*2.1.21. (22E)-23-(4-phenylphenyl)-24-nor-5α-chola-2,22-dien-6-one **(8t)***

The general procedure with *p*-phenylstyrene afforded 47 mg (32%) of the title compound **23a** as a colorless oil: IR ν (cm^{-1}) 2949, 1700, 1654, 1593, 1565, 1465, 960. ^{1}H NMR ($CDCl_3$) δ 0.72, 0.75 (both s, 3H, CH_3); 1.15 (d, 3H, J = 6.7 Hz, CH_3); 1.71–1.80 (m, 2H); 1.96–2.04 (m, 4H); 2.08 (dt, 1H, J = 12.5, J' = 3.1 Hz); 2.22–2.31 (m, 2H); 2.33–2.37 (m, 2H); 5.57 (m, 1H, H-3); 5.69 (m, 1H, H-2); 6.11 (dd, 1H, J = 15.7, J' = 8.7 Hz, H-22); 6.34 (d, 1H, J = 15.7 Hz, H-23); 7.33 (m, 1H, Ar-H); 7.39–7.44 (m, 4H, 4×Ar-H); 7.52–7.54 (m, 2H, 2×Ar-H); 7.57–7.60 (m, 2H, 2×Ar-H). ^{13}C NMR δ 12.20 (C-18), 13.50 (C-19),

20.39 (C-21), 21.10, 21.70, 23.92, 28.21, 37.68, 39.34, 39.37, 40.04, 40.47, 42.93, 46.95, 53.39, 53.82, 55.84, 56.74, 124.50 (C-3), 124.96 (C-2), 126.32 (2×C), 126.86 (2×C), 126.98, 127.12, 127.16 (2×C), 128.73 (2×C), 137.04, 137.15, 139.51, 140.83, 211.98 (C-6). HRMS: (API+) calculated for $C_{35}H_{43}O$ ($[M+H]^+$) 479.3314, Found 479.3317.

2.1.22. (22E)-23-(2-isopropoxyphenyl)-24-nor-5α-chola-2,22-dien-6-one (9)

The general procedure with different *bis-ortho substituted* styrene or without styrene afforded 20 mg (14%) of the title compound **9** as a colorless oil: IR ν (cm^{-1}) 2940, 1701, 1653, 1591, 1561, 963. ^{1}H NMR ($CDCl_3$) δ 0.72, 0.74 (both s, 3H, CH_3); 1.14 (d, 3H, J = 6.7 Hz, CH_3); 1.34 (d, 3H, J = 6.1 Hz, CH(**CH_3**)$_2$); 1.35 (d, 3H, J = 6.1 Hz, CH(**CH_3**)$_2$); 1.71–1.81 (m, 2H); 1.96–2.03 (m, 4H); 2.08 (dt, 1H, J = 12.5, J' = 3.2 Hz); 2.22–2.31 (m, 2H); 2.33–2.37 (m, 2H); 4.49 (septet, 1H, J = 6.1 Hz, O**CH**$(CH_3)_2$); 5.58 (m, 1H, H-3); 5.69 (m, 1H, H-2); 6.03 (dd, 1H, J = 15.9, J' = 8.9 Hz, H-22); 6.62 (d, 1H, J = 15.9 Hz, H-23); 6.85–6.90 (m, 2H); 7.13 (m, 1H); 7.40 (dd, 1H, J = 7.6, J' = 1.5 Hz). ^{13}C NMR δ 12.21 (C-18), 13.50 (C-19), 20.51 (C-21), 21.10, 21.70, 22.25 (2×C, CH(**CH_3**)$_2$), 23.94, 28.21, 37.69, 39.34, 39.37, 40.05, 40.70, 42.89, 46.97, 53.41, 53.82, 55.94, 56.77, 71.04 (O**CH**$(CH_3)_2$), 114.70, 120.71, 122.32, 124.51 (C-3), 124.94 (C-2), 126.31, 127.49, 128.43, 137.13, 154.79, 212.05 (C-6). HRMS: (API+) calculated for $C_{32}H_{45}O_2$ ($[M+H]^+$) 461.3420, Found 461.3423.

2.2. General procedure for dihydroxylation of dienes

To a solution of diene (0.2 mmol); hydroquinidine 4-chlorobenzoate (23 mg; 0.049 mmol); methansulfonamide (33 mg; 0.35 mmol); potassium carbonate (140 mg; 1.01 mmol); and potassium ferricyanide (350 mg; 1.06 mmol) in the mixture of *t*-butanol and water (15 mL; 1:1 v/v) was added 0.1 mL of osmium tetroxide in *t*-butanol (1 g/20 mL; 0.02 mmol). Reaction mixture was stirred at room temperature for 24 h. A saturated solution of sodium sulfite (3 mL) was then added. After an additional 30 min of stirring, the reaction mixture was diluted with ethyl acetate (30 mL) and extracted with water (2 × 20 mL). The combined organic fractions were dried over anhydrous magnesium sulfate and evaporated under reduced pressure. Column chromatography on silica gel (mobile phase – MeOH/$CHCl_3$ – 1/16) gave the desired product.

Compounds **10a** (m. p. 269–271 °C, EtOH), **10d** (m. p. 276–278 °C, EtOH), **10g** (m. p. 251–253 °C, EtOH), **10j** (m. p. 248–249 °C, *i*-PrOH), and **10m** (m. p. 271–273 °C, EtOH) were prepared according to general procedure which is also described in literature [11]. All data for these compounds are in agreement with same literature.

2.2.1. (22R, 23R)-2α,3α,22,23-tetrahydroxy-23-(2-fluorophenyl)-24-nor-5α-cholan-6-one (10b)

The general procedure for dihydroxylation of **8b** (60 mg; 0.14 mmol) afforded 54 mg (77%) of the title compound **10b** as a white solid: m. p. 288–290 °C (EtOH); IR ν (cm^{-1}) 3348 vb, 2941, 1710, 1620, 1587, 1492, 757. ^{1}H NMR (DMSO-d_6) δ 0.34 (H-18), 0.60 (H-19) (both s, 3H, CH_3); 0.86 (d, 3H, J = 6.4 Hz, H-21, CH_3); 1.80 (m, 1H); 1.87 (m, 1H); 1.98 (m, 1H); 2.07 (t, 1H, J = 12.6 Hz); 2.58 (d, 1H, J = 12.4 Hz); 3.44 (m, 1H, H-2); 3.58 (m, 1H, H-3); 3.74 (m, 1H, H-22); 4.18 (br s, 1H, OH); 4.31 (d, 1H, J = 5.8 Hz, OH); 4.62 (d, 1H, J = 1.8 Hz, OH); 4.66 (d, 1H, J = 8.3 Hz, H-23); 5.25 (br s, 1H, OH); 7.12 (m, 1H); 7.18 (m, 1H); 7.29 (m, 1H); 7.38 (m, 1H). ^{13}C NMR δ 11.32 (C-18), 12.51 (C-19), 13.30 (C-21), 20.73, 23.27, 26.73, 27.21 36.77, 36.98, 39.06, 40.10, 41.79, 42.00, 45.94, 50.25, 51.89, 52.86, 55.93, 67.08 (C-2), 67.45 (C-3), 68.48 (C-23), 75.65 (C-22), 114.92 (d, J = 22.8 Hz), 124.31 (d, J = 3.6 Hz), 128.82 (d, J = 3.6 Hz), 128.87, 130.01 (d, J = 14.2 Hz), 159.61 (d, J = 243.5 Hz, C-F), 211.39 (C-6). ^{19}F NMR {^{1}H} δ −118.49 (s, 1F). HRMS: (API+) calculated for $C_{29}H_{42}FO_5$ ($[M+H]^+$) 489.3016, Found 489.3017. Anal. Calcd for $C_{29}H_{41}FO_5$: C, 71.28; H, 8.46. Found: C, 71.30; H, 8.45%.

2.2.2. (22R, 23R)-2α,3α,22,23-tetrahydroxy-23-(3-fluorophenyl)-24-nor-5α-cholan-6-one (10c)

The general procedure for dihydroxylation of **8c** (60 mg; 0.14 mmol) afforded 59 mg (85%) of the title compound **10c** as a white solid: m. p. 278–279 °C (EtOH); IR ν (cm^{-1}) 3334 vb, 2939, 1710, 1615, 1591, 1461, 763. ^{1}H NMR (DMSO-d_6) δ 0.34 (H-18), 0.59 (H-19) (both s, 3H, CH_3); 0.86 (d, 3H, J = 6.4 Hz, H-21, CH_3); 1.80 (m, 1H); 1.86 (m, 1H); 1.98 (dd, 1H, J = 12.8, J' = 4.4 Hz); 2.07 (t, 1H, J = 12.8 Hz); 2.57 (dd, 1H, J = 12.0, J' = 3.0 Hz); 3.43–3.48 (m, 2H, H-2, H-3); 3.74 (m, 1H, H-22); 4.18 (d, 1H, J = 2.4 Hz, OH); 4.32 (d, 1H, J = 6.1 Hz, OH); 4.39 (dd, 1H, J = 7.9, J' = 3.4 Hz, H-23); 4.57 (d, 1H, J = 4.3 Hz, OH); 5.24 (d, 1H, J = 4.0 Hz, OH); 7.04–7.12 (m, 3H); 7.35 (m, 1H). ^{13}C NMR δ 11.38 (C-18), 12.53 (C-19), 13.35 (C-21), 20.78, 23.28, 26.81, 27.25 36.48, 37.01, 39.08, 40.09, 41.83, 42.05, 45.94, 50.28, 51.88, 52.85, 55.94, 67.09 (C-2), 67.48 (C-3), 74.56 (C-23), 76.17 (C-22), 113.46 (d, J = 21.6 Hz), 113.88 (d, J = 21.6 Hz), 123.05, 129.96 (d, J = 8.4 Hz), 146.53 (d, J = 7.2 Hz), 162.04 (d, J = 242.3 Hz, C-F), 211.54 (C-6). ^{19}F NMR {1H} δ -113.41 (s, 1F). HRMS: (API+) calculated for $C_{29}H_{42}FO_5$ ($[M+H]^+$) 489.3016, Found 489.3017. Anal. Calcd for $C_{29}H_{41}FO_5$: C, 71.28; H, 8.46. Found: C, 71.26; H, 8.49%.

2.2.3. (22R, 23R)-2α,3α,22,23-tetrahydroxy-23-(2-chlorophenyl)-24-nor-5α-cholan-6-one (10e)

The general procedure for dihydroxylation of **8e** (60 mg; 0.14 mmol) afforded 54 mg (78%) of the title compound **10e** as a white solid: m. p. 290–292 °C (EtOH); IR ν (cm^{-1}) 3307 vb, 2936, 1693, 1595, 1572, 1463, 760. ^{1}H NMR (DMSO-d_6) δ 0.38 (H-18), 0.60 (H-19) (both s, 3H, CH_3); 0.93 (d, 3H, J = 6.7 Hz, H-21, CH_3); 1.79 (m, 1H); 1.89 (m, 1H); 1.98 (dd, 1H, J = 13.4, J' = 4.7 Hz); 2.07 (t, 1H, J = 12.3 Hz); 2.58 (dd, 1H, J = 12.3, J' = 3.4 Hz); 3.45 (m, 1H, H-2); 3.58 (bd, 1H, J = 8.0 Hz, H-3); 3.74 (m, 1H, H-22); 4.18 (bd, 1H, J = 2.4 Hz, OH); 4.31 (d, 1H, J = 6.1 Hz, OH); 4.58 (bd, 1H, J = 3.4 Hz, H-23); 4.79 (d, 1H, J = 7.3 Hz, OH); 5.28 (bs, 1H, OH); 7.26 (m, 1H); 7.34 (td, 1H, J = 7.5, J' = 1.2 Hz); 7.39 (dd, 1H, J = 7.9, J' = 1.2 Hz); 7.44 (dd, 1H, J = 7.8, J' = 1.7 Hz). ^{13}C NMR δ 11.37 (C-18), 13.36 (C-19), 13.39 (C-21), 20.76, 23.33, 26.81, 27.40, 37.01, 37.36, 39.04, 40.10, 41.82, 42.08, 45.93, 50.26, 52.13, 52.84, 55.93, 67.09 (C-2), 67.47 (C-3), 71.00 (C-23), 75.66 (C-22), 127.19, 128.65, 128.92, 129.13, 131.92, 141.13, 211.54 (C-6). HRMS: (API+) calculated for $C_{29}H_{42}ClO_5$ ($[M+H]^+$) 505.2721, Found 505.2720. Anal. Calcd for $C_{29}H_{41}ClO_5$: C, 68.96; H, 8.18. Found: C, 68.93; H, 8.19%.

2.2.4. (22R, 23R)-2α,3α,22,23-tetrahydroxy-23-(3-chlorophenyl)-24-nor-5α-cholan-6-one (10f)

The general procedure for dihydroxylation of **8f** (60 mg; 0.14 mmol) afforded 56 mg (80%) of the title compound **10f** as a white solid: m. p. 269–270 °C (EtOH); IR ν (cm^{-1}) 3257 vb, 2936, 1709, 1600, 1576, 1461, 781, 699. ^{1}H NMR (DMSO-d_6) δ 0.35 (H-18), 0.60 (H-19) (both s, 3H, CH_3); 0.86 (d, 3H, J = 6.7 Hz, H-21, CH_3); 1.79 (m, 1H); 1.87 (m, 1H); 1.96 (dd, 1H, J = 13.4, J' = 4.6 Hz); 2.07 (t, 1H, J = 12.3 Hz); 2.58 (dd, 1H, J = 12.3, J' = 3.2 Hz); 3.43–3.46 (m, 2H, H-2, H-3); 3.74 (m, 1H, H-22); 4.18 (bd, 1H, J = 2.1 Hz, OH); 4.33 (d, 1H, J = 5.8 Hz, OH); 4.38 (bd, 1H, J = 8.3 Hz, H-23); 4.59 (d, 1H, J = 3.4 Hz, OH); 5.26 (bs, 1H, OH); 7.23 (m, 1H); 7.29–7.31 (m, 2H); 7.35 (t, 1H, J = 7.6 Hz). ^{13}C NMR δ 11.44 (C-18), 12.58 (C-19), 13.39 (C-21), 20.81, 23.33, 26.85, 27.31, 36.59, 37.06, 39.12, 40.11, 41.88, 42.09, 45.98, 50.33, 51.93, 52.90, 55.98, 67.13 (C-2), 67.52 (C-3), 74.56 (C-23), 76.16 (C-22), 125.73, 126.77, 127.12, 130.01, 132.75, 146.12, 211.64 (C-6). HRMS: (API+) calculated for $C_{29}H_{42}ClO_5$ ($[M+H]^+$) 505.2721, Found 505.2716. Anal. Calcd for $C_{29}H_{41}ClO_5$: C, 68.96; H, 8.18. Found: C, 68.95; H, 8.23%.

2.2.5. (22R, 23R)-2α,3α,22,23-tetrahydroxy-23-(2-bromophenyl)-24-nor-5α-cholan-6-one (10h)

The general procedure for dihydroxylation of **8h** (60 mg;

0.13 mmol) afforded 55 mg (81%) of the title compound **10h** as a white solid: m. p. 288–289 °C (EtOH); IR ν (cm^{-1}) 3313 vb, 2944, 1699, 1593, 1564, 1471, 786, 682. ^{1}H NMR (DMSO-d_6) δ 0.39 (H-18), 0.60 (H-19) (both s, 3H, CH_3); 0.95 (d, 3H, J = 6.7 Hz, H-21, CH_3); 1.78(m, 1H); 1.89 (m, 1H); 1.98 (dd, 1H, J = 13.1, J' = 4.6 Hz); 2.07 (t, 1H, J = 12.3 Hz); 2.58 (dd, 1H, J = 12.3, J' = 3.2 Hz); 3.46 (m, 1H, H-2); 3.58 (bd, 1H, J = 7.0 Hz, H-3); 3.74 (m, 1H, H-22); 4.20 (d, 1H, J = 1.8 Hz, OH); 4.34 (d, 1H, J = 5.8 Hz, OH); 4.58 (m, 1H, H-23); 4.73 (d, 1H, J = 7.0 Hz, OH); 5.31 (bs, 1H, OH); 7.18 (m, 1H); 7.38 (m, 1H); 7.42 (dd, 1H, J = 7.9, J' = 1.8 Hz); 7.55 (dd, 1H J = 7.9, J' = 1.2 Hz). ^{13}C NMR δ 11.45 (C-18), 13.40 (C-19), 13.69 (C-21), 20.81, 23.40, 26.86, 27.47, 37.07, 37.60, 39.10, 40.10, 41.89, 42.15, 45.98, 50.34, 52.22, 52.89, 55.97, 67.13 (C-2), 67.52 (C-3), 73.47 (C-23), 75.65 (C-22), 122.72, 127.76, 129.08, 129.46, 132.26, 142.83, 211.68 (C-6). HRMS: (API+) calculated for $C_{29}H_{42}{}^{79}BrO_5$ ([M+H]$^+$) 549.2216, Found 549.2211. Anal. Calcd for $C_{29}H_{41}BrO_5$: C, 63.38; H, 7.52. Found: C, 63.35; H, 7.56%.

2.2.6. (22R, 23R)-2α,3α,22,23-tetrahydroxy-23-(3-bromophenyl)-24-nor-5α-cholan-6-one (10i)

The general procedure for dihydroxylation of **8i** (60 mg; 0.13 mmol) afforded 54 mg (79%) of the title compound **10i** as a white solid: m. p. 264–265 °C (EtOH); IR ν (cm^{-1}) 3242 vb, 2935, 1709, 1596, 1572, 1460, 782, 700. ^{1}H NMR (DMSO-d_6) δ 0.35 (H-18), 0.60 (H-19) (both s, 3H, CH_3); 0.86 (d, 3H, J = 6.7 Hz, H-21, CH_3); 1.79 (m, 1H); 1.87 (m, 1H); 1.98 (dd, 1H, J = 13.1, J' = 4.8 Hz); 2.07 (t, 1H, J = 12.3 Hz); 2.58 (dd, 1H, J = 12.3, J' = 3.2 Hz); 3.42–3.46 (m, 2H, H-2, H-3); 3.74 (m, 1H, H-22); 4.19 (bs, 1H, OH); 4.33 (bd, 1H, J = 5.5 Hz, OH); 4.37 (bd, 1H, J = 8.3 Hz, H-23); 4.59 (bs, 1H, OH); 5.26 (bs, 1H, OH); 7.26–7.30 (m, 2H); 7.43 (m, 1H); 7.45 (m, 1H). ^{13}C NMR δ 11.44 (C-18), 12.58 (C-19), 13.38 (C-21), 20.80, 23.32, 26.84, 27.30, 36.60, 37.05, 39.11, 40.10, 41.87, 42.09, 45.97, 50.32, 51.93, 52.89, 55.97, 67.12 (C-2), 67.51 (C-3), 74.52 (C-23), 76.15 (C-22), 121.39, 126.10, 129.69, 130.00, 130.33, 146.38, 211.62 (C-6). HRMS: (API+) calculated for $C_{29}H_{42}{}^{79}BrO_5$ ([M+H]$^+$) 549.2216, Found 549.2211. Anal. Calcd for $C_{29}H_{41}BrO_5$: C, 63.38; H, 7.52. Found: C, 63.32; H, 7.59%.

2.2.7. (22R, 23R)-2α,3α,22,23-tetrahydroxy-23-(2-methylphenyl)-24-nor-5α-cholan-6-one (10k)

The general procedure for dihydroxylation of **8k** (60 mg; 0.14 mmol) afforded 53 mg (76%) of the title compound **10k** as a white solid: m. p. 269–271 °C (EtOH); IR ν (cm^{-1}) 3300 vb, 2942, 1706, 1631, 1604, 1461, 981, 759. ^{1}H NMR (DMSO-d_6) δ 0.29 (H-18), 0.55 (H-19) (both s, 3H, CH_3); 0.81 (d, 3H, J = 6.4 Hz, H-21, CH_3); 1.76 (m, 1H); 1.83 (m, 1H); 1.97 (dd, 1H, J = 13.1, J' = 4.9 Hz); 2.02 (t, 1H, J = 12.3 Hz); 2.26 (s, 3H, CH_3); 2.58 (dd, 1H, J = 12.3, J' = 3.2 Hz); 3.40 (m, 1H, H-2); 3.59 (dd, 1H, J = 8.1, J' = 2.6 Hz, H-3); 3.69 (m, 1H, H-22); 4.13 (d, 1H, J = 2.1 Hz, OH); 4.27 (d, 1H, J = 5.8 Hz, OH); 4.44 (d, 1H, J = 4.0 Hz, OH); 4.52 (dd, 1H, J = 8.3, J' = 1.8 Hz, H-23); 4.94 (d, 1H, J = 3.4 Hz, OH); 7.06–7.08 (m, 2H); 7.10 (m, 1H); 7.20 (d, 1H, J = 7.3 Hz). ^{13}C NMR δ 11.42 (C-18), 13.25 (C-19), 13.37 (C-21), 19.32 (Ar-**CH_3**), 20.79, 23.35, 26.83, 27.35, 36.79, 37.04, 39.07, 40.10, 41.86, 42.09, 45.96, 50.31, 52.21, 52.87, 55.95, 67.11 (C-2), 67.50 (C-3), 71.41 (C-23), 75.50 (C-22), 125.74, 126.82, 127.38, 130.15, 135.06, 141.42, 211.61 (C-6). HRMS: (API+) calculated for $C_{30}H_{45}O_5$ ([M+H]$^+$) 485.3267, Found 485.3268. Anal. Calcd for $C_{30}H_{44}O_5$: C, 74.34; H, 9.15. Found: C, 74.29; H, 9.19%.

2.2.8. (22R, 23R)-2α,3α,22,23-tetrahydroxy-23-(3-methylphenyl)-24-nor-5α-cholan-6-one (10l)

The general procedure for dihydroxylation of **8l** (60 mg; 0.14 mmol) afforded 55 mg (79%) of the title compound **10l** as a white solid: m. p. 276–278 °C (EtOH); IR ν (cm^{-1}) 3315 vb, 2938, 1708, 1611, 1593, 1460, 988, 707. ^{1}H NMR (DMSO-d_6) δ 0.32 (H-18), 0.60 (H-19) (both s, 3H, CH_3); 0.85 (d, 3H, J = 6.4 Hz, H-21, CH_3); 1.80 (m, 1H); 1.87 (m, 1H); 1.98 (dd, 1H, J = 13.2, J' = 4.9 Hz); 2.07 (t, 1H, J = 12.6 Hz); 2.28 (s, 3H, CH_3); 2.58 (dd, 1H, J = 12.1, J' = 3.2 Hz); 3.44–3.49 (m, 2H, H-2, H-3); 3.74 (m, 1H, H-22); 4.18 (d, 1H, J = 2.1 Hz, OH); 4.30–4.33 (m, 2H, H-23, OH); 4.46 (d, 1H, J = 4.0 Hz, OH); 5.03 (d, 1H, J = 3.4 Hz, OH); 7.03–7.05 (m, 2H); 7.07 (s, 1H); 7.19 (t, 1H, J = 7.5 Hz). ^{13}C NMR δ 11.45 (C-18), 12.51 (C-19), 13.37 (C-21), 18.59 (Ar-**CH_3**), 20.80, 23.32, 26.83, 27.23, 36.35, 37.04, 39.12, 40.10, 41.86, 42.08, 45.97, 50.31, 51.91, 52.88, 55.97, 67.11 (C-2), 67.50 (C-3), 75.15 (C-23), 76.21 (C-22), 124.15, 127.67, 127.83, 127.94, 136.99, 143.20, 211.62 (C-6). HRMS: (API+) calculated for $C_{30}H_{45}O_5$ ([M+H]$^+$) 485.3267, Found 485.3269. Anal. Calcd for $C_{30}H_{44}O_5$: C, 74.34; H, 9.15. Found: C, 74.31; H, 9.16%.

2.2.9. (22R, 23R)-2α,3α,22,23-tetrahydroxy-23-(2-trifluoromethylphenyl)-24-nor-5α-cholan-6-one (10n)

The general procedure for dihydroxylation of **8n** (60 mg; 0.13 mmol) afforded 50 mg (73%) of the title compound **10n** as a white solid: m. p. 268–270 °C (EtOH); IR ν (cm^{-1}) 3418 vb, 2939, 1701, 1613, 1589, 1315, 1114, 772. ^{1}H NMR (DMSO-d_6) δ 0.38 (H-18), 0.60 (H-19) (both s, 3H, CH_3); 0.87 (d, 3H, J = 6.4 Hz, H-21, CH_3); 1.82 (m, 1H); 1.88 (m, 1H); 1.99 (dd, 1H, J = 13.1, J' = 4.6 Hz); 2.07 (t, 1H, J = 12.6 Hz); 2.58 (dd, 1H, J = 12.2, J' = 3.1 Hz); 3.45 (m, 1H, H-2); 3.66 (m, 1H, H-3); 3.74 (bs, 1H, H-22); 4.18 (d, 1H, J = 2.1 Hz, OH); 4.31 (d, 1H, J = 6.1 Hz, OH); 4.63 (d, 1H, J = 4.3 Hz, H-23); 4.70 (d, 1H, J = 4.6 Hz, OH); 5.32 (bs, 1H, OH); 7.46 (m, 1H); 7.65–7.70 (m, 3H). ^{13}C NMR δ 11.31 (C-18), 12.83 (C-19), 13.35 (C-21), 20.75, 23.34, 26.81, 27.38, 37.00, 37.77, 39.04, 40.10, 41.82, 42.11, 45.91, 50.28, 52.32, 52.84, 55.91, 67.09 (C-2), 67.47 (C-3), 69.76 (C-23), 75.19 (C-22), 124.49 (q, J = 274.7 Hz, CF_3), 126.26 (q, J = 28.8 Hz), 125.21 (q, J = 5.0 Hz), 127.59, 129.38, 132.39, 142.56, 211.54 (C-6). ^{19}F NMR {^{1}H} δ -55.95 (s, 1F). HRMS: (API+) calculated for $C_{30}H_{42}F_3O_5$ ([M+H]$^+$) 539.2984, Found 539.2986. Anal. Calcd for $C_{30}H_{41}F_3O_5$: C, 66.89; H, 7.67. Found: C, 66.88; H, 7.69%.

2.2.10. (22R, 23R)-2α,3α,22,23-tetrahydroxy-23-(3-trifluoromethylphenyl)-24-nor-5α-cholan-6-one (10o)

The general procedure for dihydroxylation of **8o** (60 mg; 0.13 mmol) afforded 53 mg (77%) of the title compound **10o** as a white solid: m. p. 243–245 °C (EtOH); IR ν (cm^{-1}) 3257 vb, 2934, 1712, 1618, 1457, 1329, 1113, 706. ^{1}H NMR (DMSO-d_6) δ 0.32 (H-18), 0.59 (H-19) (both s, 3H, CH_3); 0.86 (d, 3H, J = 6.4 Hz, H-21, CH_3); 1.79 (m, 1H); 1.86 (m, 1H); 1.97 (dd, 1H, J = 13.1, J' = 4.6 Hz); 2.07 (t, 1H, J = 12.6 Hz); 2.57 (dd, 1H, J = 12.2, J' = 3.3 Hz); 3.42–3.49 (m, 2H, H-2, H-3); 3.74 (bs, 1H, H-22); 4.19 (d, 1H, J = 1.8 Hz, OH); 4.33 (d, 1H, J = 5.5 Hz, OH); 4.50 (d, 1H, J = 7.9 Hz, OH); 4.65 (bs, 1H, H-23); 5.35 (bs, 1H, OH); 7.54–7.62 (m, 4H). ^{13}C NMR δ 11.30 (C-18), 12.59 (C-19), 13.36 (C-21), 20.78, 23.30, 26.83, 27.22, 36.54, 37.01, 39.08, 40.10, 41.84, 42.09, 45.94, 50.31, 51.93, 52.86, 55.94, 67.11 (C-2), 67.50 (C-3), 74.52 (C-23), 76.23 (C-22), 123.38 (q, J = 4.0 Hz), 123.84 (q, J = 4.0 Hz), 124.37 (q, J = 272.3 Hz, CF_3), 128.77 (q, J = 31.8 Hz), 129.14, 131.12, 145.01, 211.59 (C-6). ^{19}F NMR {^{1}H} δ -60.97 (s, 1F). HRMS: (API+) calculated for $C_{30}H_{42}F_3O_5$ ([M+H]$^+$) 539.2984, Found 539.2985. Anal. Calcd for $C_{30}H_{41}F_3O_5$: C, 66.89; H, 7.67. Found: C, 66.85; H, 7.71%.

2.2.11. (22R, 23R)-2α,3α,22,23-tetrahydroxy-23-(4-trifluoromethylphenyl)-24-nor-5α-cholan-6-one (10p)

The general procedure for dihydroxylation of **8p** (60 mg; 0.13 mmol) afforded 54 mg (78%) of the title compound **10p** as a white solid: m. p. 265–266 °C (EtOH); IR ν (cm^{-1}) 3224 vb, 2937, 1710, 1621, 1461, 1331, 1121, 838. ^{1}H NMR (DMSO-d_6) δ 0.33 (H-18), 0.59 (H-19) (both s, 3H, CH_3); 0.86 (d, 3H, J = 6.4 Hz, H-21, CH_3); 1.80 (m, 1H); 1.86 (m, 1H); 1.98 (dd, 1H, J = 13.1, J' = 4.6 Hz); 2.07 (t, 1H, J = 12.6 Hz); 2.58 (dd, 1H, J = 12.2, J' = 3.1 Hz); 3.44 (m, 1H, H-2); 3.50 (d, 1H, J = 7.6 Hz, H-3); 3.74 (bs, 1H, H-22); 4.19 (bs, 1H, OH); 4.33 (d, 1H, J = 4.6 Hz, OH); 4.47 (d, 1H, J = 8.3 Hz, H-23); 4.66 (bs, 1H, OH); 5.34 (bs, 1H, OH); 7.50 (d, 2H, J = 8.1 Hz); 7.68 (d, 2H,

J = 8.1 Hz). ^{13}C NMR δ 11.41 (C-18), 12.57 (C-19), 13.36 (C-21), 20.80, 23.29, 26.83, 27.26, 36.61, 37.03, 39.11, 40.10, 41.85, 42.07, 45.96, 50.31, 51.92, 52.87, 55.95, 67.11 (C-2), 67.50 (C-3), 74.68 (C-23), 76.10 (C-22), 124.40 (q, J = 271.9 Hz, CF_3), 124.96 (q, 2×C, J = 3.6 Hz), 127.70 (q, J = 31.2 Hz), 127.80 (2×C), 148.29, 211.59 (C-6). ^{19}F NMR {^{1}H} δ -60.59 (s, 1F). HRMS: (API+) calculated for $C_{30}H_{42}F_3O_5$ ([M+H]$^+$) 539.2984, Found 539.2986. Anal. Calcd for $C_{30}H_{41}F_3O_5$: C, 66.89; H, 7.67. Found: C, 66.86; H, 7.70%.

2.2.12. (22R, 23R)-2α,3α,22,23-tetrahydroxy-23-(2,4-dimethylphenyl)-24-nor-5α-cholan-6-one ***(10q)***

The general procedure for dihydroxylation of **8q** (60 mg; 0.14 mmol) afforded 52 mg (75%) of the title compound **10q** as a white solid: m. p. 253–255 °C (EtOH); IR ν (cm^{-1}) 3350 vb, 2943, 1693, 1617, 1502, 1458, 987, 817. ^{1}H NMR (DMSO-d_6) δ 0.35 (H-18), 0.60 (H-19) (both s, 3H, CH_3); 0.85 (d, 3H, J = 6.4 Hz, H-21, CH_3); 1.78–1.88 (m, 2H); 1.98 (dd, 1H, J = 13.1, J' = 4.6 Hz); 2.07 (t, 1H, J = 12.5 Hz); 2.22 (s, 3H, Ar-**CH_3**); 2.26 (s, 3H, Ar-**CH_3**); 2.58 (dd, 1H, J = 12.1, J' = 3.0 Hz); 3.44 (m, 1H, H-2); 3.63 (dd, 1H, J = 8.3 Hz, H-3); 3.74 (m, 1H, H-22); 4.19 (bs, 1H, OH); 4.33 (bs, 1H, OH); 4.46 (bs, 1H, OH); 4.52 (d, 1H, J = 8.3 Hz, H-23); 4.89 (bs, 1H, OH); 6.93 (s, 1H); 6.96 (d, 1H, J = 7.9 Hz); 7.12 (d, 1H, J = 7.9 Hz). ^{13}C NMR δ 11.50 (C-18), 13.13 (C-19), 13.38 (C-21), 19.25 (Ar-**CH_3**), 20.65 (Ar-**CH_3**), 20.80, 23.36, 26.84, 27.36, 36.79, 37.05, 39.09, 40.10, 41.87, 42.09, 45.98, 50.31, 52.17, 52.88, 55.97, 67.12 (C-2), 67.51 (C-3), 71.26 (C-23), 75.44 (C-22), 126.41, 127.40, 130.84, 134.94, 135.62, 138.30, 211.64 (C-6). HRMS: (API+) calculated for $C_{31}H_{47}O_5$ ([M+H]$^+$) 499.3423, Found 499.3426. Anal. Calcd for $C_{31}H_{46}O_5$: C, 74.66; H, 9.30. Found: C, 74.63; H, 9.32%.

2.2.13. (22R, 23R)-2α,3α,22,23-tetrahydroxy-23-(2,5-dimethylphenyl)-24-nor-5α-cholan-6-one ***(10r)***

The general procedure for dihydroxylation of **8r** (60 mg; 0.14 mmol) afforded 49 mg (71%) of the title compound **10r** as a white solid: m. p. 281–283 °C (EtOH); IR ν (cm^{-1}) 3332 vb, 2933, 1707, 1616, 1506, 1461, 985. ^{1}H NMR (DMSO-d_6) δ 0.36 (H-18), 0.60 (H-19) (both s, 3H, CH_3); 0.86 (d, 3H, J = 6.4 Hz, H-21, CH_3); 1.82 (m, 1H); 1.87 (m, 1H); 1.98 (dd, 1H, J = 13.1, J' = 4.6 Hz); 2.07 (t, 1H, J = 12.6 Hz); 2.23 (s, 3H, Ar-**CH_3**); 2.24 (s, 3H, Ar-**CH_3**); 2.58 (dd, 1H, J = 12.0, J' = 3.1 Hz); 3.45 (m, 1H, H-2); 3.61 (bd, 1H, J = 7.9 Hz, H-3); 3.74 (bs, 1H, H-22); 4.20 (bs, 1H, OH); 4.34 (d, 1H, J = 5.2 Hz, OH); 4.47 (bs, 1H, OH); 4.52 (d, 1H, J = 7.9 Hz, H-23); 4.94 (bs, 1H, OH); 6.92 (dd, 1H, J = 7.6, J' = 1.2 Hz); 6.99 (d, 1H, J = 7.6 Hz); 7.06 (bs, 1H). ^{13}C NMR δ 11.47 (C-18), 13.29 (C-19), 13.40 (C-21), 18.92 (Ar-**CH_3**), 20.81, 20.85 (Ar-**CH_3**), 23.42, 26.86, 27.32, 36.91, 37.08, 39.10, 40.11, 41.88, 42.13, 45.98, 50.34, 52.25, 52.91, 55.98, 67.14 (C-2), 67.52 (C-3), 71.45 (C-23), 75.53 (C-22), 127.46, 127.99, 130.08, 131.91, 134.34, 141.26, 211.68 (C-6). HRMS: (API+) calculated for $C_{31}H_{47}O_5$ ([M+H]$^+$) 499.3423, Found 499.3425. Anal. Calcd for $C_{31}H_{46}O_5$: C, 74.66; H, 9.30. Found: C, 74.61; H, 9.33%.

2.2.14. (22R, 23R)-2α,3α,22,23-tetrahydroxy-23-(4-t-butylphenyl)-24-nor-5α-cholan-6-one ***(10s)***

The general procedure for dihydroxylation of **8s** (60 mg; 0.13 mmol) afforded 53 mg (77%) of the title compound **10s** as a white solid: m. p. 255–256 °C (EtOH); IR ν (cm^{-1}) 3234 vb, 2938, 1709, 1616, 1512, 1461, 1084, 991, 831. ^{1}H NMR (DMSO-d_6) δ 0.32 (H-18), 0.59 (H-19) (both s, 3H, CH_3); 0.85 (d, 3H, J = 6.4 Hz, H-21, CH_3); 1.26 (s, 3H, *t*-Bu); 1.77–1.88 (m, 2H); 1.98 (dd, 1H, J = 13.1, J' = 4.6 Hz); 2.07 (t, 1H, J = 12.6 Hz); 2.58 (dd, 1H, J = 12.3, J' = 3.4 Hz); 3.44 (m, 1H, H-2); 3.51 (d, 1H, J = 8.3 Hz, H-3); 3.74 (m, 1H, H-22); 4.20 (vb s, 2H, 2×OH); 4.32 (d, 1H, J = 8.3 Hz, H-23); 4.42 (vb s, 1H, OH); 4.99 (vb s, 1H, OH); 7.18 (d, 2H, J = 8.5 Hz); 7.33 (d, 2H, J = 8.5 Hz). ^{13}C NMR δ 11.44 (C-18), 12.48 (C-19), 13.33 (C-21), 20.77, 23.29, 26.81, 27.13, 31.19 (3×C, *t*-Bu), 34.21, 36.33, 37.00, 39.10, 40.10, 41.81, 42.03, 45.94, 50.27, 51.95, 52.84, 55.92, 67.08 (C-2), 67.47 (C-3), 74.95 (C-23), 76.05 (C-22), 124.72 (2×C), 126.75 (2×C), 140.23, 149.41, 211.53 (C-6). HRMS: (API+) calculated for $C_{33}H_{51}O_5$ ([M+H]$^+$) 527.3736, Found 527.3734. Anal. Calcd for $C_{33}H_{50}O_5$: C, 75.25; H, 9.57. Found: C, 75.22; H, 9.61%.

2.2.15. (22R, 23R)-2α,3α,22,23-tetrahydroxy-23-(4-phenylphenyl)-24-nor-5α-cholan-6-one ***(10t)***

The general procedure for dihydroxylation of **8t** (40 mg; 0.08 mmol) afforded 33 mg (72%) of the title compound **10t** as a white solid: m. p. 251–253 °C (EtOH); IR ν (cm^{-1}) 3328 vb, 2955, 1705, 1643, 1600, 1580, 1465, 967, 763. ^{1}H NMR (DMSO-d_6) δ 0.34 (H-18), 0.59 (H-19) (both s, 3H, CH_3); 0.89 (d, 3H, J = 6.4 Hz, H-21, CH_3); 1.78–1.89 (m, 2H); 1.97 (dd, 1H, J = 13.1, J' = 4.6 Hz); 2.07 (t, 1H, J = 12.6 Hz); 2.58 (dd, 1H, J = 12.1, J' = 3.0 Hz); 3.44 (m, 1H, H-2); 3.54 (d, 1H, J = 8.6 Hz, H-3); 3.74 (bs, 1H, H-22); 4.19 (bs, 1H, OH); 4.33 (bs, 1H, OH); 4.41 (d, 1H, J = 8.9 Hz, H-23); 4.55 (bs, 1H, OH); 5.15 (bs, 1H, OH); 7.33–7.37 (m, 3H); 7.45 (t, 2H, J = 7.6 Hz); 7.64 (d, 2H, J = 8.3 Hz); 7.68 (t, 2H, J = 8.3 Hz). ^{13}C NMR δ 11.49 (C-18), 12.52 (C-19), 13.35 (C-21), 20.79, 23.29, 26.81, 27.27, 36.52, 37.02, 39.12, 40.09, 41.83, 42.05, 45.95, 50.28, 51.92, 52.86, 55.95, 67.10 (C-2), 67.48 (C-3), 74.87 (C-23), 76.17 (C-22), 126.26 (2×C), 126.51 (2×C), 127.38, 127.67 (2×C), 128.96 (2×C), 138.74, 139.80, 142.58, 211.57 (C-6). HRMS: (API+) calculated for $C_{35}H_{47}O_5$ ([M+H]$^+$) 547.3423, Found 547.3421. Anal. Calcd for $C_{35}H_{46}O_5$: C, 76.89; H, 8.48. Found: C, 76.85; H, 8.53%.

3. Molecular docking

Docking was performed prediction of conformation and energy ranking between BRI1 receptor (PDBID: 3RGZ) and. Docking studies was carried out using AutoDock Vina 1.05 [13]. All 3D structures of BRI1 ligands were prepared with Marvin 5.10.3 [10], software which can be used for drawing, displaying and characterization of chemical structure, substructures and reactions. Ligands were prepared as derivatives of natural ligand brassinolide (BLD). Polar hydrogens were added to all ligands and proteins with the AutoDock Tools (ADT) [14] program prior to docking with Autodock Vina program. Grid box with size of 40 Å were centered on active site of protein. Exhaustiveness parameter was set to 20 (default 8). After docking we compared docked ligand with brassinolide crystal-like poses and the best crystal-like poses of each ligand were analyzed.

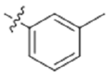
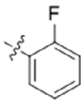
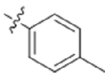
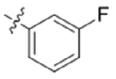
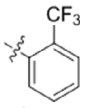
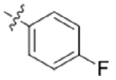
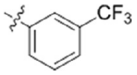
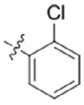
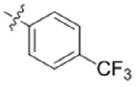
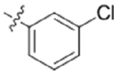
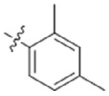
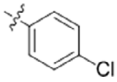
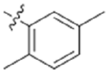
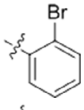
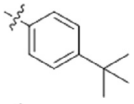
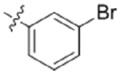
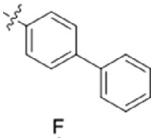
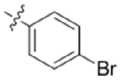
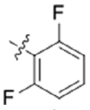
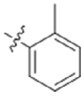
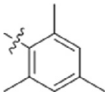
4. The pea inhibition biotest

Pea seedlings (*Pisum arvense* L. sort Arvica) germinating for 2 days were selected for uniformity from a large population and then transferred into pots containing perlite and 1/10 diluted Hoagland solution (half concentration, pH 5.7) After 24 h in a dark cultivation room (24 °C, humidity 75%) the seedlings were treated with different amounts of tested compounds in 5 µl fractionated lanolin. The substances were applied as microdrops to the scar left after the removal of bract. The control plants were treated with lanolin alone. At least seven plants were used for each experiment and the assays were repeated at least three times. The inhibition of etiolated pea stems were measured after 4 days and the difference in length between treated and control plants provided a measure of activity. For each treatment, 8 seedlings were analyzed in two biological replicates. The mean values were subjected to the statistical analysis using the Student's *t* test.

5. Determination of ethylene production

For measurement of ethylene production, pea seedlings (8 plants/tested amount of substance) were placed in a 0.5 L glass container for 24 h in the dark. One milliliter of headspace gas from the chamber was withdrawn for each measurement and injected into a gas chromatograph (Agilent Technologies, GC System, USA) equipped with a flame

Table 1
Isolated yields of products in alkene cross-metathesis.

Entry	Ar	Yield (%)	Entry	Ar	Yield (%)
a		81	l		69
b		65	m		71
c		73	n		57
d		75	o		66
e		64	p		71
f		70	q		61
g		75	r		58
h		59	s		60
i		66	t		32
j		73	u		na
k		65	v		na

na – compound not isolated.

ionic detector (FID) and a capillary column (HP-AL/S stationary phase, 15 lm, i.d. = 0.535). The chromatographic analytical parameters were as follows: column temperature: 150 °C; detector temperature: 220 °C; and helium was used as carrier gas. The area under the resultant peak (*y*-axis) *versus* sensitivity (*x*-axis; $nL.mL^{-1}$) was representing a quantitative measure of ethylene concentration. The measurements were done in triplicates and data were statistically analyzed using the Student's *t* test.

6. Arabidopsis brassinosteroid sensitivity assay

Arabidopsis thaliana L. (Heyhn.) (Columbia ecotype, Col-0; referred to Arabidopsis) seedlings were stratified for 2 d at 4 °C and germinated on vertical half-strength Murashige and Skoog (1% w/v sucrose) agar plates with different concentrations of 24-epiBL and BR derivatives at 22 °C in a 16 h/8 h light-dark cycle for 5 d. Roots were then straightened on solid media plates, scanned with an Epson high-resolution scanner and the entire root length measured with ImageJ (http://rsbweb.nih.gov/ij/). For each treatment, more than 15 seedlings were analyzed in two biological repeats. *P* values were calculated with a two-tailed Student *t*-test using Excel software.

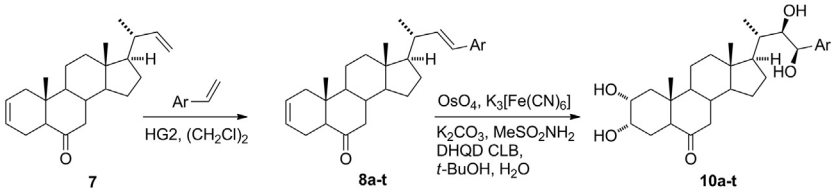
7. Cytotoxicity

Calcein AM cytotoxicity assay was performed exactly as described earlier [11].

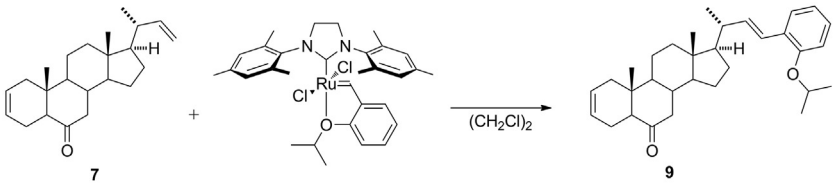
8. Results and discussion

8.1. Chemistry

For the preparation of above mentioned compounds we started with the known [15] 24-nor-5α-chola-2,22-dien-6-one (**7**). With this compound cross-metathesis were carried out using twenty commercially available styrenes substituted with fluorine, chlorine, bromine, trifluoromethyl, alkyls and phenyl group. The reaction was catalyzed by Hoveyda-Grubbs second generation catalyst in refluxing dichloroethane. This catalyst has proven to be efficient for different steroid side chain cross-metatheses [15–18]. Due to the fact that compound **7** and used styrenes belongs to the group of type I olefins (non-, meta- and para- substituted styrenes) and type II olefins (ortho- substituted styrenes) if Hoveyda-Grubbs second generation catalyst is used [19], the reactions proceed in two steps. Firstly, the homodimerization of styrene with strong release of ethylene takes place followed by the secondary cross-metathesis of steroid and *in-situ* formed stilbene. This is the main reason of long reaction time. In most cases, the cross-metathesis gave the corresponding 23-aryl products in good isolated yields 60–80% (see Scheme 1). Lower yield is typical for ortho-substituted styrenes as their homodimers are sparingly consumable during secondary cross-metathesis. In the case of 4-phenylstyrene the low yield (32%) is caused by formation of poorly soluble 4,4′-diphenylstilbene



Scheme 1. Preparation of aryldienones and aryl-tetraols. For Ar see Table 1.



Scheme 2. Formation of isopropoxy derivative **9** if bis-*ortho* substituted or no styrenes are used in the reaction.

Table 2
IC_{50} (mol/L) values obtained from the pea inhibition biotest. Compounds **10a**, **10d**, **10g**, **10j**, and **10m** are presented for comparison; the results were published [11].

Compound	IC_{50} (mol/L)
24-epibrassinolide	1.66×10^{-5}
10f	1.80×10^{-5}
10b	2.15×10^{-5}
10n	2.30×10^{-5}
10a	2.52×10^{-6}
10d	2.0×10^{-6}
10g	1.8×10^{-5}
10j	2.7×10^{-4}
10m	1.8×10^{-4}

during homodimerization step. No desired products were observed if bis-*ortho* substituted styrenes were used. In these cases we were able to isolate only very small amount of 2-isopropoxyphenyl derivative **9** as a product of reaction between steroid olefin and Hoveyda-Grubbs catalyst (see Scheme 2). This was probably caused by steric hindrance near steroidal terminal double bond. All products **8a-t** were obtained as *trans* double bond isomers as indicated by NMR analysis.

Next, Sharpless simultaneous dihydroxylation of both double bonds was used to minimize formation of unnatural configuration of 22 and 23 hydroxy groups. Hydroquinidine 4-chlorobenzoate was used as chiral ligand. The reaction rate was increased by addition of methansulfonamide [20]. Such reaction conditions led only to the desired 22*R*,23*R*-isomers **10a-t** in very good yields (75–83%).

All compounds were characterized by NMR, IR and MS techniques together with elemental analysis for all tetraols. Compounds **10a**, **10d**, **10g**, **10j**, and **10m** were also prepared by different synthetic strategy [11] (see Table 1).

8.2. Biological activity and docking

Biological activity of new BRs derivatives was monitored by pea inhibition biotest. This test is based on that BRs inhibit the growth of etiolated seedlings at high concentration and the inhibition is probably caused by ethylene production which is mediated by BRs. Dose response curves for all prepared BRs derivatives are shown in SFig. 1. The IC_{50} values obtained from the pea inhibition biotest are summarized in Table 2. The most active BRs derivatives were **10f**, **10b**, and **10n** (IC_{50} 1.8×10^{-5}–2.3×10^{-5} mol.L^{-1}) compared to 24-epibrassinolide (IC_{50} 1.6×10^{-5} mol.L^{-1}), used as a positive control (Fig. 2).

The crosstalk of BRs and ethylene regulates various aspects of plant growth and development. Hansen et al. [21] showed that exogenously

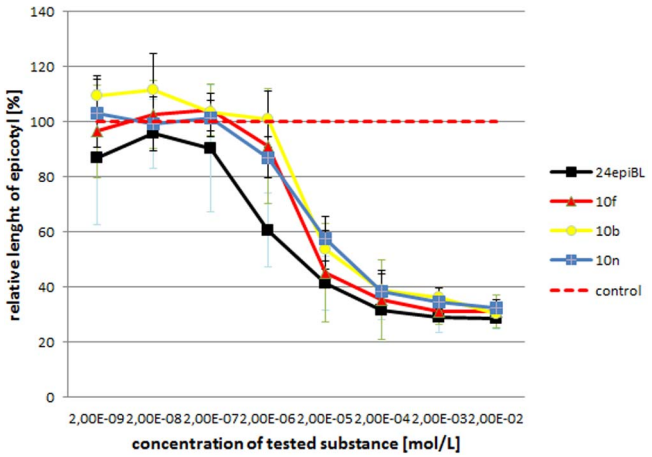


Fig. 2. Effect of selected brassinosteroid derivatives on the inhibition of etiolated pea seedlings. Error bars represent s.d.

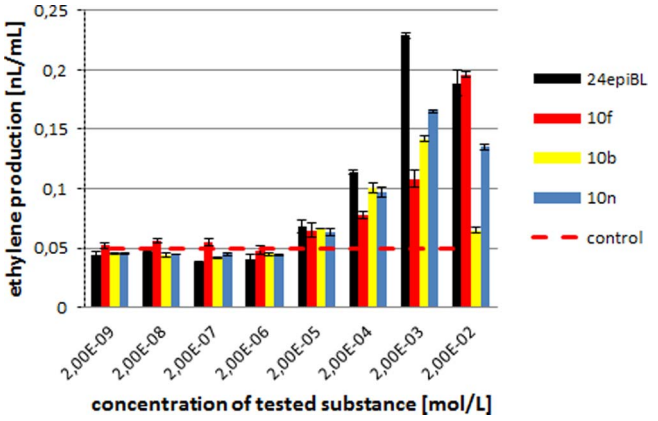


Fig. 3. Effect of selected brassinosteroid derivatives on ethylene production (nL.mL^{-1}) in etiolated pea seedlings determined by GC-FID 24 h after ventilation. Error bars represent s.d.

applied BRs stimulate ethylene production by stabilizing the ACS protein. When low levels of ethylene are applied to etiolated pea seedlings, the characteristic triple response can be observed: inhibition of stem elongation, radial swelling of the stem and the absence of a normal geotropic response [22]. Production of ethylene was measured in cultivation vessels during the incubation of etiolated pea plants after treatment of different BR derivatives (SFig. 2). The high concentrations of ethylene (196, 142 and 165 nL.L^{-1}) were determined after treatment of **10f**, **10b and 10n** and compared to 229 nL.L^{-1} for 24-epiBL treatment. While level of this gaseous plant hormone produced by untreated control pea plants, was found to be significantly lower (about 60 nL.L^{-1}, Fig. 3).

BRs derivatives were further assessed for biological activity based on their inhibitory effect on Arabidopsis root growth [23]. The effects of compounds **10f**, **10b** and **10n** on the Arabidopsis roots are shown in Fig. 4. Results of all prepared analogues are summarized in SFig. 3.

Molecular docking into BRI1 receptor shows that several compounds binds with very high binding energy. In some cases the binding energy was even better than for naturally occurring brassinolide (−10.6 kcal/mol) [11] (see SI for molecular docking of all new compounds). The high binding affinity of compounds **10f** (−11.0 kcal/mol), **10b** (−10.7 kcal/mol), and **10n** (−11.9 kcal/mol) also proves the good biological activities of these analogues (Fig. 5).

Relationship between biological activity and substitution pattern in the phenyl group shows that no substitution or substitution with one small group (fluorine, chlorine) led to promising compounds with high

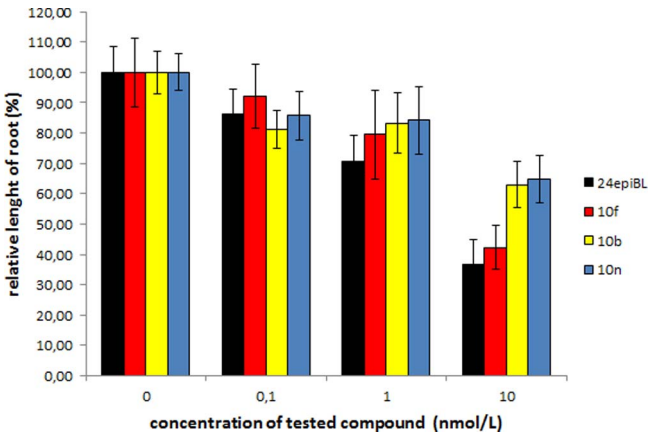


Fig. 4. Effect of selected brassinosteroid derivatives on the inhibition of Arabidopsis root lenght. 5 days old *Arabidopsis thaliana* seedlings (Columbia ecotype, Col-0) were treated by DMSO/24epiBL/BR analogues. For each treatment more than 25 seedlings were analyzed in two biological repeats.

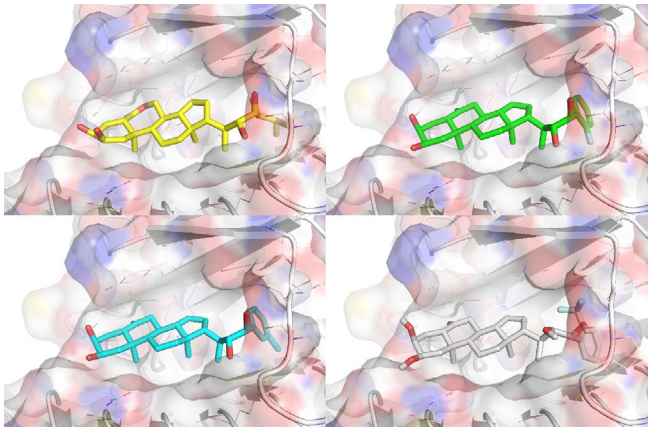


Fig. 5. Poses of 24-epibrassinolide (yellow) and three new analogues of brassinosteroids, **10b** (green), **10f** (cyan), and **10n** (grey) within BRI1 binding site.

plant activities. The most active are compounds **10a** (no substitution), **10b** and **10c** (fluorine in *ortho* and *para* position), and **10f** (chlorine in *meta* position). On the other hand, substitution with bulky groups (phenyl, *t*-butyl, methyl or trifluoromethyl) or substitution with more than one group (e.g. **10q** and **10r**) causes significant decrease or complete loss of plant activity. The only exception from bulky groups is compound **10n** which showed good activity in two of the three assays. This may be explained by smaller interaction of substituents in *ortho* position with receptor cavity – the group can be oriented out of the cavity.

Antiproliferative activity of BRs derivatives was screened towards various tumor cell lines and normal cells, including T-lymphoblastic leukemia CEM, breast carcinoma (MCF7) and cervical carcinoma (HeLa) and human foreskin fibroblasts (BJ). All tested BR analogues had no detectable cytotoxic activity, even when tested in concentrations up to 50 μM (data not shown).

9. Conclusions

Overall 15 novel and 5 known phenyl analogues of brassinosteroid were synthesized *via* alkene cross metathesis. The metathesis showed as an effective method for preparation of new brassinosteroid derivatives with plant growth promoting activities comparable with natural brassinosteroids. The results of biological screenings showed that molecular docking into BRI1 is a powerful tool for prediction and design of new compounds with strong brassinosteroid activities. Very potent brassinosteroids analogues prepared by this synthesis can be also used for potential application in agriculture to improve growth and yield or to increase the resistance of plants against various biotic and abiotic stresses.

Acknowledgments

This work was supported by grant GJ15-08202Y of the Grant Agency of the Czech Republic and by the by project of the Ministry of Education, Youth and Sports CR NPUI LO1204. This work was also supported by student project IGA_PrF_2017_013 and IGA_PrF_2017_028 of the Palacky University in Olomouc.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2017.08.010.

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International Journal of
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Review

Crosstalk between Brassinosteroids and Ethylene during Plant Growth and under Abiotic Stress Conditions

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Received: 30 September 2018; Accepted: 20 October 2018; Published: 22 October 2018

Abstract: Plant hormones through signaling networks mutually regulate several signaling and metabolic systems essential for both plant development and plant responses to different environmental stresses. Extensive research has enabled the main effects of all known phytohormones classes to be identified. Therefore, it is now possible to investigate the interesting topic of plant hormonal crosstalk more fully. In this review, we focus on the role of brassinosteroids and ethylene during plant growth and development especially flowering, ripening of fruits, apical hook development, and root and shoot growth. As well as it summarizes their interaction during various abiotic stress conditions.

Keywords: brassinosteroid; ethylene; plant growth; stress tolerance

1. Introduction

To date, nine groups of plant hormones have been identified, i.e., auxins, brassinosteroids, cytokinins, gibberellins, ethylene, jasmonic acid, strigolactones, abscisic acid, and salicylic acid. Genetic and physiological studies have revealed the critical roles of these phytohormones in plant growth and development, as well as plant responses to various biotic and abiotic stresses [1].

Brassinosteroids (BRs) are a class of polyhydroxylated steroidal hormones that regulate various aspects of plant growth and development. They were initially identified based on their growth promoting activities, but subsequent physiological and biochemical studies have revealed additional functions of BRs in regulating a wide range of processes, including seed germination, senescence, polarization of cell membranes and photosynthetic efficiency. Recently, it has been reported that BRs increase plant tolerance to stress factors, e.g., salt, drought, temperature, and heavy metals [2,3].

Ethylene, the first identified gaseous plant hormone, has a simple two-carbon structure. Nevertheless, it has been shown to regulate many diverse developmental and physiological processes in plants. In etiolated seedlings, ethylene causes a typical "triple response", consisting of exaggerated curvature of the apical hook, inhibition of stem elongation and radial swelling of the hypocotyl. Besides the triple response, ethylene is involved in every phase of the plant life cycle, e.g., seed germination, root hair development, root nodulation, flower senescence, abscission, and ripening of fruit. Moreover, ethylene acts as a stress hormone during biotic and abiotic stress conditions [4].

Plant hormone crosstalk is a complex topic of broad and current interest. In this review, we provide a comprehensive overview of the interaction of BRs and ethylene during plant development and under abiotic stress conditions (Figure 1).

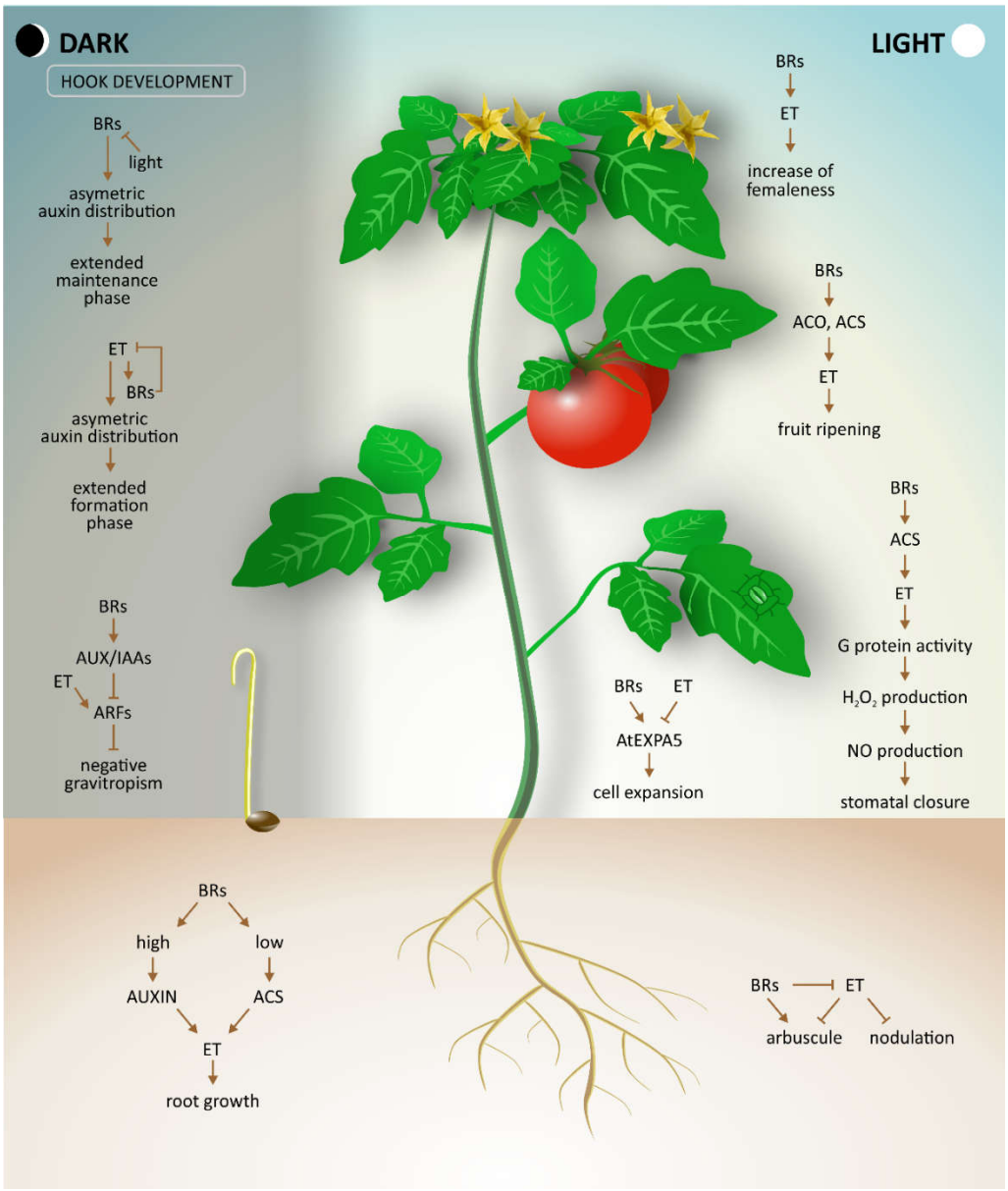

Figure 1. Simplified model of brassinosteroids and ethylene crosstalk showing the effects of these two hormones during plant growth and development. Arrows indicate stimulatory effect and blunted lines indicate inhibitory effect. See corresponding sections of the text for details and references.

2. Root Growth

Roots are an important underground part of vascular plants with two main functions—fixing plants in a soil and absorption of water and nutrients. Hence, well-developed roots are crucial for proper growth and development of the whole plant. In higher plants, control of root growth is mainly associated with auxins and cytokinins as positive and negative regulators, respectively [5]. However, other plant hormones and their interactions play an important role in diverse growth processes in roots. In addition, other signal molecules, such as reactive oxygen species (ROS), play valuable roles in root development [6].

Interaction between BRs, ethylene, and ROS has been examined by Lv et al., 2018 [7]. In their study, an *Arabidopsis* mutant (*det2-9*) with a defect in BR synthesis was identified based on its short-root phenotype by EMS mutant screening. Because both ROS and ethylene signaling were enhanced in the *det2-9* mutant, it was suggested that the short-root phenotype resulted from hyper-accumulation

of ethylene and superoxide anions (O_2^-). Exogenous application of BRs showed that they either positively or negatively regulated the biosynthesis of ethylene depending on the applied concentration. In seedlings treated with a low concentration (10 or 100 nM) of 24-epibrassinolide (EBL), ethylene production was greatly reduced, whereas treatment with higher concentrations of EBL ($\geq$ 500 nM) caused a strong increase. Accordingly, BRs at low concentrations (10–100 nM) inhibited expression of ethylene response factors (ERFs), whereas high concentrations ($\geq$500 nM) enhanced ERF expression, consistent with the observed changes in ethylene levels after treatment with BRs. Chromatin immunoprecipitation (ChIP)/qPCR analysis confirmed direct interaction of ACSs (1-aminocyclopropane-1-carboxylic acid synthases, crucial enzymes in the ethylene biosynthetic pathway) by BES1 or BZR1 (brassinosteroid-regulated transcription factors). This interaction appeared as inhibition because over-expression of both BES1 and BZR1 strongly suppressed the activity of ACS promoters. qRT-PCR results using BR-insensitive mutants indicated increased expression of ACSs. Altogether, these findings suggest that at physiological levels, BRs regulate the repression of ethylene biosynthesis via the BES1 and BZR1 transcription factors, whereas at high levels, BRs induce ethylene biosynthesis by increasing the stability of ACSs and influencing auxin signaling, increasing ethylene production. It was also shown that BRs (via the peroxidase pathway) inhibited the synthesis of O_2^-, thereby controlling root growth, because of hyper-accumulation of O_2^- contributed to the short-root phenotype in the *det2-9* mutant [7].

Not only is the regulation of longitudinal growth, but also directional growth, important for proper root development. Gravitropism and the elongation of roots can be modulated by various environmental signals. Singh and co-workers [8] showed that enrichment of the medium with glucose (Glc) broadly modulates seedling root growth direction and simultaneous application of BRs dramatically enhances this modulation. In particular, Glc caused root deviation from straight vertical growth and this deviation was dose-dependent on Glc content in the medium. Experiments suggested that Glc may enhance BR signaling via enhancing BRI1 endocytosis from the plasma membrane to early endosomes. Follow-up work [9] focused on the interplay of other phytohormones and Glc in controlling root directional growth. The main findings of this work were that the presence of cytokinins and ethylene could abolish deviation of roots growing on medium enriched with Glc/BRs and they (cytokinins and ethylene) could also act antagonistically with BRs in the case of directional growth regulation. Further experiments with various mutants suggested that cytokinin signaling works downstream to BRs and antagonizes the Glc induced root directional response via ethylene-mediated machinery [9].

3. Shoot Growth and Apical Hook Development

The growth of shoots is the direct result of cell elongation, which is controlled by a complex system of phytohormone interaction. BRs are plant hormones with strong cell-promoting activity. In 2014, Bergoci et al. [10] described one of many mechanisms by which BRs promote cell elongation. Their proposed model scheme included interference between BRs and a rapid alkalization factor (RALF) comprising peptides belonging to compounds with inhibitory activity on growth. Simultaneous treatment with AtRALF1 and brassinolide (BL) induced lower levels of AtRALF1-inducible cell wall remodeling genes *AtPRP1*, *AtPRP3*, and *AtHRGP2*, which are responsible for cell wall hardening and inhibition of further elongation. In additional experiments, it was observed that plants with a partially silenced *AtRALF1* gene showed increased levels of the expansine gene *AtEXPA5* involved in cell expansion [10]. A previous study [11] showed that exogenously applied BRs increase levels of AtEXPA5, suggesting an antagonistic effect between AtRALF1 and BR in the regulation of expansine genes. In contrast, ethylene was found to reduce AtEXPA5 expression levels, thereby regulating growth of the hypocotyl [12]. These results suggest that AtRALF1 and ethylene may act together to achieve the same effect [10].

Another study dealing with the influence of ethylene and BRs on hypocotyl development was published in 2013 [13]. The study involved screening and identifying mutant plants of *Arabidopsis*

with an altered response to acsinone7303, which is a small molecule that can act like an uncompetitive inhibitor of ACS. Treatment of etiolated *eto1* mutant seedlings with acsinone reduced ethylene levels and suppressed the triple response. Several *ret* mutants with reduced sensitivity to acsinone7303 were identified and two of them (*ret8* and *ret41*) were characterized. Map-based cloning revealed that *ret8* carried a mutation in CESA6 (cellulose synthase 6, part of the primary wall CESA complex), whereas *ret41* carried a mutation in DET2 (de-etiolated-2, an enzyme catalyzing the reduction of campesterol to campastanol in the BR biosynthetic pathway). Etiolated seedlings of both mutants exhibited short hypocotyls and roots even when the *eto1* mutation was removed, indicating that the hypocotyl phenotype did not entirely depend on elevated levels of ethylene. Furthermore, addition of chemical inhibitors of ethylene biosynthesis and perception did not effectively suppress the triple response in $cesa6^{ret8}$ and $det2^{ret41}$ mutants. This indicates that the short hypocotyls in etiolated $cesa6^{ret8}$ and $det2^{ret41}$ mutants were probably caused by loss-of-function mutations of CESA6 and DET2, respectively, which both play an independent role in seedling development. However, an abundance of ethylene in eto1 enhanced the short hypocotyl phenotype in *cesa6* and *det2*. Additional experiments with EBL treatment of *eto1*, *det2-1*, and $det2^{ret41}$ showed that the balance between levels of ethylene and BRs was important for proper regulation of hypocotyl growth [13].

Not only cell elongation, but also gravitropic growth is also crucial for proper shoot development. Vandenbussche et al. [14] have shown that ethylene and BRs have opposing effects in regulating shoot gravitropism in darkness—ethylene enhances and BRs reduce gravitropic growth. Experiments in the presence of ethylene inhibitors showed that a lack of ethylene signaling enhances BR sensitivity, suggesting that endogenous ethylene may stimulate shoot gravitropism by reducing the sensitivity to BRs. It is probable that ethylene and BRs control the same downstream components even though they act in opposite ways. Additional analysis showed that both hormones regulate overlapping sets of *AUX/IAA* genes, implying that the effect of both hormones is performed through auxin signaling [14].

Early development of the *Arabidopsis* hypocotyl is accompanied by the formation of an apical hook, which protects the shoot apical meristem cotyledons as the seedling grows through the soil. As apical hook development is an important process following seed germination, all phases of hook development, such as hook formation, hook maintenance, and opening of the hook, are tightly regulated by the complex crosstalk of multiple hormones [15]. Both BRs and ethylene have been demonstrated to be indispensable for hook development [16]. Experiments have indicated that ethylene prolongs the formation phase of the hook development, whereas BRs prolong the maintenance phase, thereby delaying the hook opening phase [16]. Moreover, additional observations of the hook development process in plants treated with ethylene precursor ACC, EBL, (Figure 2) and an inhibitor of BR biosynthesis, brassinazole (BRZ, Figure 2), showed that ethylene-induced exaggeration of the apical hook curvature and shortening of the maintenance phase require normal BR biosynthesis. These findings were confirmed by various experiments investigating diverse BR biosynthesis as well as ethylene signaling mutants.

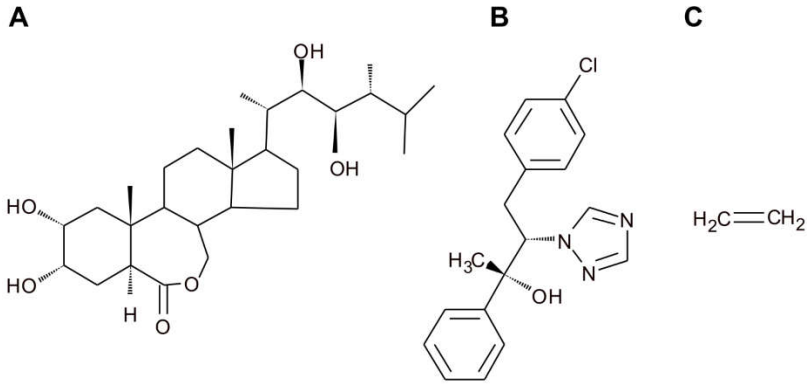

Figure 2. Structures of 24-epibrassinolide (**A**); brassinazole (**B**); and ethylene (**C**).

4. Flowering

The formation of flowers is a critical developmental stage because it has a direct influence on plant reproduction and yield. The *Cucurbitaceae* family is well-known for its diversity of sex expression phenotypes. Generally, in these plants, male flowers are produced early during plant development, followed later by female or bisexual flower production. Popadopoulou and Grumet [17] investigated whether BRs are involved in this process of cucurbit sex expression. They chose three different species (cucumber, melon, and zucchini) as experimental model plants. After treatment of cucumber plants with BRs, a shorter duration before appearance of the first female flower and increased production of female buds were observed. At the same time, ethylene production rose, suggesting that the effect of BRs was mediated by ethylene. Although zucchini and melon plants showed a similar increase in ethylene production as cucumber, increased femaleness was not observed in these plants after treatment with BRs. It was deduced that this was possibly because different species have different sensitivity to ethylene. Thus, in the proposed mechanism of interplay between BRs and ethylene during flower development in *Cucurbitaceae*, BRs were assumed to act indirectly via increased ethylene production with an increase in femaleness dependent on the sensitivity of specific species to ethylene.

Similar results demonstrating that ethylene has a major effect on sexual expression and flower development were shown in a more recent study from 2011. Manzano and co-workers [18] studied the effect of ethylene and BRs on flower development in different lines of *Cucurbita pepo* plants, i.e., *Bolognese (Bog)* and *Vegetable Spaghetti (Veg)*, which differ in ethylene production and sensitivity. The results showed variation in the sensitivity to ethylene among the analyzed genotypes. In the *Veg* line, ethephon (ethylene-releasing compound) induced earlier and higher production of female flowers, whereas in the *Bog* line, treatment with ethephon did not significantly alter the sexual expression. Additional data showed that the *Bog* line produced more ethylene and was more sensitive to this hormone, whereas the *Veg* line was characterized by lower production of and less sensitivity to ethylene, suggesting that this was the reason why both lines differed in their sexual expression. Further results indicated that BRs play a minor role in the control of sexual expression in *Cucurbita pepo* in comparison with ethylene [18]. The authors suggested that BR-induced ethylene may be dependent on the ethylene response, since treatment with BRZ (an inhibitor of BR biosynthesis) reduced ethylene production in the *Bog* line but increased it in the ethylene insensitive *Veg* line. Taken together, these results agreed with the previous study that the differential effect of BRs on the sexual expression of the different genotypes of *Cucurbita pepo* is probably due to the different sensitivity of these lines to ethylene [18].

5. Ripening and Postharvest Development of Fruit

The terminal stage of plant development is ripening of fruit, which makes fruit attractive and palatable to many seed-dispersing organisms. Because ripe fruit also represents a large proportion of the human diet, ripening makes fruit a valuable agricultural commodity. The process of ripening includes biochemical and physiological changes, such as modification of cell wall structure, conversion of starch to sugars, alterations in pigment biosynthesis, and heightened levels of flavor and aromatic volatiles. Based on respiration and ethylene biosynthesis rates, two major classifications of ripening fruit can be distinguished, i.e., climacteric and non-climacteric. Ripening of climacteric fruits, such as tomatoes, cucurbits, avocados, and bananas, is accompanied by increased respiration and ethylene biosynthesis. In contrast, non-climacteric fruits, such as citrus, do not require ethylene for their ripening [19].

The effect of BRs on quality attributes of ripening fruits and ethylene synthesis has been investigated in a recent study [20]. Tomatoes, typical climacteric fruits, were used as a model system for studying the role of BRs and ethylene during ripening. Changes in gene expression of BR synthesis were observed during tomato fruit development, suggesting that BRs might play an important role in this process This was confirmed by other experiments, in which BL-treated tomato fruits showed decreased total chlorophyll content and increased lycopene content, whereas fruits treated with BRZ

displayed minor degradation of chlorophyll and lower lycopene content than the control or BR treated tomatoes. Overall, BL treatment accelerated ripening of tomato fruit, whereas treatment with BRZ delayed ripening. The same study showed that BRs can accelerate postharvest ripening of tomatoes, probably via increased ethylene production. This was demonstrated by gene expression analysis, which showed a sharp increase in the expression of genes involved in the regulation of ACS and ACO protein synthesis (LeACS2, LeASC4, LeACO1, and LeACO4) in BR treated fruit. In contrast, transcript levels of these genes were significantly depressed in tomatoes treated with BRZ [20].

A very recent study dealing with roles of BRs and ethylene during the fruit ripening point out, that at least in case of bananas, endogenous and exogenous BRs can play opposite roles in the process of ripening. In this work, authors proved that application of different concentrations of BRs promote the ripening of bananas, possibly via up-regulation of ethylene biosynthetic genes and consequently the acceleration of ethylene production. Furthermore the authors characterized three *BZR* genes in bananas (*MaBZR1, MaBZR2, and MaBZR3*). These genes encode proteins (MaBZR1-3) which belong to BZR1/BES1 transcription factors family with a central role in BR response. Both the continuous decrease of *MaBZR1-MaBZR3* expression in process of ripening as well as the suppression of a *MaBZR1-3* promoter activity indicate that MaBZR1-MaBZR3 play negative role in banana ripening. In addition, MaBZR1/2 act like a transcription inhibitors with a binding activity to element present in the promoters of ethylene biosynthetic genes (*MaASC1, MaACO13, and MaACO14*). For better understanding how high levels of BRs affect the BZR1/BES1 module regulating ethylene biosynthetic genes that turn in increase ethylene production more research is still needed [21].

Another recent article [22] focused on non-climacteric fruits and the effect of BRs/ethylene on their ripening. In this work, strawberries were used as a model study of non-climacteric fruits and they were treated with an exogenous spray of ethylene (ethephon) and EBL. The results showed that the level of phenolic compounds was influenced by both phytohormones: application of BRs tended to reduce the phenolic compound content, whereas ethylene treatment increased it. High levels of phenolic compounds caused by ethylene treatment resulted in senescence, whereas reduction of the phenolic content by BRs promoted fruit conservation as a result of increased antioxidant activity.

6. Stress Response

Both hormones (BRs and ethylene) not only play a role in plant growth and development but are also well known as hormones involved in plant responses to biotic and abiotic stresses [23–26]. The main interactions between BRs and ethylene during various abiotic stress conditions are presented in Table 1 and Figure 3.

One example by which these two hormones interact during abiotic stresses is by inducing an alternative respiratory pathway, as suggested in a recent work [27]. In this study, cucumber seedlings were exposed to salt, drought, and cold stress conditions. Pretreatment with BL (the most active BR) resulted in enhanced ethylene biosynthesis and capacity of the alternative oxidase pathway (AOX) in cucumber seedlings under stress conditions. After additional experiments investigating the relationship between ethylene and ROS (H_2O_2), a hypothetical model describing the function of BL, ethylene and ROS in the BL-induced AOX capacity was proposed. In this model, BRs induced ethylene and ROS generation, which subsequently enhanced AOX capacity. Enhanced activity of AOX can eliminate excess ROS generation to avoid oxidative damage in plant cells and improve their stress tolerance.

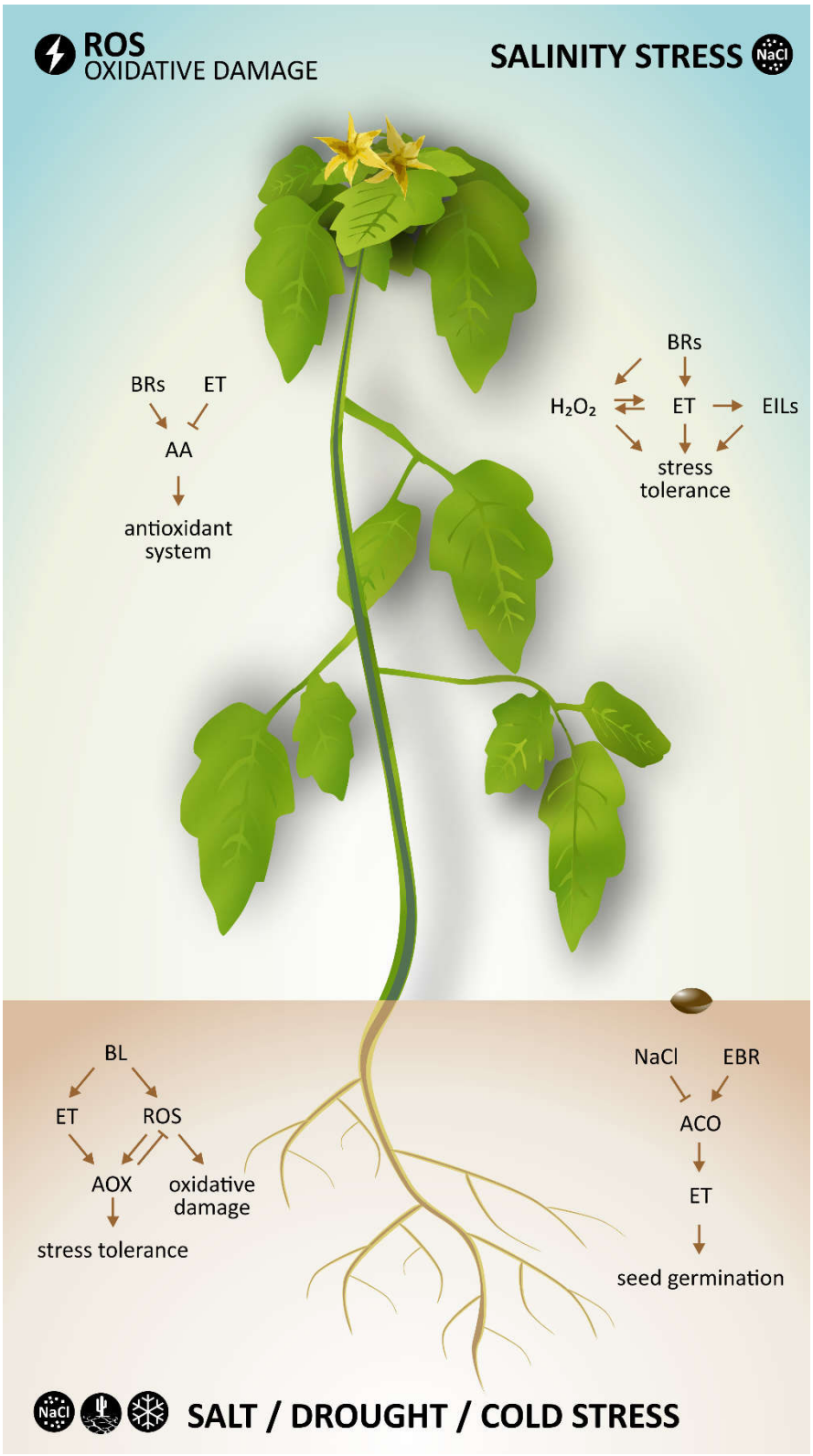

Figure 3. A general simplify model of BRs and ethylene interaction during abiotic stresses. Arrows indicate stimulatory effect and blunted lines indicate inhibitory effect. See corresponding sections of the text for details and references.

The rate of transpiration and plant water loss is regulated by the opening and closing of stomata, microscopic pores on the surfaces of leaves and stems which are bounded by two guard cells. Hence,

stomata play a key role in a plant's protection against water stress and pathogens. Stomatal opening and closing relies on reversible fluctuations of turgor and osmotically induced water flow in the guard cells. This crucial movement is triggered by various endogenous and exogenous stimuli. Thus, investigation of this opening/closing mechanism is important for understanding how plants defend against water stress and pathogens [28]. Stomatal movement is regulated by multiple plant hormones participating in a complex network of signaling pathways. The best known plant hormone linked with stomatal closure is ABA (abscisic acid), but a recent study has shown that BRs and ethylene also influence this process [29]. The results also indicated that BRs close stomata in a dose- and time-dependent manner. Experiments with *bri1-301* mutant plants containing a mutation in the BRI1 kinase domain, which leads to reduced sensitivity to BR, showed that BRs have a specific effect on stomata closure and that functional BRI1 receptor is essential for this process. Because treatment with EBR was found to significantly increase ethylene production, the study also tested whether ethylene was involved in BR-induced stomatal closure. In further experiments, EBR-induced stomatal closure was shown to be completely abolished in Arabidopsis ethylene-insensitive mutants (*etr1-1* and *etr1-3*), suggesting that ethylene plays an essential role in mediation of BR-induced stomatal closure. Furthermore, it was shown that both H_2O_2 (a form of ROS) and NO (nitric oxide) are involved as signaling molecules in BR-induced stomatal closure and that the BRI1 receptor is required for generation of H_2O_2 and NO induced by BRs in guard cells in *Arabidopsis*. Additional experiments with ethylene synthesis and ethylene perception inhibitors, as well as with mutants exhibiting a lesion in producing H_2O_2 and NO, suggested that ethylene mediates BR-induced stomatal closure by inducing the synthesis of H_2O_2 and NO in guard cells. Finally, the study presented genetic evidence that Gα (G protein α-subunit) acts as a positive regulator and mediates the action of ethylene in BR-induced stomatal closure upstream of H_2O_2 and NO production. Moreover, it indicated that H_2O_2 induces production of NO in BR-induced stomatal closure. Based on all these findings, a model of BR-induced stomatal closure was proposed, whereby binding of BR into the BRI1 receptor induces ACS expression and ethylene synthesis. Additionally, increased ethylene activates Gα, which stimulates production of H_2O_2 and subsequent production of NO, culminating in stomata closure [29].

One of the most crucial processes, seed germination, is affected by various stress conditions. For instance, salinity stress suppresses seed germination. Wang et al. [30] studied the effect of BRs and ethylene on the germination of cucumber seeds under salinity stress and showed that the inhibitory effect of salt (due to the presence of NaCl) on seed germination was significantly ameliorated by addition of EBR or ACC into the incubation medium. Moreover, seed germination was greater in the presence of EBR and ACC together, suggesting that these hormones may have combined alleviating effects on seed germination under salinity stress. Changes in ethylene production were also observed in this work. In the presence of NaCl in the incubation medium, imbibed seeds produced less ethylene. Addition of EBR to the medium significantly alleviated the salt-induced suppression of ethylene production of imbibed seeds. It was also shown that the suppression of ethylene production under salt stress was caused by the inhibitory effect of NaCl on the ethylene biosynthetic enzyme ACO (ACC oxidase) and that ACO activity could be reversed by treatment with EBR. Based on these findings, it was concluded that EBR affects seed germination under saline stress conditions by regulating ethylene production via recovery of NaCl-induced suppression of ACO activity [30].

An important study dealing with salt stress and crosstalk between BRs and ethylene was recently published by Zhu et al. [31]. In this work, the mechanism by which BRs induce salt tolerance in tomato plants was investigated. An increase in H_2O_2 and ethylene production in tomato seedlings treated with BL was observed, indicating that H_2O_2 and ethylene are involved in BR-induced stress tolerance. The results also demonstrated that both BRs and ethylene could promote H_2O_2 generation. Based on the results, a model for interactions between BRs, ethylene and ROS during salt stress was proposed. The model considered that BRs affect ethylene biosynthesis and signaling by increasing ACS (ethylene synthesis hormone) activity and stabilizing EILs (ethylene-insensitive3-like, ethylene

transcription factor family), respectively, which is at least partially caused by BR-induced generation of H_2O_2. Further, increased levels of both ethylene and H_2O_2 lead to salt stress tolerance [31].

In plants, many stress conditions can cause oxidative damage. Thus, plant cells need a sophisticated central antioxidant system. Interaction of ascorbic acid (AA) and glutathione (GSH) play a crucial role in this antioxidant system to protect plants against oxidative damage. Ascorbic acid also has other physiological roles, e.g., regulation of photosynthesis and cell growth in plants [32]. Both BRs and ethylene have been shown to alter ascorbic acid-glutathione (AA-GSH) levels in tomato plants. Using a combination of genetics and chemical application, Mazorra et al. [33] showed that BRs and ethylene signaling pathways act antagonistically during regulation of AA content in tomato leaves, i.e., BRs promote AA accumulation in tomato leaves, whereas ethylene suppresses it. However, this antagonistic regulation of AA content seems to occur via independent mechanisms, i.e., normal ethylene signaling is not required for the BR effect and endogenous BRs are not critical for ethylene action [33].

Table 1. Interactions of brassinosteroids and ethylene during various abiotic stresses.

Type of Stress	Species	Applied Regulator	Hormonal Interactions	Physiological Effect	References
salt drought cold	*Cucumis sativus*	BL	BL enhanced ET biosynthesis	BRs induced ET and ROS generation, which subsequently enhanced AOX capacity leading to increase of stress tolerance	Wei et al., 2015 [27]
salt	*Cucumis sativus*	EBR	EBR ameliorated the inhibitory effect of salt on ethylene production	EBR affects seed germination under saline stress conditions by regulating ethylene production via recovery of NaCl-induced suppression of ACO activity	Wang et al., 2011 [30]
salt	*Solanum lycopersicum*	BL	BRs affect ethylene biosynthesis and signaling by increasing ACS and stabilizing EILs respectively	BRs induce generation of ET and H_2O_2 and increased levels of ET and H_2O_2 lead to salt stress tolerance	Zhu et al., 2016 [31]
oxidative	*Solanum lycopersicum*	EBL 1-MCP	BRs and ET signaling pathways act antagonistically during regulation of AA content in leaves	BRs promote AA accumulation in tomato leaves, whereas ET suppresses it.	Mazorra et al., 2014 [33]
salt	*Lactuca sativa*	DI-31	DI-31 caused a decrease in ethylene synthesis	Pretreatment with DI-31 decrease the negative effect of salinity on the fresh weight and prevent the reduction in weight of lettuce plants	Serna et al., 2015 [34]

Another study into the BR-ethylene interplay during protective processes against salt stress used DI-31, a BR analogue with a spiroketalic ring instead of the typical BR side chain as a cheaper alternative of BR. Lettuce plants were chosen as an experimental model of a moderately salt tolerant vegetable. After saline treatment (100 mM NaCl), a decrease in the fresh weight of both roots and shoots was observed. Pretreatment with DI-31 decreased the negative effect of salinity on the fresh weight and prevented the reduction in weight of lettuce plants. The effect of this BR analogue on ethylene emission was also examined. Without pretreatment with DI-31, plants produced more ethylene, whereas treatment with DI-31 caused a decrease in ethylene synthesis. A high correlation between the fresh weight and ethylene level caused by salt stress and possible DI-31 treatment was also observed, indicating that the synthesis of ethylene and reduction of plant weight were the result of salinity stress and that BR treatment enabled better tolerance to salinity. In addition, free ACC levels highly correlated with ethylene emission caused by NaCl treatment, which may signify that activation of ACO and ACS activity due to NaCl. DI-31 pretreatment decreased the free ACC content in tested

lettuce plants. It was suggested that this BR analogue may cause lower activity of ethylene biosynthetic enzymes, e.g., ACC synthase or ACC oxidase, thus decreasing ACC and ethylene production during salinity stress and helping to protect lettuce plants against salinity [34].

Various abiotic stress conditions, such as drought or salinity, also influence symbioses between plants and microorganisms, and subsequently the uptake of essential nutrients. A recently identified ethylene signaling mutant of pea *Psein2* [35] has been studied to examine whether the interaction between BRs and ethylene may influence mycorrhizal development. Compared with wild-type pea plants, *Psein2* mutants exhibited a significant increase in the number of nodules formed for a given root mass. Moreover, these nodules were smaller and more closely spaced. After treatment with ethephon, an ethylene-releasing compound, elevated ethylene levels, which can occur in plants under stress, were achieved. In wild-type plants, ethephon treatment caused a significant reduction of fungal colonization of roots, whereas this response was absent in *ein2* mutants. These results suggest that ethylene is a negative regulator of mycorrhizal colonization. A reduced number of nodules was also characteristic for the brassinosteroid-deficient mutant *lk* [36]. To examine the interaction between BRs and ethylene on nodulation, phenotypes of the double mutant *lk ein2* were examined. Compared to *lk* single mutants, *ein2* background dwarf *lk* mutants showed considerably increased numbers of nodules and reduced nodule spacing. Nodules on the double mutant were found to be pink and appeared functional. These data suggest that BRs may stimulate initiation of nodules by affecting ethylene levels but do not affect following nodule development. With regard to arbuscular mycorrhizas, the *lk* mutation was found to reduce total root colonization by the fungus. Using the *lk ein2* double mutant, interaction of BRs and ethylene during this process could be tested. It was observed that the decrease in mycorrhizal colonization in *lk* mutant plants was comparable with low arbuscular colonization of *lk ein2* mutants, indicating that BRs have a primary effect on mycorrhizal colonization rather than acting indirectly via altered ethylene production. In summary, by using genetic studies, it was shown that ethylene influences both nodule number and arbuscular mycorrhizal colonization. However, further experiments with *lk ein2* double mutants suggested that a major part of the BR effect on modulation number may be due to elevated ethylene production, whereas the effect of BRs on colonization by mycorrhizal fungi is likely direct rather than indirect via ethylene signaling [37]. Another example of using symbiosis to overcome biotic and abiotic stresses is a microbial association of endophytic bacterium (*Enterobacter* sp. SA 187) and the desert pioneer plant *Indigofera argentea* Burm.f. (*Fabaceae*) [38]. Following experiments have shown that *Enterobacter* sp. SA 187 enhances yield of important crop plant—alfalfa (*Medicago sativa* L.) and also growth of *Arabidopsis thaliana*. Together with an ability to induce salt stress tolerance in *Arabidopsis*, *Enterobacter* sp. SA 187 has a high potential as a biological solution for improving crop production [39]. In Zélicourt et al. [39] authors further revealed that this induction of salt stress tolerance is caused via production of bacterial 2-keto-4-methylthiobutyric acid (KMBA) which is known for its conversion into ethylene, corresponding with opinion that ethylene plays positive roles in salinity response. However few studies present that some mutant plants with knock-out mutation in ACSs (crucial enzymes in ethylene biosynthesis) show increased salinity-tolerance. This discrepancy and more about the role of ethylene in plants during salinity stress is summarized in a recent review [40].

Rice is the most important basic food crop for the world's population, so in recent years, there have been intensively explored mechanisms to increase the resistance of this plant to stress. Kumar and co-authors [41] characterized a gene *OsSta2* whose overexpression increases a tolerance of rice plants to oxidative and salt stresses. Because plants with the overexpressed *OsSta2* also showed increased responsiveness to exogenous abscisic acid (ABA), authors suggest that this gene plays a role in the ABA signaling pathway during the stress response [41]. This observation could help to complete a proposed model of ABA-dependent gene regulation mediated by OsPYL/RCAR5 in rice proposed by Kim et al. [42]. Further research dealing with stress tolerance of rice plants has revealed the importance of dehydrin gene *OsDhn1*. Rice plants overexpressing this gene show higher tolerance to drought

and salt stress. This advantage is probably caused by ROS scavenging and reducing the oxidative damage [43].

For better understanding of stress response mechanism and facilitating molecular breeding it is very important to provide the genome-wide studies identifying gene families with important roles in stress responses. These studies are for example recently providing in the *Cucurbitaceae* species, where AP2/EREBP (APETALA2/ethylene responsive element binding protein) one of the largest gene families were identified and classified [44]. These genes play important roles in dealing with various environmental stresses. Further genome-wide identification provided in the *Cucurbitaceae* species was focused on the dehydrin genes encoding dehydrines—hydrophilic proteins act like molecular chaperons playing crucial role in the process of abiotic stress tolerance [45]. Both of these studies could be essential for future breeding of new *Cucurbitaceae* cultivars with stress tolerance.

Mentioning transcription factors involved in plant responses to various stresses, a novel orthologue (*MsERF11*) of ethylene response factor gene has been isolated from alfalfa (*Medicago sativa* L.), this gene encodes a nuclear located protein which as a transcription factor plays important roles during biotic or abiotic stress conditions. Because in additional experiments transgenic *Arabidopsis* plants with transferred *MsERF11* gene showed enhanced tolerances to salt stress, Chen et al. propose the potential of MsERF11 in agriculture for improving crop's salt tolerance [46].

7. Summary

To summarize, the interplay between BRs and ethylene plays an important role during all developmental phases of the plant life cycle, as well as during biotic and abiotic stresses. In this article, we reviewed the synergistic effect of these hormones on root growth, seed germination under salt stress, stomatal closure, fruit ripening, and sex expression in the *Cucurbitaceae* family. The antagonistic effect of BRs and ethylene was also discussed, namely expression of the expansine gene *AtEXPA5* in *Arabidopsis* during hypocotyl growth and gravitropic growth of hypocotyls in darkness. Clearly, phytohormonal crosstalk is a complex area in which plenty of interactions remain unknown and requires further investigation by using novel approaches such as genome-wide epigenetic analyses or next-generation transcriptome sequencing of plants after BR or ethylene treatment could help clarify the mechanism of interaction between these essential plant growth regulators. The possibly understanding of the synergistic and antagonistic cross-talks of crucial plant hormones such as brassinosteroids and ethylene give us the huge potential to improve stress tolerance and yield of important agricultural crops.

Author Contributions: P.J. and J.O. were involved in the writing of this article. M.S. reviewed the structure of the article.

Acknowledgments: This work was supported by the Ministry of Education Youth and Sports, Czech Republic (grant LO1204 from the National Program of Sustainability I). The authors would like to thank Ota Blahoušek for drawing the figures using CorelDRAW 2017 and Joelle Hoggan (Sees-Editing Ltd., UK) for English editing.

Conflicts of Interest: The authors declare no conflict of interest.

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BRASSINOSTEROIDS INDUCE STRONG, DOSE-DEPENDENT INHIBITION OF ETIOLATED PEA SEEDLING GROWTH CORRELATED WITH ETHYLENE PRODUCTION

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Abstract
We have recently discovered that brassinosteroids (BRs) can inhibit growth of etiolated pea seedlings dose-dependently, in a similar manner to the 'triple response' induced by ethylene. We demonstrate here that the growth inhibition of etiolated pea shoots strongly correlates with increases in ethylene production, which also responds dose-dependently to applied BRs. We assessed biological activities of two natural BRs on pea seedlings, which are excellent material as they grow rapidly, and respond both linearly and uni-phasically to applied BRs. We then compared the BRs' inhibitory effects on growth, and induction of ethylene and ACC (1-aminocyclopropane-1-carboxylic acid) production, to those of representatives of other phytohormone classes (cytokinins, auxins and gibberellins). Auxin induced ca. 50-fold weaker responses in etiolated pea seedlings than brassinolide, and the other phytohormones induced much weaker (or opposite) responses. Following optimization of conditions for determining ethylene production after BR treatment, we found a positive correlation between BR bioactivity and ethylene production. Finally, we optimized conditions for pea growth responses and developed a new, highly sensitive and convenient bioassay for BR activity.

Keywords: brassinosteroid, growth inhibition, bioassay, *Pisum sativum (*var. *arvense)* sort. *Arvica*, ethylene, 1-aminocyclopropane-1-carboxylic acid

Abbreviations: ACC, 1-Aminocyclopropane-1-carboxylic acid; ACO,1-Aminocyclopropane-1-carboxylic acid oxidase; ACS, 1-Aminocyclopropane-1-carboxylic acid synthase; BAP, 6-Benzylaminopurine; BL, Brassinolide; BRs, Brassinosteroids; GA, Gibberellin; GA3, Gibberellic acid; IAA, Indole-3-acetic acid; TDZ, Thidiazuron; *t*ZR, *Trans*-zeatin riboside; 24-epiBL, 24-epibrassinolide;

1. Introduction

Brassinosteroids (BRs) are a family of naturally occurring plant steroids that are involved in diverse developmental and physiological processes, including cell elongation, cell division, leaf senescence, vascular differentiation, flowering time control, male reproduction, photomorphogenesis and responses to both biotic and abiotic stresses [1-3]. As potent plant growth regulators, BRs have been used to enhance the growth and yields of important agricultural crops [4]. Since BRs are present in plants in extremely low concentrations and have potent biological activities, their identification requires highly sensitive bioassays, based on responses to BRs that are not influenced by other endogenous plant hormones.

Ethylene, the simplest plant hormone (a gaseous compound consisting of two carbon and four hydrogen atoms) is produced in most plant tissues and cell types. It also affects diverse processes in plants, including seed germination, growth, apical hook formation, organ senescence, fruit ripening, abscission, gravitropism, and stress responses [5,6]. Application of ethylene at low levels to etiolated seedlings typically causes a 'triple response': inhibition of stem elongation, radial swelling of the stem, and impairment of the normal geotropic response (formation of an exaggerated apical hook). This seedling phenotype has been used for identifying ethylene-related mutants [7,8] . Ethylene biosynthesis involves three main steps. The first is conversion of the amino acid methionine to S-adenosyl-methionine (SAM), catalyzed by the specific enzyme SAM synthetase (SAMS). The next (generally rate-limiting step) in ethylene biosynthesis is conversion of SAM to 1-Aminocyclopropane-1-carboxylic acid (ACC) catalyzed by 1-Aminocyclopropane-1-carboxylic acid synthase (ACS). The last step is conversion of ACC to ethylene, catalyzed by ACC oxidase (ACO) [9] . Interestingly, Tsang et al. [10] found that ACC, the direct precursor of ethylene, can act as an active signaling molecule itself, independently of ethylene.

Several studies have shown that BRs stimulate ethylene production in various plant tissues [11-13]. One of the main mechanisms how BRs could positively influence ethylene biosynthesis is via stabilization of ACC synthase the crucial enzyme in ethylene biosynthesis [14]. However, in a recent study of BRs' effects on root growth, Lv et al. [15] found that they can have either of two effects on ethylene synthesis in Arabidopsis roots, depending on the applied concentration. Ethylene production was greatly reduced in seedlings treated with a low concentration (10 or 100 nM) of 24-epibrassinolide (24-epiBL), while a higher concentration (≥ 500 nM) strongly enhanced ethylene production. Chromatin immunoprecipitation (ChIP)/qPCR analysis showed that interactions of BES1 and BZR1 (BR-regulated transcription factors) with the promoter of *ACSs* play important roles in these responses. The interactions are inhibitory, because expression of *ACS* is strongly suppressed when the BR transcription factors are over-expressed, and vice versa *ACS* expression is increased in BR-insensitive mutants. Altogether these results suggest that at physiological levels BRs repress ethylene biosynthesis via interaction with BES1 and BZR1 transcription factors and the promoters of *ACSs*, encoding the key ethylene biosynthetic enzyme, while at high levels BRs and auxins synergistically induce ethylene production in Arabidopsis roots [15].

We recently discovered that brassinolide (BL) application has strong effects on etiolated pea seedlings, including all three phenotypic elements of the classical 'triple response' to ethylene (elongation and radial swelling of the hypocotyl, and exaggerated apical hook formation). Thus, in the study presented here we tested the hypothesis that BRs' biological activities may be mediated by ethylene, and the specificity of their activities, by examining corresponding activities of other plant hormones. We also developed a robust, sensitive and convenient bioassay, the pea seedling growth inhibition test (in which ethylene production could also be monitored), for evaluating hormonal activities of new synthetic BR derivatives with potential agricultural uses.

2. Results and Discussion

2.1. Effects of brassinosteroids on growth of etiolated pea seedlings

First we analyzed effects of two exogenously applied BRs (BL and 24-epiBL) at various concentrations on growth of etiolated pea seedlings and found that BRs change their growth pattern. After treatment with brassinosteroids in higher concentration than 0.2 µM we observed the reducing rate of elongation (Fig. 1a, b). This effect is also accompanied with declining weight of epicotyls biomass (Fig. 1d). Except the inhibition and loosing biomass of epicotyls we also observed in these plants increasing lateral expansion (Fig. 1c), leading to swelling of the regions bellow the hook. It is evident from IC_{50} values (Table 1) that this response of pea hypocotyls is highly sensitive to BRs. The results with 24-epibrassinolide (BL IC_{50} – 2.20E-05 M; 24-epiBL IC_{50} – 1.86E-05 M) showed that it is a bit less active than brassinolide. Inhibitory effects of BRs on hypocotyl elongation of dark-grown plants have also been observed by Tanaka et al. [17], who found that BL inhibited elongation of etiolated Arabidopsis plants' hypocotyls at concentrations higher than 0.01 µM. In addition to inhibiting the growth and inducing swelling of etiolated pea seedlings, BRs also caused curvature of their etiolated stems, leading to an exaggerated apical hook (Fig. 1a). These are three phenotypic elements of the typical 'triple response' of etiolated plants to ethylene observed in most dicots, including Arabidopsis [8]. Therefore, we examined BRs' effects on ethylene production in the seedlings.

Table 1 IC50 (mol/l) values of selected brassinosteroids and other phytohormones obtained from the pea growth inhibition biotest.

IC 50	concentration [mol/l]
BL	2.20E-05
24-epiBL	1.86E-05
tZR	2.99E-02
IAA	1.78E-03
TDZ	2.59E-02
GA3	no inhibition

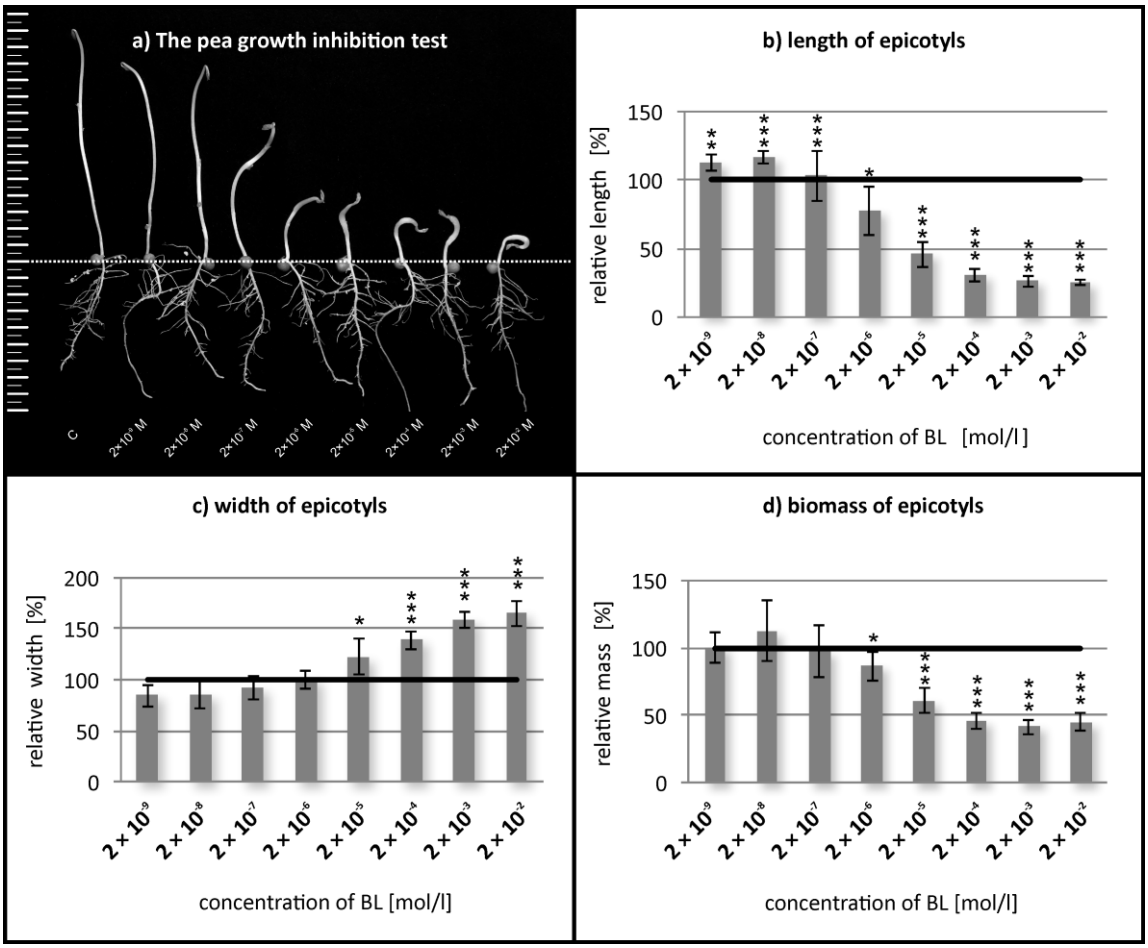

Fig. 1. Visual effects of BL on etiolated pea plants (a) and quantified effects on the length (b), width (c) and biomass (d) of epicotyls treated with BL at indicated concentrations. Error bars represent S.D. Asterisks represent significant changes (t-test), *represents p value <0.05, **represent p value <0.01, ***represent p value <0.001.

2.2. Inhibitory effects of other plant growth regulators on epicotyl growth

To gauge the BR-specificity of the observed inhibitory effects on growth of etiolated plants, we tested effects of exogenous applications of representatives of the other main phytohormonal groups: auxin (indole-3-acetic acid, IAA), gibberellin (gibberellic acid, GA3), and cytokinins (*trans*-zeatin riboside, tZR, and thidiazuron, TDZ). Structures of these compounds are shown in Figure 2. Auxin had much stronger inhibitory effects on pea hypocotyl growth and elongation than the gibberellin and cytokinins (Fig. 3), but only at substantially higher concentrations than the BRs (IC_{50} values for IAA, BL and 24-epiBL: 1.78E-03 M, 2.20E-05 M, and 1.86E-05 M, respectively). Thus, ca. 80-fold more IAA than BL was required. The cytokinins also inhibited elongation, but their IC_{50} values were ca. 1000-times higher than those of the BRs. Similarly, Chory et al. [18] found that the natural cytokinin N^6-isopentenyladenine inhibited hypocotyl elongation of etiolated Arabidopsis at a much higher concentration (3.10^{-6} M) than BRs. Finally, treatment of the plants with GA3 had the opposite

effect, causing etiolated pea stems to lengthen, in accordance with findings by Cowling and Harberd [19] that 14-day-old Arabidopsis plants treated with 10^{-6} M GA4 had longer hypocotyls than non-treated controls. Data presented in Table 1 clearly show that the seedlings responded highly sensitively and dose-dependently to the applied BRs. As already mentioned, in addition to inhibiting growth, BRs caused swelling and curvature of the seedlings' etiolated stems.

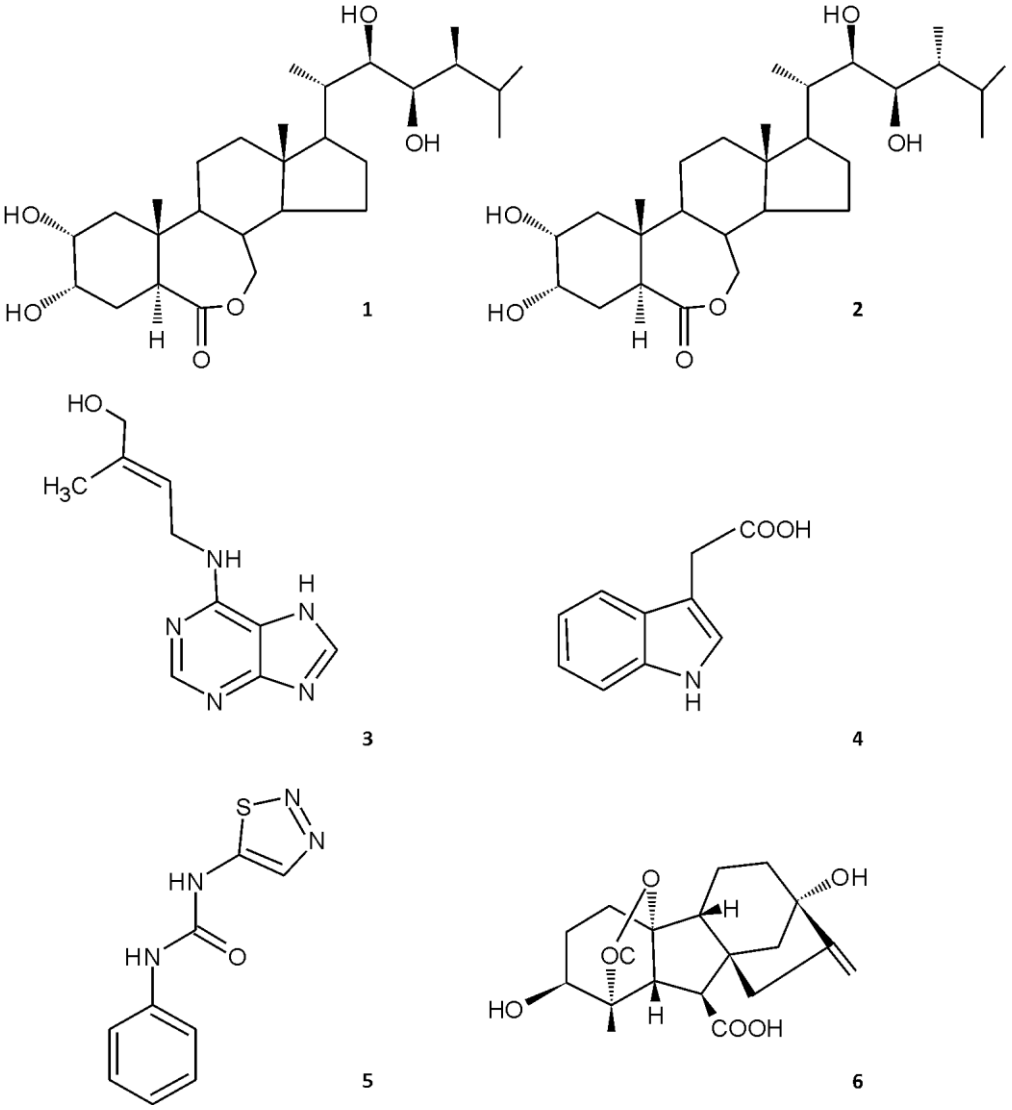

Fig. 2. Structures of tested growth regulators: brassinolide (1), 24-epibrassinolide (2), *trans*-zeatin (3), indole-3-acetic acid (4), thidiazuron (5), gibberellic acid (GA3) (6).

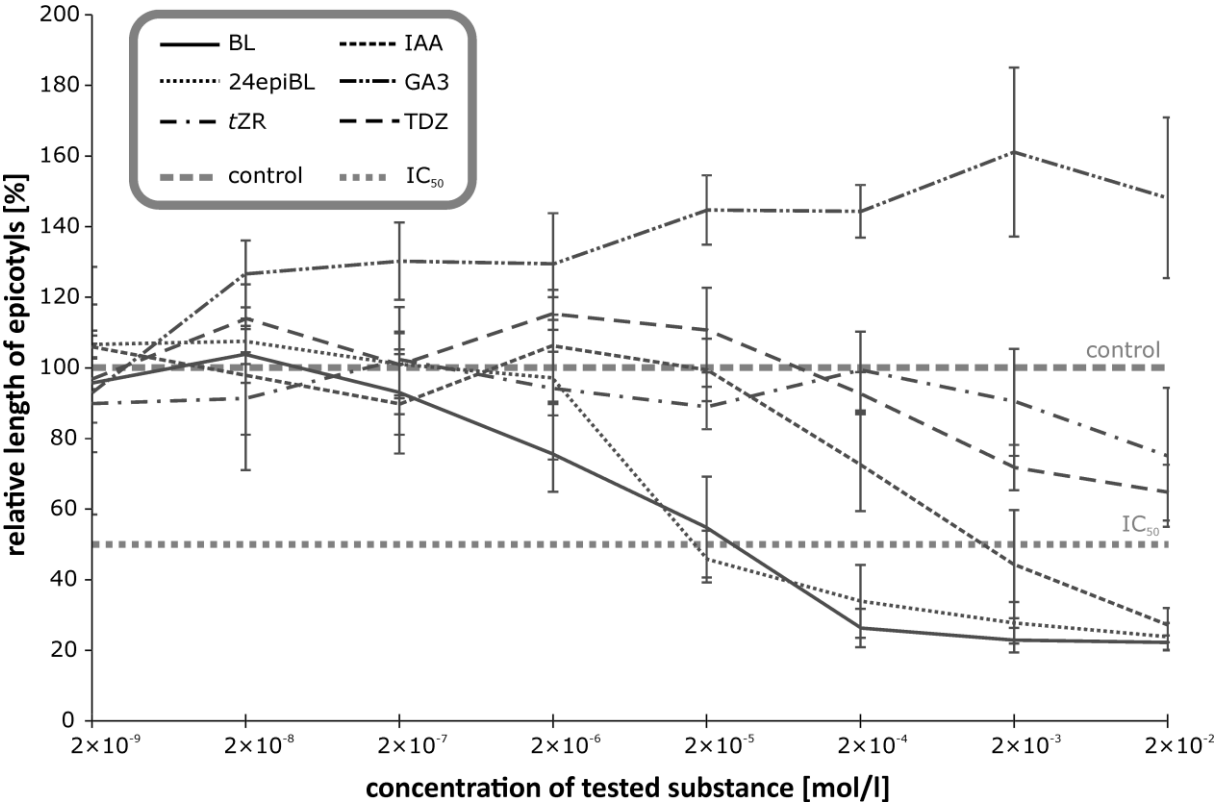

Fig. 3. Effect of selected growth regulators on inhibition of etiolated pea seedlings' growth. Error bars represent standard deviations of the means. (For statistical data see SuppTable1).

2.3. Effects of BRs and other phytohormones on ethylene production in etiolated pea seedlings

The results presented above clearly indicate that the inhibitory effect of BRs is mediated by endogenous ethylene biosynthesis. Thus, we determined ethylene production using a method that had been optimized with respect to treatment duration and temperature. Seedlings treated with a BR (or other phytohormone) at a given concentration are hermetically sealed in an Erlenmeyer flask, incubated in the dark at 22 °C and ethylene levels in the flask are measured after 24 hours (when ethylene levels peaked in optimization tests; Fig. 4).' Note, however, Fig. 4 shows that ethylene levels were higher after 24 hours than after 12 and 6 hours, but not that they peaked then. The largest amounts of ethylene were produced by plants treated with 20 mM IAA or BRs (Fig. 5), supporting the hypothesis that BRs' inhibitory effects on etiolated pea seedlings are mediated by increases in ethylene production. Moreover, the minimum concentrations of BL (or 24-epiBL) and IAA required to elicit significant effects on ethylene production were ca. 20 nM and 20 µM, respectively. Thus, ethylene production in pea stems clearly responds much more sensitively to BRs (apps.100-times) than to IAA.

High ethylene production in plants treated with auxins is not surprising as auxin-induced ethylene production has been observed in numerous plant species [14,20,21]. BRs have also

been shown to induce production of ethylene, both alone and synergistically with other phytohormones in etiolated mung bean seedlings [12,22]. However, etiolated pea seedlings appear to be the most sensitive systems tested to date, responding detectably to as little as 100 fmol of BL. Mechanistic evidence that BR and auxin promote ethylene production has been provided by Joo et al. [23], who showed that 24-epiBL induces expression of the auxin-responsive ACC synthase gen AtACS4 in Arabidopsis. In addition, cross-talk of BRs with ethylene is important for germination of seeds under salinity stress [24]. All this published information is consistent with our observations that BRs inhibit growth of pea seedlings' stems and promote ethylene production in them.

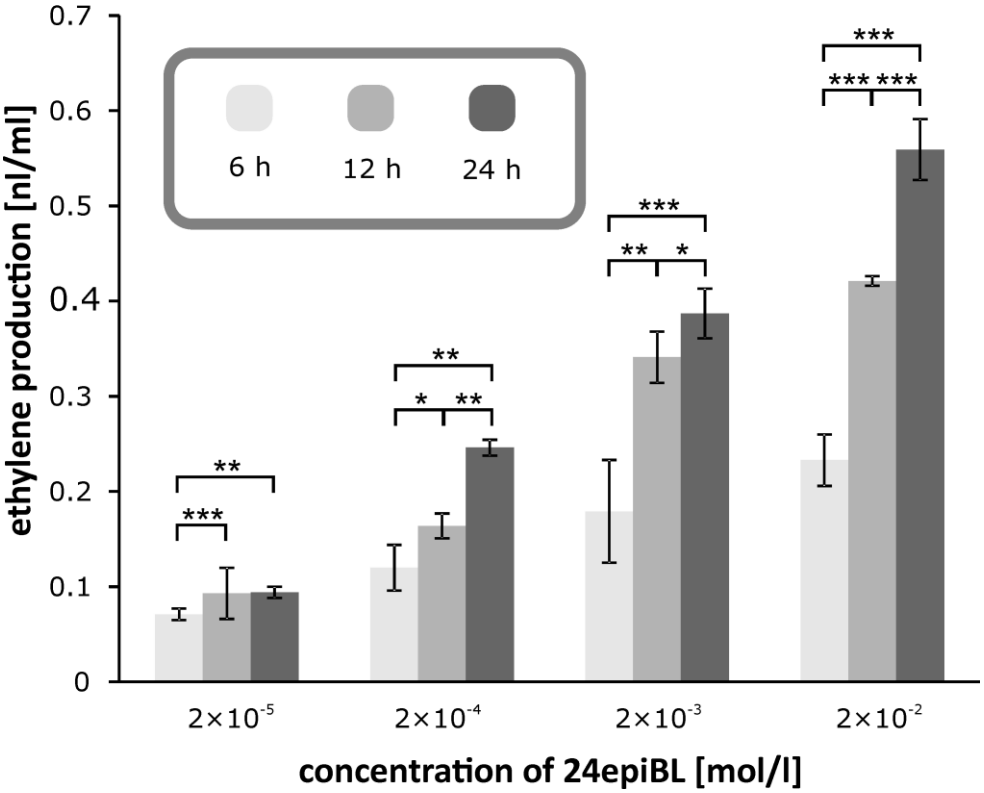

Fig. 4. Effects of 24-epiBL on ethylene production (nl/ml) by etiolated pea seedlings determined by GC-FID 6 , 12 and 24 h after ventilation. Error bars represent standard deviations of the means. Error bars represent S.D. Asterisks represent significant changes (t-test), *represents p value <0.05, **represent p value <0.01, ***represent p value <0.001.

Application of TDZ also induced an increase in ethylene production, but only at the strongest (very high) concentration used (20 mM). These results are consistent with demonstrations that TDZ promotes ethylene evolution in several plant species [25,26], and is used for this purpose in cotton defoliation. Similarly, Lorteau et al. [27] found that the cytokinin 6-benzylaminopurine (BAP) stimulated ethylene production in pea roots (ethylene production was measured 6 hours after the cytokinin treatment) The time between the administration of

cytokinin and the ethylene determination appears to be decisive for the final amount of ethylene measured. James Rushing's work [28] shows that ethylene production in broccoli florets treated by BAP or zeatin peaked on 2nd day later, and dropped to control levels after 4 days. In stark contrast, ethylene production was considerably lower in our seedlings treated with *t*ZR. GA3 also repressed ethylene production. Many studies have shown that ethylene can modulate gibberellin action or concentration [29-31], but the reverse interaction has received much less attention. However, Ferguson et al. [32] found that GA1 can probably suppress ethylene production because GA1-deficient pea mutants produced nearly twice as much ethylene as wild-type plants, in accordance with our observations of GA3's effects on pea seedlings (Fig. 5).

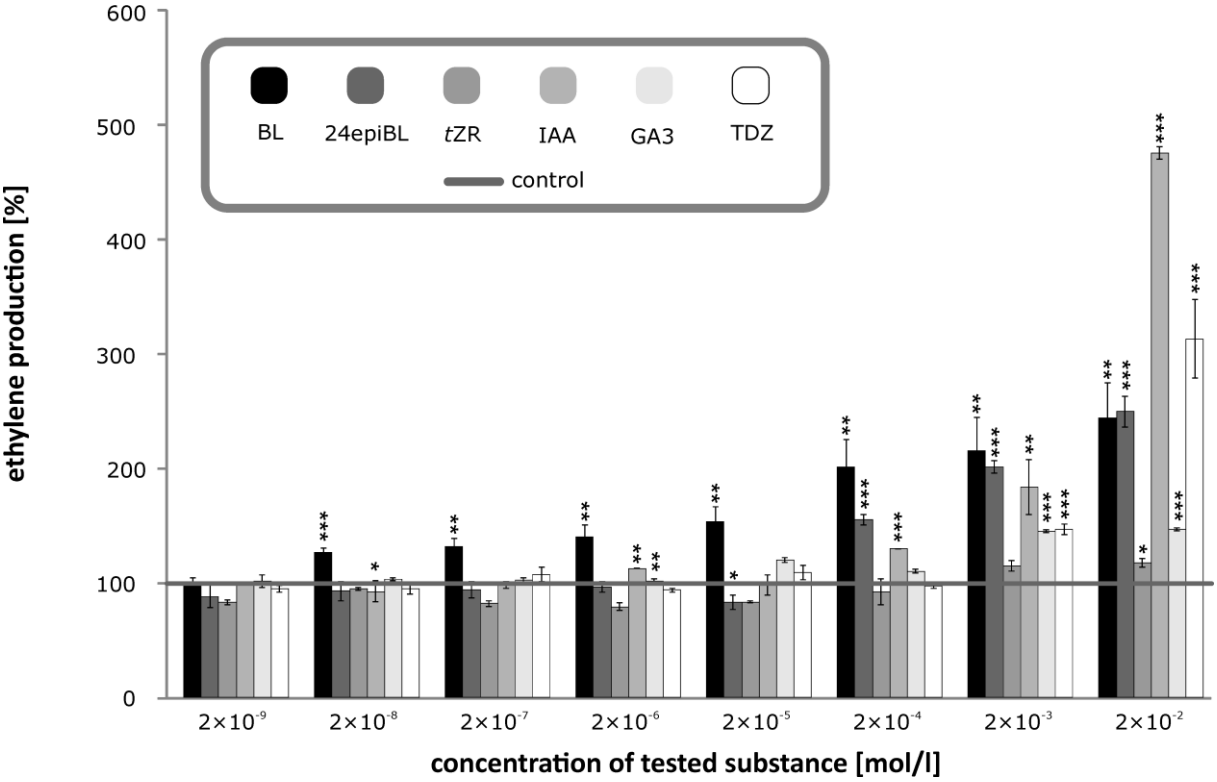

Fig. 5. Effects of selected growth regulators on ethylene production (nl/ml) by etiolated pea seedlings determined by GC-FID 24 h after ventilation. Error bars represent standard deviations of the means. Error bars represent S.D. Asterisks represent significant changes (t-test), *represents p value <0.05, **represent p value <0.01, ***represent p value <0.001.

2.4. Determination of ACC, a direct biosynthetic precursor of ethylene, in plants treated with BRs in time

As already mentioned, there are strong indications that BRs promote ethylene biosynthesis in seedlings by stimulating transcription of ACS genes and increasing the stability of ACS5 and ACS9 proteins [33]. Alternatively, BRs may suppress ethylene biosynthesis through interaction with BES1 and BZR1 transcription factors and the promoters of *ACSs* genes, encoding the key

ethylene biosynthetic enzyme at BR levels below some threshold, but at higher levels induce ethylene production in conjunction with auxins [15]. To elucidate whether the increased ethylene production we observed after BR treatment was due to increases in ACC biosynthesis, we measured time courses of changes in concentrations of ACC and ethylene in BR (24-epiBL)-treated pea seedlings. As shown in Figure 6, ethylene production increased over time and peaked 36 hours after the treatment, in accordance with previous findings that BRs may enhance ethylene production in etiolated plants treated with BR at times ranging from 8 hours [34] to 3 days [35]. ACC levels in 24-epiBL-treated plants also peaked 36 hours after treatment, and strongly correlated with ethylene production. These data corroborate the finding by Hansen et al. (2009) that induction of ethylene production by BR treatment is strongly linked to ACC biosynthesis.

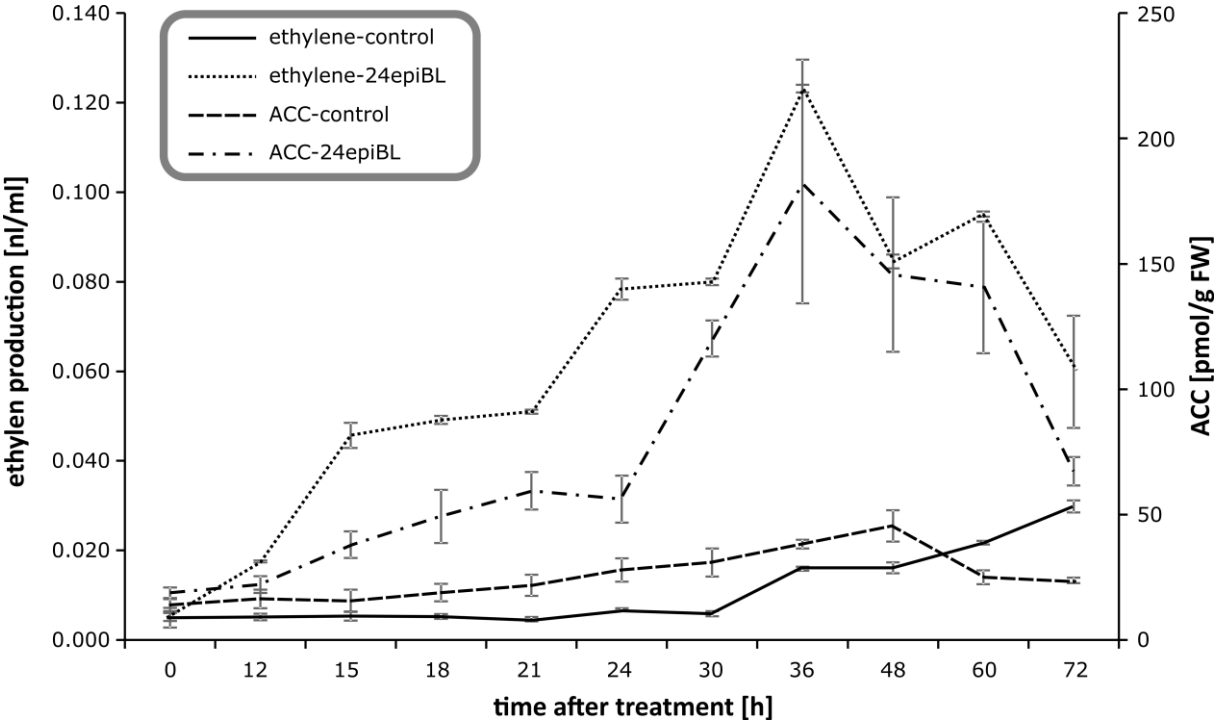

Fig. 6. Effects of 24-epiBL on ethylene production (nl/ml) and concentration of ACC (pmol/g FW) in etiolated pea seedlings determined by GC-FID resp. MS. Error bars represent standard deviations of the means.

2.5. Development of a new bioassay

Several bioassays for BRs have been developed. In past the two most commonly used are the bean second internode elongation (BSIE) assay and rice leaf lamina inclination test (RLIT). In the BSIE assay, elongation of the second internode of bean (*Phaseolus vulgaris*) seedlings is recorded. This elongation is characteristically accompanied by curvature, swelling and splitting, effects sometimes referred to as 'the brassin response'. In this bioassay auxins are inactive and gibberellins only cause elongation of the treated and upper internodes [36]. In

the RLIT, explants (each consisting of a leaf lamina, lamina joint and leaf sheath) are excised from etiolated rice seedlings and floated on test solutions, then the inclination angle induced by test compounds is recorded [37]. In a modified version of the RLIT, intact dwarf rice (*Oryza sativa*) seedlings are used and test solution is applied as a microdrop at the junction between the lamina and the sheath. In the RLIT auxins are active, but at much higher concentrations than BRs. Gibberellins induce a straight growth response without bending of the leaf. Another assay is based on fluorometric measurement of nitric oxide production by tomato suspension-cultures, which is induced by BL [38].

Table 2 Sensitivity of the pea growth inhibition biotest and three previously described bioassays for BRs.

Bioassay	**Detection limit**	**Reference**
BSIE	20 pmol	[40]
RLIT	0.1 pmol	[40]
NO production bioassay	0.5 pmol	[38]
Pea inhibition bioassay	0.1 pmol	This study

Based on the data presented in the previous sections, we developed a new bioassay, 'the pea growth inhibition biotest', for testing BRs' biological activity. This biotest is highly specific for BRs (Fig. 3) and one of the most sensitive BR assays, because as little as 100 fmol of BL can induce the monitored responses (Tab. 2). The elongation of the stems is linearly dependent on the logarithm of BL concentration over four orders of magnitude (Fig. 7) and inter-assay variability is about 8 %. We found that several factors affect this biotest's sensitivity. Firstly light: as etiolated plants are used it essential to perform all operations in the dark or in green light (540 nm). Another important factor is the application of BRs to the plants in droplets of fractionated lanolin (Fig. 8), because the BRs must be in continual contact with the plants' tissues. The sensitivity is also dependent on the pea cultivar. We compared responses of numerous cultivars and found that *Pisum sativum (*var. *arvense)* sort. *Arvica* is highly suitable, because it grows rapidly and its elongation response to BRs is uni-phasic [39].

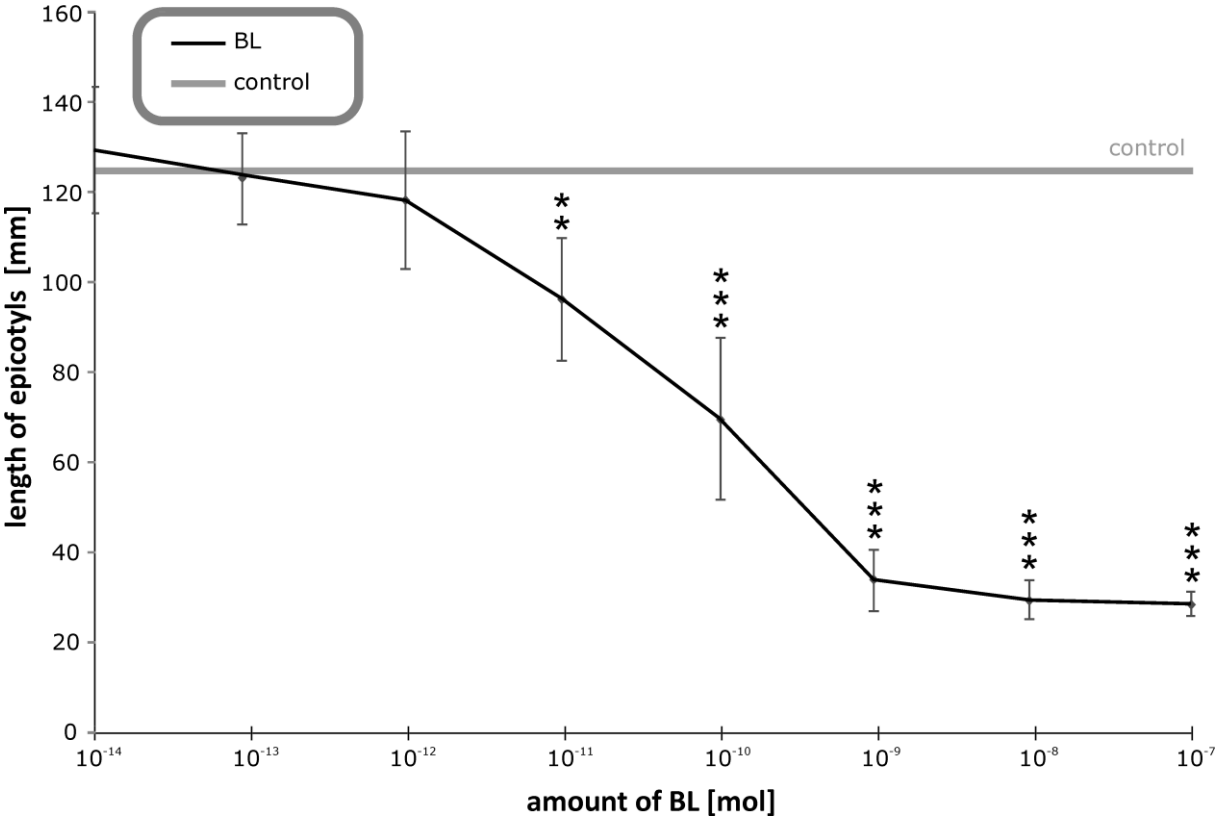

Fig. 7. Inhibitory effect of brassinolide (BL) on etiolated pea seedlings' growth. Error bars represent S.D. Asterisks represent significant changes (t-test), *represents p value <0.05, **represent p value <0.01, ***represent p value <0.001.

3. Materials and Methods

3.1 General information

All chemicals and solvents were purchased commercially and used without further purification. Chemical compounds applied in this study were brassinolide, 24-epibrassinolide, indole-3-acetic acid, gibberellin GA3, *trans*-zeatin, thidiazuron and [$^{2}H_{4}$]1-aminocyclopropanecarboxylic acid (PubChem CID: 115196, 443055, 802, 9819600, 449093, 40087and 84392-07-4 respectively). All these compounds were obtained from Olchemim s.r.o. (Czech Republic). The experimental plants were etiolated pea *Pisum sativum (*var. *arvense)* sort. *Arvica* seedlings.

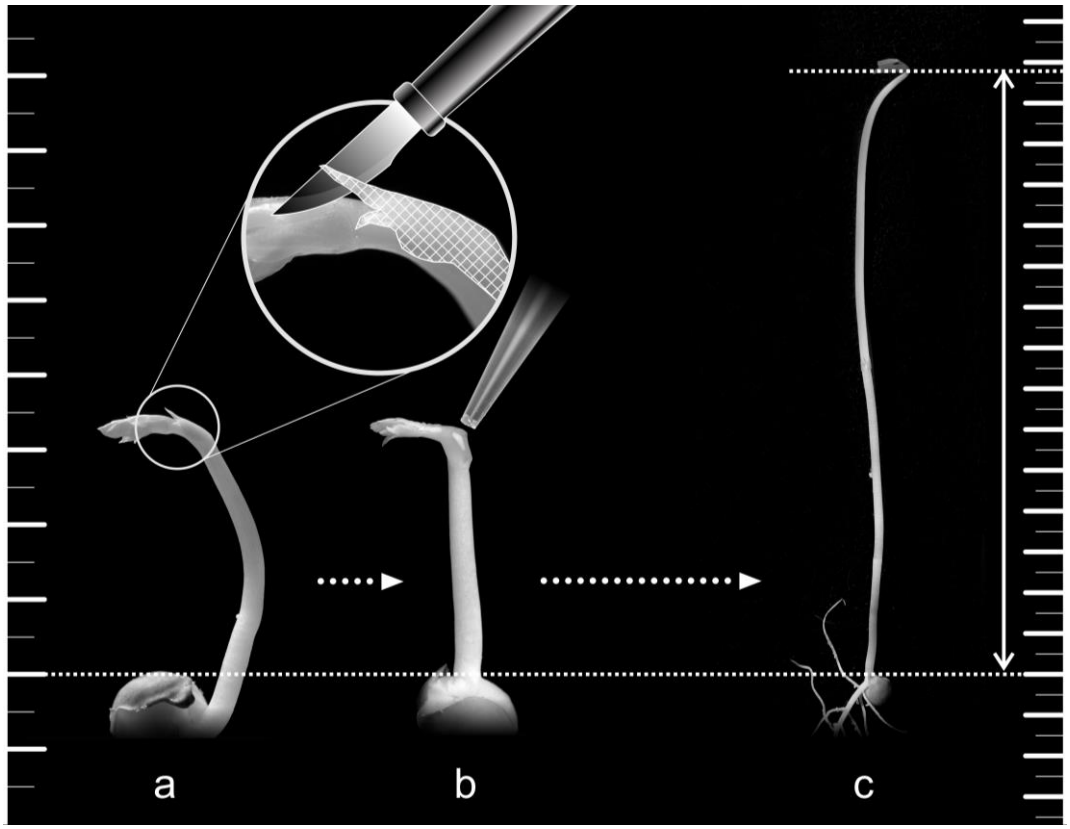

Fig. 8. Scheme of the pea inhibition assay – cutting of bract (a), application of tested compound in microdrop of lanolin on the scar formed by bract removal (b) measurement of epicotyl length (c)

3.2 Pea seedling cultivation

Pea seeds were germinated for 2 days on moist filter paper in the dark, then uniform seedlings from a large population were transferred into pots containing perlite and 1/10 diluted Hoagland solution (half-concentration, pH 5.7). The pots were placed in a dark cultivation room (24 °C, relative humidity 75%) and 24 hours later the seedlings were treated with various amounts of test compounds in 5 µl fractionated lanolin. The substances were applied in microdrops to the scar left after bract removal (Fig. 8). Control plants were treated with lanolin alone. The length of etiolated pea stems was measured after 4 days (Fig. 8) and the difference in length between treated and control plants was used as a measure of activity. Sets of eight seedlings were subjected to each treatment (exposure to one of the test compounds at one of the concentrations) in each of three independent experiments, p-values were calculated with two-tailed Student t-test using Excel software.

3.3 Determination of ethylene production

To measure ethylene production, pea seedlings (eight per treatment) were placed in 0.5 l glass containers for 24 h in the dark. A portion (1 ml) of headspace gas was withdrawn from each

container by syringe for each measurement and injected into a GC System gas chromatograph equipped with a flame ionic detector (FID) and HP-AL/S capillary column (50 m × 0.535 mm × 15 µm), all from Agilent Technologies. The chromatographic settings were: column temperature, 150 °C; detector temperature, 220 °C; carrier gas. The area under the resultant peak (*y*-axis) *versus* sensitivity (*x*-axis; $nl.ml^{-1}$) was representing a quantitative measure of ethylene concentration, p-values were calculated with two-tailed Student t-test using Excel software.

3.4 ACC determination

The tissue (50 mg of etiolated pea plants) was homogenized in 1 ml of H_2O:methanol:chloroform (1:2:1), 50 pmol of internal standard ([D_4]ACC) was added to each sample, and after centrifugation (4 °C, 15 000 rpm) the supernatant was collected and evaporated to dryness. The samples were derivatized using an AccQ-Tag Ultra kit (Waters) and subsequently analyzed by an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) system consisting of an ACQUITY UPLC® I-Class system (Waters, Milford, MA, USA) and a Xevo™ TQ-S MS triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK) [16].

4. Conclusion

The etiolated plants treated with brassinosteroids in higher concentration than 0.2 µM showed declining weight of epicotyls biomass and increasing lateral expansion, leading to swelling of the regions bellow the hook. Because inhibited plants had signs of “triple response” to ethylene, we also developed method for ethylene measurement and examined its production together with its biosynthetic precursor ACC. Ethylene production increased with time after treatment and peaked in 36 hours; these results correlate with ACC accumulation in these plants. Based on these results, a new sensitive bioassay which using etiolated pea plants has been developed. The biotest is highly specific and sensitive for BRs; as little as 100 fmol of BR can be detected.

Supplementary Materials: Supplementary data to this article can be found online at www……. **Table S1:** statistical data for Fig. 4 – length of epicotyls after treatment, *represents p value <0.05, **represent p value <0.01, ***represent p value <0.001.

Funding: The work was supported by an ERDF grant for the project "Plants as a tool for sustainable global development" (No. CZ.02.1.01/0.0/0.0/16_019/0000827) and the Internal Grant Agency of Palacky University (no. IGA_PrF_2019_020).

Acknowledgments: The authors would like to thank Sees-Editing Ltd., UK, for English correction and Ota Blahoušek for graphic editing.

Author Contributions: JO, MS planned the research. PJ, JM, ON performed the experiments and data analysis. PJ, JO and MS wrote the manuscript.

Conflict of interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

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SuppTable1 statistical data for Fig. 3 – length of epicotyls after BR treatment

ttest	compound	c [mol/L]	p value		ttest	compound	c [mol/L]	p value	
BL		2,00E-09	0,51911	-	IAA		2,00E-09	0,360875	-
		2,00E-08	0,522542	-			2,00E-08	0,747311	-
		2,00E-07	0,325809	-			2,00E-07	0,161763	-
		2,00E-06	0,002028	**			2,00E-06	0,414765	-
		2,00E-05	2,54E-05	***			2,00E-05	0,918297	-
		2,00E-04	1,64E-09	***			2,00E-04	0,001709	**
		2,00E-03	4,96E-10	***			2,00E-03	2,25E-06	***
		2,00E-02	3,35E-10	***			2,00E-02	3,82E-10	***
24epiBL		2,00E-09	0,087668	-	GA3		2,00E-09	0,695175	-
		2,00E-08	0,084818	-			2,00E-08	0,017927	*
		2,00E-07	0,829234	-			2,00E-07	0,009965	**
		2,00E-06	0,526198	-			2,00E-06	0,015807	*
		2,00E-05	6,07E-09	***			2,00E-05	0,00052	***
		2,00E-04	3,46E-09	***			2,00E-04	0,000427	***
		2,00E-03	3,88E-11	***			2,00E-03	0,000324	***
		2,00E-02	5,02E-12	***			2,00E-02	0,001915	**
tZR		2,00E-09	0,318369	-	TDZ		2,00E-09	0,571013	-
		2,00E-08	0,449671	-			2,00E-08	0,009998	**
		2,00E-07	0,847271	-			2,00E-07	0,856863	-
		2,00E-06	0,6008	-			2,00E-06	0,000577	***
		2,00E-05	0,229864	-			2,00E-05	0,069406	-
		2,00E-04	0,929079	-			2,00E-04	0,07741	-
		2,00E-03	0,365523	-			2,00E-03	3,88E-06	***
		2,00E-02	0,04463	*			2,00E-02	8,11E-07	***

Chapter

THE CROSSTALK OF BRASSINOSTEROIDS AND OTHER HORMONES IN ABIOTIC STRESS TOLERANCE

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ABSTRACT

Brassinosteroids (BRs) are a class of steroid plant hormones that participate in the regulation of numerous developmental processes, including root and shoot growth, vascular differentiation, fertility and seed germination, and tolerance to biotic and abiotic stresses. The utilization of BRs in protecting plants from adverse environmental stress has positive prospects because these compounds are non-toxic, non-genotoxic, biosafe and eco-friendly. In this way, they could have diverse applications in agri- and horticulture. However, the hormonal control of plant development and stress adaptation relies on a complex network of synergistic and antagonistic interactions between various hormones.

We focus on how the crosstalk between brassinosteroids and other hormones, such as auxin, abscisic acid, gibberellins, ethylene, cytokinins, jasmonate and salicylic acid, influences the adaptation of plants to various abiotic stress.

Keywords: abiotic stress, abscisic acid, auxins, brassinosteroids, cytokinins, ethylene, gibberellins, jasmonate, salicylic acid

INTRODUCTION

Brassinosteroids (BRs) are endogenous steroidal plant hormones essential for various plant growth and development processes, such as cell elongation and division, seed germination, seed size control, xylem differentiation, stem and root growth, development of

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flowers and fruits, leaf senescence, and resistance to various biotic and abiotic stresses (Oklestkova et al. 2015, Singh and Savaldi-Goldstein 2015, Divi and Krishna 2009). This class of steroidal plant hormones was first identified in 1970, when Mitchell and co-workers isolated an oil fraction from rape pollen (*Brassica napus* L.), termed "brassins", that had a strong influence on plant growth (Mitchell et al. 1970). A few years later, about 5 mg of a solid crystalline compound, named brassinolide (BL), was purified from pollen grains and identified by X-ray crystallography to be a polyhydroxylated derivative of 5α-cholestan, namely (22R,23R,24S)-2 α,3 α,22,23-tetrahydroxy-24-methyl-β-homo-7-oxa-5 α -cholestan-6-one (Figure 1) (Grove et al. 1979). To date, more than 70 structurally and functionally related steroids have been isolated from plant materials, including pollen, anthers, seeds, leaves, stems, roots, flowers, and grains, as well as from insect and crown gall tissues (Bajguz and Tretyn 2003).

Brassinosteroid-mediated signal transduction has been extensively studied in the last decade. BRs are perceived by three leucine-rich repeat receptor-like kinases (LRR-RLKs), including the main receptor BRASSINOSTEROID-INSENSITIVE 1 (BRI1) and its two paralogs, BRL1 and BRL3 (Clouse et al. 1996, Li and Chory 1997, She et al. 2011). The interaction of BL and BRI1 results in a conformational change that allows the receptor-ligand complex to interaction with its co-receptor, BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1). The BRI1-BL-BAK1 complex then modulates a cellular cascade of kinases, culminating in the dephosphorylation and activation of two transcription factors, BRASSINAZOLE-RESISTANT1 (BZR1) and BRI1-EMS SUPPRESSOR1 (BES1) (He et al. 2005). BES1 and BZR1 can activate or repress the expression of hundreds of target genes to mediate many aspects of plant growth and development (Sun et al. 2010).

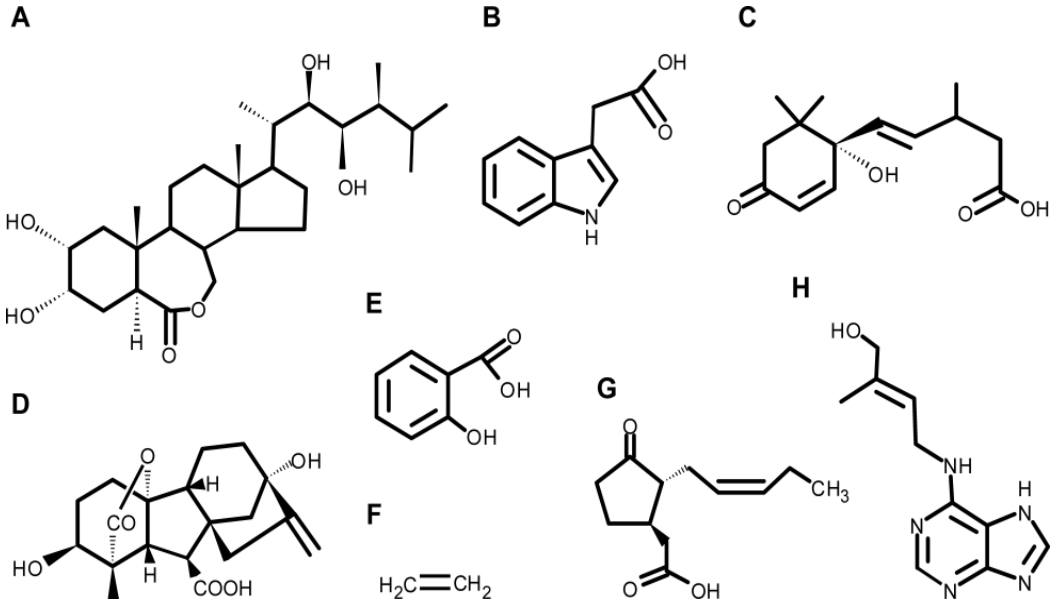

Figure 1. Structures of the major classes of phytohormones: Brassinolide (A), 3-Indoleacetic acid (B), Abscisic acid (C), Gibberellin-GA_1 (D), Salicylic acid (E), Ethylene (F), Jasmonic acid (G), Cytokinin zeatin (H).

In addition to their role in plant development, BRs can protect plants from a variety of environmental stresses, including extreme temperatures, drought, salinity, heavy metals and pathogen attack (Bajguz and Hayat 2009, Fariduddin et al. 2014). The BR-induced molecular changes related to stress tolerance include an increase in the expression of stress-responsive genes, maintenance of protein synthesis, increased activities of antioxidant enzymes, a greater accumulation of osmoprotectants, and crosstalk with other plant hormones (Dhaubhadel et al. 2002, Ozdemir et al. 2004, Ahammed et al. 2015). This chapter comprehensively reviews how BRs crosstalk with other plant hormones to confer abiotic stress tolerance.

1. INTERACTION WITH AUXIN

Auxin (IAA) plays fundamental roles in plant growth and development. IAA regulates plant morphogenesis through tissue-specific concentration gradients, which are formed by auxin biosynthesis, conjugation and degradation processes (Normanly 2010), as well as its intercellular (Petrášek and Friml 2009) and intracellular distributions (Mravec et al. 2009). IAA also participates in the transduction of a wide spectrum of external and internal signals. The concentration gradients of IAA in plant tissues are important for embryogenesis (Friml et al. 2003), as well as shoot and root organogenesis, more specifically, apical dominance (Benkova et al. 2003), vascular tissue development (Mattson et al. 2003), differential growth during tropism, and apical hook development. IAA gradients may also be decisive in senescence (Ellis et al. 2005), plant–pathogen interactions, abiotic stress responses and and other interactions between plants and the environment (Kazan and Manners 2009, Simon and Petrášek 2011).

Auxin and brassinosteroids are essential regulators of plant growth and have been known to influence both cell division and cell elongation in various developmental contexts. Observations based on the external simultaneous application of both hormones to plant tissues suggest that these compounds act in an interdependent, and possibly synergistic, manner. It has been shown that this growth-promoting effect is dependent on both the sequence of treatment, as a stimulatory effect was observed only when IAA was applied together with or after brassinosteroids, and the hormone concentration, as low concentrations promote cell elongation while higher concentrations have an inhibitory effect (Oh and Clouse 1998, Clouse and Zurek 1991, Hardtke 2007). Studies of ethylene production have provided further evidence for synergism between BRs and IAA (Arteca et al. 1983, Schlagnhaufer et al. 1984). In these studies, plants that were treated with both BRs and IAA showed a significant increase in ethylene production when compared to control, untreated plants, as well as plants that had been treated with only BRs or IAA. Furthermore, it has been shown that both BRs and IAA affect the stage of ethylene production when S-adenosylmethionine is transformed to aminocyclopropane-1-carboxylic acid (Schlagnhaufer et al. 1984).

Another connection between IAA and BR pathways is suggested by reports of BR effects on polar auxin transport. The expression of *PIN* genes, which encode the auxin efflux carriers that are crucial for the rate and direction of polar auxin transport, is partly controlled by BRs. Moreover, BRs modulate the localization of a PIN protein that is implicated in plant tropisms (Nemhauser et al. 2004, Li et al. 2005, Nakamura et al. 2004). For example, after wild-type plants were treated with brassinolide, the abundance of PIN4 and PIN7 transcripts decreased

in a dose-dependent manner, but no obvious decrease was observed for PIN2 (Nakamura et al. 2004). However, brassinolide has been shown to enhance plant tropic responses by promoting the accumulation of PIN2 from the root tip to the elongation zone, thus implying an altered distribution of endogenous IAA (Li et al. 2005).

The direct interaction between the BR signaling component BIN2 and the auxin signaling component ARF2 also demonstrates the synergistic effects of BRs and IAA in photomorphogenesis. The phosphorylation of ARF2 by BIN2 inhibits the DNA-binding and repression activities of ARF2. This indicates that BIN2 increases the expression of auxin-induced genes by directly inactivating the repressor ARF2, leading to the synergistic enhancement of transcription (Vert et al. 2008).

Kim and co-workers (Kim et al. 2006) found that the expression of *Aux/IAA* genes *AXR3/IAA17, AXR2/IAA7, SLR/IAA14* and *IAA28* significantly induced in roots upon treatment with 24-epiBL. In addition, the transcription of *Aux/IAA* genes involved in root development significantly decreased in the brassinosteroid biosynthetic mutant *det2* and in the brassinosteroid-signaling mutant *bri1*, leading the authors to suggest that *Aux/IAA* genes are the point at which BR- and IAA-signaling pathways converge during root development.

2. INTERACTION WITH ABSCISIC ACID

Abscisic acid (ABA) is a C_{15} weak acid and appears to be present in all vascular plants and mosses (Nambara and Marion-Poll 2005). ABA is one of the most important stress hormones, and plays crucial roles in various physiological processes during the plant life cycle, including seed dormancy, germination, stomatal movement, fruit development, and responses to biotic and abiotic stresses (Chernyrs and Zeevaart 2000). Cellular ABA levels fluctuate constantly in response to changing physiological and environmental conditions, and these concentrations determine ABA function in plant physiology and development (Zhu 2002). The interaction between BRs and ABA co-regulates the expression of many genes involved in biological processes such as seed germination, stomatal closure, and response to environmental stresses (Steber and McCourt 2001, Haubrick at al. 2006, Kagale et al. 2007). While ABA establishes seed dormancy during embryo maturation and inhibits seed germination (Finkelstein et al. 2008), BRs promote seed germination, possibly by enhancing the embryo growth potential to antagonize the inhibitory effect of ABA (Steber and McCourt 2001, Zhang et al. 2009). It has been reported that Arabidopsis BR-related mutants (the BR biosynthetic mutant *det-2*, the BR responsive mutant *bri1* and BKI1 overexpression line *BKI1-OX*) demonstrate increased sensitivity to the inhibitory effects of ABA during seed germination in comparison to the wild-type (Steber and McCourt 2001). On the other hand, the overexpression of BRI1 leads to an enhanced resistance to ABA-mediated inhibition of seed germination (Zhang et al. 2009).

Zhang and co-workers (Zhang et al. 2009) used the biochemical and molecular markers of both BR signaling and ABA biosynthetic mutants to demonstrate that exogenous ABA inhibits the expression of BR signaling-related genes. An analysis of the BR signaling mutant, *bri1-116*, and the subcellular localization of BKI1 further revealed that the BR receptor complex is not required for ABA to affect BR signaling outputs. However, when the BR downstream signaling component BIN2 was inhibited by LiCl, ABA was not able to inhibit

BR signaling outputs. Furthermore, the researchers used a set of ABA insensitive mutants to show that the ABA-mediated regulation of BR signaling depends on two ABA early signaling components, ABI1 and ABI2. Hence, they proposed that the BR – ABA crosstalk occurs after BR perception, but before transcriptional activation. This model may explain why many BR-responsive genes are also regulated by ABA, and also presents certain molecular mechanisms through which BRs can interact with ABA (Zhang et al. 2009).

The production of H_2O_2 is critical for BR- and ABA-induced stress tolerance in plants. Zhou et al. (2014) used tomatoes to study how BR and ABA promote H_2O_2 production, and to elucidate their roles in heat and oxidative stress responses. In RBOH1-silenced tomato plants, the application of 24-epiBL was unable to induce H_2O_2 production and ABA accumulation, yet the application of ABA was able to induce stress tolerance. A BR biosynthetic mutant $d^{\wedge im}$ showed decreased levels of ABA, but these levels increased following the exogenous application of BRs. The authors concluded that BR-induced stress tolerance involves a positive feedback mechanism through which BR stimulates NADPH oxidase for rapid and transient apoplastic H_2O_2 production. This process then increases ABA biosynthesis, which further increases H_2O_2 production and leads to prolonged stress tolerance. It was shown that ABA induces H_2O_2 production in both the apoplastic and chloroplastic compartments (Zhou et al. 2014).

Stomatal aperture regulation is crucial for minimizing water loss during stress. The effects of BRs on the stomatal apertures of tomatoes were recently studied (Xia et al. 2014). It was shown that treatment with high concentrations of BRs induced stomatal closure, whereas treatment with low concentrations of BRs led to stomatal opening. Moreover, both BR-induced stomatal responses were dependent on the reactive oxygen species (ROS) generated by NADPH oxidase; however, the kinetics of ROS induction are different at high and low levels of BRs. The results suggest that transient H_2O_2 production is essential for maintaining a balance in the cellular redox status of glutathione, which plays an important role in BR-induced stomatal opening. A prolonged increase in H_2O_2 levels, however, activates ABA signaling and mediates stomatal closure (Xia et al. 2014). Nitric oxide (NO) is involved in signal transduction when a plant responds to water stress, iron deficiency, or an ABA stimulus (Farooq et al. 2009). Zhang et al. (2011) used maize (Zea mays L.) to investigate the relationship between BRs, NO and ABA under water stress. Their results showed that BRs enhance oxidative stress tolerance by increasing ABA biosynthesis, and that NO mediates the BR-induced ABA biosynthesis (Zhang et al. 2011).

3. INTERACTION WITH ETHYLENE

Ethylene, which is the simplest olefin and exists in the gaseous state under normal physiological conditions, regulates diverse metabolic and developmental processes in plants, including seed germination, growth, apical hook formation, organ senescence, fruit ripening, leaf abscission, gravitropism, and stress responses. Ethylene can be produced by almost all parts of higher plants, but the production ratio depends on several factors, like the type of tissue or the stage of development (Abeles et al. 1992). Moreover, ethylene is biologically active in plants in trace amounts, making its effects commercially important (for example, to control the onset and rate of climacteric fruit ripening) (Bleecker and Kende, 2000). When

low levels of ethylene are applied to etiolated seedlings, the characteristic triple response can be observed; ethylene causes inhibition of stem elongation, radial swelling of the stem, and absence of a normal geotropic response (Guzmán and Ecker, 1990).

Ethylene biosynthesis can be induced by any type of wounding or physiological stress (flooding, drought, temperature stress, or pathogens) (Taiz and Zeiger, 2010). Regarding biosynthesis, S-adenosyl-methionine (S-AdoMet) and the cyclic non-protein amino acid 1-aminocyclopropane-1-carboxylic acid (ACC) have been established as precursors of ethylene. The two key enzymes involved in ethylene production in plants are ACC synthase and ACC oxidase. The enzyme ACC synthase (ACS) catalyzes the conversion of S-AdoMet to ACC, which is then converted into ethylene by ACC oxidase (ACO) (Bleecker and Kende, 2000). In this way, developmental and environmental cues could modulate ethylene production in plants by targeting either ACS or ACO (Wang et al. 2002). For instance, Hansen et al. (2009) showed that brassinosteroids stimulate ethylene production in Arabidopsis by stabilizing the ACS protein.

The last few years have seen an explosion in ethylene research, which has led to some important developments. Plenty of ethylene-signaling and ethylene-response pathways have been identified, but most of them are very complex. These pathways can be stimulated by many factors, including brassinosteroids (Stepanova and Alonso, 2005). BRs have been shown to induce ethylene production both independently and synergistically with IAA in etiolated mung-bean seedlings (Arteca et al. 1983; Yi et al. 1999). Already ten years ago, Joo et al. (2006) showed that 24-epibrassinolide (24-epiBL) stimulates the auxin-responsive ACC synthase gene *AtACS4* in Arabidopsis.

The crosstalk between BRs and ethylene is also important for the germination of seeds under salinity stress. Seed germination is suppressed by ABA (Abscisic acid), but both gibberellic acid (GA) and ethylene can reduce the ABA-induced inhibition of seed germination (Matilla and Matilla-Vazquez, 2008). Previous research has demonstrated that an exogenous supply of ethylene can mitigate the inhibition of seed germination caused by salt stress (Chang et al. 2010). BRs also participate in plant responses to abiotic stresses such as salinity, drought or low temperature (Bajguz and Hayat, 2009). Wang and co-workers (Wang et al. 2011) examined how 24-epibrassinolide affected ethylene production and ACC oxidase activity in cucumber seeds under salt stress. Their results suggest that the promotion of ethylene production may underlie the effect of 24-epiBL on seed germination under salt stress. This argument is supported by the observation that addition of aminoethoxyvinylglycine (AVG), which is an inhibitor of ethylene synthesis, significantly decreased the effect of 24-epiBL on seed germination under conditions of salt stress (Wang et al. 2011).

Another example of the crosstalk between brassinosteroids and ethylene was presented in Serna et al. (2015). This study investigated the correlation between DI-31 (brassinosteroid analogue) and ethylene synthesis in lettuce plants under salinity stress. DI-31 is a brassinosteroid analogue also known as BB-16 and has been tested in field crops for its potential to increase lettuce production (Serna et al., 2012). A variety of stress conditions, such as salinity, are characterized by an increase in ethylene production, and high levels of ethylene could aggravate the stress effect (Siddikee et al. 2012). In the study by Serna et al. (2015), the application of DI-31 reduced the negative effect of salinity stress on the fresh weight of lettuce and decreased ethylene synthesis. In this way, BRs induce tolerance to

salinity stress if high levels of ethylene and a reduction in fresh weight are assumed to stem from stress.

4. INTERACTION WITH GIBBERELLINS

Gibberellins (GAs), a group of tetracyclic diterpenoids, are plant hormones that are involved in many developmental processes in plants, including seed germination, stem elongation, leaf expansion, trichome development, pollen maturation, and the induction of flowering (Daviere and Achard 2013). Hence, GA-deficient mutant plants exhibit a dwarf and late-flowering phenotype, which can be reverted through GA treatment. GA was first identified in the pathogenic fungus *Gibberella fujikuroi* as the causal agent of 'foolish-seedling' disease in rice, which is characterized by excessive elongation of the infected plants (Yabuta and Sumiki, 1938). To date, more than 130 GAs have been identified in plants, fungi and bacteria, although only a few GAs have biological activity. The major bioactive GAs, which include GA_1, GA_3, GA_4, and GA_7, are derived from a basic diterpenoid carboxylic acid skeleton, and share a common C3-hydroxyl group. Many of the non-bioactive GAs that exist in plants are either de-activated metabolites or act as precursors for the bioactive GA forms (Yamaguchi, 2008).

Crosstalk between BRs and Gas exists over a wide range of biological processes, including plant development and responses to environmental stimuli (Wang et al. 2009, De Vleesschauwer et al. 2012, Unterholzner et al. 2015). It has also been shown that BRs regulate the biosynthesis of GAs in Arabidopsis and rice (Unterholzner et al. 2015, Tong et al. 2014). Evidence for this was provided by a study that showed that the production of bioactive GA was severely compromised and the expression of genes encoding enzymes of the GA20ox and GA3ox families was reduced in BR signaling-deficient Arabidopsis mutants. However, the application of GAs, as well as the reconstitution of GA20ox expression, rescued several of the developmental defects in the BR signaling-defective mutant bri1-301. Furthermore, the BR-regulated transcription factor BES1 binds to a regulatory motif present in the promoters of GA biosynthesis genes, including *GA20ox1* and *GA3ox1*, and induces their expression in a BR-promoted manner (Unterholzner et al. 2015). Tong and co-workers (Tong et al. 2014) studied the crosstalk between BRs and GA in rice (*Oryza sativa* L.), demonstrating that BR regulates cell elongation by modulating GA metabolism. Under physiological conditions, BR promotes GA accumulation by regulating the expression of GA metabolic genes that stimulate cell elongation. BR greatly induces the expression of *D18/GA3ox-2*, one of the GA biosynthetic genes, leading to increased levels of GA_1, an bioactive GA. Consequently, both D18 and loss-of-function GA-signaling mutants show decreased BR sensitivity. Interestingly, excessive active brassinolide application leads to GA inactivation, which is induced through the upregulation of *GA2ox-3*, a GA inactivation gene, and reduced BR biosynthesis, resulting in decreased hormone levels and growth inhibition (Tong et al. 2014). Studies in rice also show that *OsGSR1*, a member of the GAST (GA-stimulated transcript) gene family, is induced by GA and repressed by BR. RNA interference (RNAi) transgenic rice plants with reduced *OsGSR1* expression showed phenotypes similar to plants deficient in BR, including short primary roots, erect leaves and reduced fertility. In addition, the *OsGSR1* RNAi transgenic rice showed a reduced level of endogenous BRs, and

the dwarf phenotype could be rescued by the application of brassinolide. The results suggest that *OsGSR1* interacts with DIM/DWF1, an enzyme that directly regulates BR biosynthesis through the conversion of 24-methylenecholesterol to campesterol (Wang et al. 2009).

5. INTERACTION WITH CYTOKININS

Cytokinins (CKs) are hormones that regulate many developmental and physiological processes in plants. They play a crucial role in regulating the proliferation and differentiation of plant cells (Efroni et al. 2013, Schaller et al. 2014), and also control various processes in plant growth and development, including promotion of shoot growth (Zhao et al. 2010), inhibition of root development (Bielach et al. 2012), fruit and seed development, delay of senescence (Zwack and Rashotte 2013), the transduction of nutritional signals, as well as the response to abiotic and biotic stresses (O'Brien and Benková 2013, Zwack and Rashotte 2015). The cytokinins are divided into two categories: the isoprenoid cytokinins, exemplified by zeatin and isopentenyladenine, (are the most abundant type) and the naturally occurring adenine derivatives with aromatic substituents- the topolins (are considered less abundant) (Spichal et al. 2012).

It has been demonstrated in rice (*Oryza sativa*) that the expression of *isopentenyltransferase* (*IPT*) genes, driven by P_{SARK} (a stress-and maturation-induced promoter), affects plant hormone homeostasis and alters the source/sink balance during water stress (Peleg et al. 2011). The research also noted a CK increase, which coincided with the upregulation of several BR-related genes with roles in BR-biosynthesis (*DWF4*, *HYD1*) and BR-signaling (*BAK1, SERK1, BRI1*). This suggests that the crosstalk between BRs and CKs may contribute to the modification of the source-sink balance, leading to increased drought tolerance. The interaction between BRs and CKs was further supported by experiments that measured the expression of major BR-related genes in P_{SARK}*:IPT* and wild-type plants treated with exogenous CK. *DWF4*, a gene encoding a sterol C-22 hydroxylase that mediates a key reaction during BR biosynthesis, was shown to be up-regulated in the P_{SARK}*:IPT* plants under water stress conditions (Peleg et al. 2011). This overexpression of *DWF4* in rice has also been shown to result in increased grain weight (Wu et al. 2008).

6. INTERACTION WITH SALICYLIC ACID AND JASMONATES

Salicylic acid (SA) is a secondary metabolite produced by a wide range of prokaryotic and eukaryotic organisms, including plants. Chemically, it belongs to a group of phenolic compounds that possess an aromatic ring with either a hydroxyl group or its functional derivative (An and Mou 2011). In plants, SA has a well-established role of being a signaling molecule in immune response (Vlot et al. 2009) and the exogenous application of SA affects diverse plant processes such as thermogenesis (Raskin et al. 1987), seed germination (Rajou et al. 2006), cell growth (Vanacker et al. 2001), respiration (Norman et al. 2004), stomatal responses (Manthe et al. 1992; Lee 1998), senescence (Rao et al. 2002), thermotolerance (Clarke et al. 2004), and nodulation (Stacey et al. 2006). However, because SA is heavily

involved in crosstalk with other plant hormones it may only indirectly influence some of the mentioned processes (Robert-Seilaniantz et al. 2007; Pieterse et al. 2009).

In Arabidopsis, 24-epiBL positively regulates SA pathway components NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) and transcription factor WRKY70 to mediate thermotolerance and defense gene expression. NPR1 may likely be a critical component of BR-mediated effects on thermo- and salt tolerance, as BRs exert anti-stress effects also through interaction with salicylic acid (Divi et al. 2010).

Jasmonates (JAs) belong to a group of plant oxylipins and modulate many essential roles in plant development, ranging from germination to vegetative growth to senescence. They are directly involved in a number of physiological processes such as stamen and trichome development, vegetative growth, cell cycle regulation, senescence, anthocyanin biosynthesis regulation, and fruit ripening (Wasternack and Hause, 2013, Sharma and Laxmi 2016). In addition, JAs activate plant defense mechanisms following insect-driven wounding, pathogenic attack, and environmental stress, such as low temperature, salinity, or heavy metal toxicity (Pauwels and Goossens 2011, Sharma and Laxmi 2016). Studies in Arabidopsis revealed that BR treatment induces the expression of the OPR3 gene, which encodes a protein that plays an important role in JA biosynthesis by converting 12-oxo-phytodienoic acid (OPDA) to 12-oxo-phytenoic acid (OPC-8:0). *OPR3* mRNA expression levels were positively influenced by BRs, providing a potential link between BRs and JA action (Schaller et al. 2000).

ACKNOWLEDGEMENTS

This work was funded by the Ministry of Education, Youth and Sports of the Czech Republic – NPU I program with project LO1204, by grant GJ15-08202Y and by Internal Grant Agency of Palacky University- IGA_PrF_2016_018.

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FACULTY OF SCIENCE
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Effect of brassinosteroids on ethylene production in plants

Summary of the Ph.D. thesis
P1527 Biology – 1501V019 Experimental biology

Supervisor – Mgr. Jana Oklešťková, PhD.

Olomouc
2019

This PhD. thesis was carried out in the Laboratory of Growth Regulators within the framework of internal PhD. Study of Experimental Biology, guaranteed by the Laboratory of Growth Regulators, Faculty of Science, Palacký University in Olomouc, between the years 2015-2019.

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The oral defence will take place on **26.8.2019** before the Commission for the Ph.D. thesis of the Study Program Experimental Biology, **room……..**, Šlechtitelů 27, Olomouc – Holice.

The PhD. thesis and expert reviews will be avaiable 14 days before the defence in the Study Department of Faculty of Science (Mgr. M. Karásková), Palacky University, 17. listopadu 12, Olomouc.

After the defense, the Ph.D. thesis will be stored in the Library of the Biological Departments of Faculty of Science, Palacky University, Šlechtitelů 27, Olomouc – Holice.

Prof. Ing. Miroslav Strnad, CSc. DSc.
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CONTENT

1. INTRODUCTION

Plant hormones affect various aspects of plant development and play key roles in plant resistance to diverse environmental stresses. While extensive research has uncovered the effects of the main phytohormone classes - auxins, cytokinins, gibberellins, abscisic acid and ethylene, newer classes of plant hormones have been discovered like brassinosteroids, jasmonic acid, salicylic acid and polyamines. It is now recognized that plant growth and development are controlled by the mutual interactions among plant hormones. This thesis focuses on plant hormone crosstalk as an emergent area of this research. Brassinosteroids (BRs) are a family of naturally occurring steroid plant hormones that regulate various processes of growth and development, including cell elongation, cell division, leaf senescence, vascular differentiation, flowering time control, male reproduction, photomorphogenesis and responses to biotic and abiotic stresses. A number of these effects are tightly linked with almost all other classes of plant hormones.

This work looks closer at the relationship between brassinosteroids and ethylene, which is the only gaseous plant hormone with a simple structure. Ethylene plays an important role in a number of developmental processes in plants like opening of flowers, ripening of fruits and abscission of leaves. It is also a stress hormone and such is involved in most plant responses to biotic and abiotic stresses. The structure-activity relationship of biosynthetic precursors of brassinolide and a series of new brassinosteroid analogues were also studied in this research to elucidate which structural motifs are important for BR induced biological activity. These structures could be then used as a template for synthesis of new BR analogues with growth promoting activity for agricultural usage.

2. AIMS AND SCOPE

Brassinosteroids (BRs) as an important group of steroidal plant hormones involved in a variety of crucial physiological processes, are interesting compounds for further investigation and potential usage in agriculture. Because plant growth and development is a complex subject in which more than one group of plant hormones is involved, it is important to take the crosstalk between plant hormones into account and think more broadly about the topic.

The overall aims of the work described in this thesis were as follows:

1. To write a review about crosstalk between brassinosteroids and ethylene

2. To examine and assess the effect of new BR synthetic derivatives on ethylene production in plants

3. To evaluate the biological activity of these compounds in different bioassays

4. To investigate the biological activity of BR biosynthetic precursors and their effect on ethylene production

3. MATERIALS AND METHODS

3.1 Biological material

Pea seedlings - *Pisum sativum* var*. arvense* sort Arvica

Arabidopsis WT - *Arabidopsis thaliana* (Columbia ecotype, Col-0)

Arabidopsis ethylene signaling mutant lines - *Arabidopsis thaliana ein 2-5, etr 1-1, ein 3-1eil 1-1*

Arabidopsis BR mutant lines - *Arabidopsis thaliana dwf4, cpd, det2, rot3/cyp90d1, cyp85a1/cyp85a2, bri1/brl1/brl3*

3.2 Chemicals

All chromatographic solvents were of analytical grade or higher purity (Merck KGaA)

Chemical compounds - brassinolide, 24-epibrassinolide, indole-3-acetic acid, gibberellin GA3, trans-zeatin, thidiazuron and 1- aminocyclopropanecarboxylic acid, [D_4]ACC standard (Olchemim s.r.o. Czech Republic)

Brassinosteroid biosynthetic precursors were synthetized at the Laboratory of Growth Regulators Olomouc, Czech Republic)

3.3 Equipment

GC System gas chromatograph (Agilent Technologies, GC Systems) equipped with a flame ionic detector (FID) and HP-AL/S capillary column (50 m × 0.535 mm × 15 µm)

Laser-based photo-acoustic detector (ETD-300 ethylene detector, Sensor Sense, The Netherlands)

Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) system consisting of an ACQUITY UPLC® I-Class system (Waters, Milford, MA, USA) and a XevoTM TQ-S MS triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK).

3.4 Methods

3.4.1 The pea inhibition biotest

Pea seedlings (*Pisum sativum* var*. arvense* sort Arvica) germinating for 2 days were selected for uniformity from a large population and then transferred into pots containing perlite and 1/10 diluted Hoagland solution (half concentration, pH 5.7). After 24 h in a dark cultivation room (24 °C, humidity 75%) the seedlings were treated with different amounts of tested compounds in 5 µl fractionated lanolin. The substances were applied as microdrops to the scar left after the removal of the bract. The control plants were treated with lanolin alone. The inhibition of etiolated pea stems was measured after 4 days and the difference in length between the treated and control plants provided a measure of activity. The mean values were statistical analysed using the Student's t test (for more details see Supplement I, Supplement II, Supplement IV).

3.4.2 Determination of ethylene (The pea inhibition biotest)

For measurement of ethylene production, pea seedlings (8 plants/tested amount of substance) were placed in a 0.5 L glass container for 24 h in the dark. 1 ml of headspace gas was withdrawn from each container by syringe for each measurement and injected into a GC System gas chromatograph (Agilent Technologies, GC Systems) equipped with a flame ionic detector (FID) and HP-AL/S capillary column (50 m × 0.535 mm × 15 µm). The chromatographic analytical parameters were as follows: column temperature: 150 °C; detector temperature: 220 °C; and helium was used as a carrier gas. The area under the resultant peak (y-axis) versus sensitivity (x-axis; nl.ml-1) represented a quantitative measure of ethylene concentration; data were statistically analyzed using the Student's t test (for more details see Supplement I, Supplement II, Supplement IV).

3.4.3 Arabidopsis growth sensitivity assay

Arabidopsis thaliana (Columbia ecotype, Col-0; referred to as Arabidopsis) seedlings or Arabidopsis ethylene signaling mutant seedlings (*ein 2-5, etr 1-1, ein 3-1eil 1-1*) were stratified for 2 days at 4 °C and germinated on vertical half-strength Murashige and Skoog (1% w/v sucrose) agar plates with different concentrations of tested compound at 22 °C in a 16 h/8 h light–dark cycle for 7 days. The plates were then scanned with an Epson high-resolution scanner and the entire root and hypocotyl length measured with ImageJ

(http://rsbweb.nih.gov/ij/). P values were calculated with a two-tailed Student t-test using Excel software (see Supplement I, II).

3.4.4 Arabidopsis growth rescue assay

Arabidopsis *cyp85a1/cyp85a2* heterozygous mutant seedlings were stratified for 2 days at 4 °C and germinated on free vertical half-strength Murashige and Skoog (1% w/v sucrose) agar plates for 6 days. Then *cyp85a1/cyp85a2* homozygous plants were transferred to ½ Murashige and Skoog (1% w/v sucrose) agar plates containing DMSO or tested compound. 3 days after transfer, the plates were scanned with an Epson high-resolution scanner and the root length was measured with ImageJ (http://rsbweb.nih.gov/ij/).

3.4.5 Determination of ethylene production with photo-acoustic detector

Approximately 35 sterilized seeds of *Arabidopsis thaliana* (Columbia ecotype, Col-0; referred to as Arabidopsis) were placed in a 10 ml chromatography vials containing 5 ml of half-strength Murashige and Skoog (1% w/v sucrose) agar medium. Seeds in vials were vernalized for 2 days at 4°C in darkness and then vials were incubated at 22°C under long-day conditions (16 hours light/8 hours dark). After 4 days, vials were sealed and after another 24 hours, they were flushed at a flow rate of 2.5 Lh-1 and ethylene was measured with the laser-based photo-acoustic detector (ETD-300 ethylene detector, Sensor Sense, The Netherlands).

3.4.6 ACC determination

The tissue (50 mg of etiolated pea plants/50 pcs of 7day old light-grown Arabidopsis roots/hypocotyls) was homogenized in 1 ml of H_2O:methanol:chloroform (1:2:1), 50 pmol of internal standard ([D_4]ACC) was added to each sample, and after centrifugation (4 °C, 15 000 rpm) the supernatant was collected and evaporated to dryness. The samples were derivatized using an AccQ-Tag Ultra kit (Waters) and subsequently analyzed by an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) system consisting of an ACQUITY UPLC® I-Class system (Waters, Milford, MA, USA) and a XevoTM TQ-S MS triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK) (see Supplement IV).

4. SURVEY OF RESULTS

4.1 Design, synthesis and biological activities of new brassinosteroid analogues with a phenyl group in the side chain (Supplement I)

The aim of this study was to prepare a series of new brassinosteroid derivatives with a p-substituted phenyl group in the side chain. These novel brassinosteroid analogues were synthetized based on results from *in silico* molecular docking into the BRI1 receptor. To validate the docking method and to screen these new compounds in order to find compounds with strong brassinosteroid activity which could be potentially used in agriculture to improve growth and yield. Synthetized derivatives were tested in different brassinosteroid bioassays (the pea inhibition biotest, Arabidopsis growth bioassay, BES1 dephosphorylation assay) and the results were compared with naturally occurring brassinosteroids. Both types of brassinosteroids (6-ketones and B-lactones) were synthetized and 3 compounds (lactone 8f, 6-oxo derivatives 8c and 9c) with biological activity comparable with natural brassinosteroids were identified. It has been also shown that molecular docking into the BRI1 can be used as a powerful tool for prediction and design of new compounds with brassinosteroid activities, because analogues with similar or better binding energies than for brassinosteroids predicted by molecular docking also showed the strongest brassinosteroid activities. As a result of this work, new active compounds could be good candidates for application in agriculture. Progress in the chemical synthesis could help to overcome the economic restrictions which currently constrain the use of BRs in a large scale in agriculture. Finally *in silico* molecular docking appears to be a useful tool for predicting the brassinosteroid activity of new compounds.

Author contribution – co-author – screening of biological activities of new brassinosteroid analogues (performing the pea inhibition test, measurement of ethylene production in treated pea plants)

4.2 Synthesis of novel aryl brassinosteroids through alkene cross-metathesis and preliminary biological study (Supplement II)

The subsequent study is again aiming to synthesis and to test the biological activity of new phenyl analogue of brassinosteroids. In this study, a series of new aryl analogues of brassinosteroids was synthesized via alkene cross-metathesis which has been shown to be an efficient method for construction of the new side chains in the brassinosteroid structure. The biological activities of these newly prepared derivatives were established using different plant bioassays (the pea inhibition biotest, Arabidopsis growth bioassay). The ethylene production in pea seedlings treated with these compounds was also monitored and in order to compare the results with theoretical studies, *in silico* molecular docking into the BRI1 receptor was performed. Based on data from biological studies, 3 newly synthetized aryl analogues (10f, 10b, 10n) were identified as compounds with similar biological activity to 24-epiBL. Molecular docking into the BR receptor showed high binding affinity of these compounds which also predict good biological activity. In general, the results showed that the relationship between biological activity and substitution pattern in the phenyl group can be suggested as follows – no substitution or substitution with one small group like fluorine or chlorine leads to compounds with high brassinosteroid activity. In contrast, substitution with bulky groups (phenyl, methyl or others) or substitution with more than one group significantly reduces the biological activity of the berssinosteroid-like compound. Overall, alkene cross metathesis has been shown to be one prospective method for preparation of new brassinosteroid derivatives with potential use in agriculture to improve plant growth, enhance yield and/or increase the resistance of plants against various stresses.

Author contribution – first author - screening of biological activities of new brassinosteroid analogues (performing the pea inhibition test, Arabidopsis growth assays, measurement of ethylene production in treated pea plants)

4.3 Brassinosteroids induce strong, dose-dependent inhibition of etiolated pea seedling growth correlated with ethylene production (Supplement IV – submitted article)

The aim of this study was to examine the effect of brassinosteroids on the growth of etiolated pea seedlings. The results reported in this work, showed that BRs inhibit the etiolated growth of pea seedlings in a similar manner to the 'triple response' induced by ethylene. There was a correlation between growth inhibition and increase in ethylene/ACC levels which suggests that BRs' biological activities may be mediated by ethylene. And finally the data confirm the development of a new, highly sensitive and convenient bioassay for BR activity (in which ethylene production could also be monitored). This bioassay could be routinely used for evaluating the hormonal activities of new synthetic BR derivatives with potential agricultural uses.

Author contribution – first author - bioassay development, optimization, performing of the experiments, writing of manuscript

5. CONCLUSION AND PERSPECTIVES

This thesis focuses mainly on steroidal plant hormones – brassinosteroids, their effects on etiolated pea plants, the biological activity of BR precursors and novel synthetic analogues. The effects of BR described in this thesis are considered in relation to the interaction between BRs and the gaseous plant hormone, ethylene.

The most important outcomes of the work described are:

1. Treatment of etiolated pea plants with brassinosteroids induces all phenotypic elements of the ethylene 'triple response' to ethylene and the level of both ethylene and ACC increases in these plants in a dose-dependent manner. The results confirm that a highly sensitive bioassay for BRs using etiolated pea seedlings has been developed (Supplement IV).
2. Three compounds from the first series of brassinosteroid derivatives with p-substituted phenyl group in the side chain were identified as compounds with biological activity comparable with naturally occurring brassinosteroids (Supplement I).
3. It has been shown that molecular *in silico* docking into the BRI1 receptor can be used as a powerful tool for prediction and design of new compounds with brassinosteroid activities (Supplement I and II).
4. Through screening the second series of newly synthetized aryl BR analogues another 3 compounds with BR-like activity have been identified and the connection between the substitution pattern in the phenyl group and biological activity of the compound has been revealed (Supplement II).
5. Examination of BR biosynthetic precursors in the pea inhibition test and Arabidopsis growth test showed that BR biological activity occurs dose-dependently in downstream intermediates of the BR biosynthetic pathway
6. Comprehensive reviews on the interactions of brassinosteroids and ethylene during plant development and under various stress conditions have been published (Supplement III, Supplement V)

Plant hormone crosstalk is a complex topic of broad and current interest and the data presented in this thesis may form a basis for further research on a molecular level leading to elucidation of brassinosteroids-ethylene crosstalk. Newly synthetized BR analogues with high biological activity may have potential agriculture application by themselves or be used as a template for the synthesis of new compounds. *In silico* docking has also been shown to be a useful tool for the design of new bioactive compoundsand the newly developed sensitive bioassay for brassinosteroid is routinely used for examining BR biological activity.

6. LIST OF PAPERS & CONTRIBUTION REPORT

- Kvasnica M., Oklestkova J., Bazgier V., Rárová L., **Korinkova P**., Mikulík J., Budesinsky M., Béres T., Berka K., Lu Q., Russinova E., Strnad M. (2016). Design, synthesis and biological activities of new brassinosteroid analogues with a phenyl group in the side chain. Organic & Biomolecular Chemistry 14, 8691-8701. (Supplement I)
    - Co-author – screening of biological activities of new brassinosteroid analogues (performing the pea inhibition test, measurement of ethylene production in treated pea plants)

- **Korinkova P**., Bazgier V., Oklestkova J., Rarova L., Strnad M., Kvasnica M. (2017). Synthesis of novel aryl brassinosteroids through alkene cross-metathesis and preliminary biological study. Steroids 127, 46-55. (Supplement II)
    - First author - screening of biological activities of new brassinosteroid analogues (performing the pea inhibition test, measurement of ethylene production in treated pea plants, Arabidopsis root and hypocotyl sensitivity assays)

- **Jiroutová P**., Oklestkova J., Strnad M., (2018) Crosstalk between Brassinosteroids and Ethylene during Plant Growth and under Abiotic Stress Conditions. Int. J. Mol. Sci 19 (10), 3283. (Supplement III)
    - First author – writing of manuscript

- **submitted paper** - **Jiroutová P**., Mikulík J., Novák O., Oklestkova J., Strnad M., (2019) Brassinosteroids induce strong, dose-dependent inhibition of etiolated pea seedling growth correlated with ethylene production. Biomolecules, special issue – Phytohormones. (Supplement IV)
    - First author - bioassay development, optimization, performing of the experiments, writing of manuscript

<u>Chapter in book</u>

- J. Oklestkova, **P. Korinkova**, M. Strnad: The Crosstalk of Brassinosteroids and Other Hormones in Abiotic Stress Tolerance, in: Mechanisms Behind Phytohormonal Signalling and Crop Abiotic Stress Tolerance, NOVA SCIENCE PUBLISHERS, INC., NY 11788-3619, USA, 2017. (Supplement V)
    - Co-author – participation in writing of the manuscript

Published abstracts:

Kořínková P., Oklešťková J., Mikulík J., Strnad M. New Bioassay for Brassinosteroids – Pea inhibition Test. TNRP - Trends in Natural Products Research Conference 2014, Olomouc Czech republic

Kořínková P., Oklešťková J., Mikulík J., Strnad M. Cross-talk between brassinosteroids and ethylene in etiolated pea (Pisum arvense L. sort Arvica). KEBR - Conference of Plant Experimental Biology, 2015, Brno, Czech Republic

Kořínková P., Oklešťková J., Mikulík J., Kvasnica M., Strnad M. Cross-talk between brassinosteroids and ethylene in etiolated pea seedlings. Growth regulators on the way, 2016, Malá Morávka, Czech Republic

Kořínková P., Oklešťková J., Mikulík J., Kvasnica M., Strnad M. Biological activity of brassinosteroids and their synthetic derivatives. TNPR– Trends in Natural Products Research Conference PSE Young Scientists Meeting 2016, Pulawy, Poland

Kořínková P., Oklešťková J., Mikulík J., Strnad M. New Bioassay for Brassinosteroids – The Pea inhibition Test. 23th Conference on Isoprenoids 2016, Minsk, Belarus

Kořínková P., Oklešťková J., Bittnerová L., Mikulík J., Strnad M. Structure activity relationship of Brassinolide biosynthetic precursors and their effect on ethylene production. International CEPLAS SUMMER SCHOOL „Emerging Frontiers in Plant Sciences“ 2017, Hennef, Germany

Jiroutová P., Oklešťková J., Kvasnica M., Strnad M. Biological activity of brassinosteroid precursors and new derivatives. CBPRS – Chemistry and biology of Phytohormones and related substances 2018, Luhačovice, Czech Republic

Jiroutová P., Oklešťková J., Depaepe T., Van Der Straeten D., Strnad M. Interaction of brassinosteroid biosynthetic precursors with other Phytohormones. The 3rd INTERNATIONAL BRASSINOSTEROID CONFERENCE 2018, San Diego, USA

7. SUMMARY (Souhrn, In Czech)

Název disertační práce: Vliv brassinosteroidů na produkci ethylenu u rostlin

Disertační práce se zabývá účinkem rostlinných hormonů brassinosteroidů na produkci ethylenu v rostlinách. Brassinosteroidy jsou látky steroidní povahy, které mají zásadní vliv na řadu vývojových procesů v rostlinách. Názorným příkladem nepostradatelosti brassinosteroidů na správný růst a vývoj rostlin, jsou mutantní rostliny, které nemají schopnost syntetizovat vlastní steroidní hormony. Tyto mutantní rostliny se již na první pohled značně liší od normálních rostlin a to především svým výrazně zakrnělým růstem. Naopak exogenní aplikací brassinosteroidů na rostliny je možné stimulovat jejich růst a především odolnost rostlin na biotický a abiotický stres, tím se brassinosteroidy stávají velmi zajímavou skupinou hormonů s potencionálním využitím v zemědělství ke zvýšení úrodnosti a odolnosti polních plodin. Nevýhodou brassinosteroidů a překážkou jejich plošnému použití v zemědělství je jejich složitá syntéza a s ní související vysoká pořizovací cena.

V rámci disertační práce byly otestovány dvě série nových syntetických brassinosteroidních derivátů, ze kterých šest nově připravených látek vykazovalo srovnatelnou biologickou aktivitu s přirozeně se vyskytujícími brassinosteroidy. Mimo nových brassinosteroidních derivátů byly také testovány biosyntetické prekurzory brassinolidu. Biologicky aktivní prekurzory by následně mohly sloužit jako předloha k syntéze nových derivátů, které by si zachovaly vlastnosti brassinosteroidů a zároveň by se zjednodušila jejich syntéza, čímž by se snížila i pořizovací cena těchto látek a výrazně by vzrostl potenciál využití brassinosteroidů v zemědělství.

Všechny látky byly testovány v několika biologických testech se zaměřením na jejich biologickou aktivitu a vliv na produkci etylenu.