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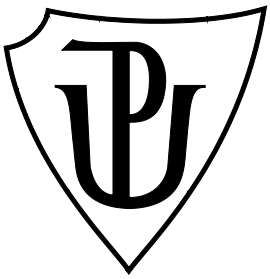

Ph.D. Thesis

Modifications of double bonds of lupane triterpenoids with anticancer activity

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Declaration

I hereby declare that this thesis is my original work, written under the supervision of doc. RNDr Milan Urban, Ph.D. All used literature sources are cited in the References section.

In Olomouc, ...

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Jan Pokorný

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Abstract

Pentacyclic triterpenoids are a large group of natural substances with a broad palette of biological activities. Many researchers are interested in their synthetic modifications in order to exploit their promising potential in drug development. One of the best-known compounds from the group of triterpenes is betulinic acid, which is known for its strong anticancer properties. Oxidation of betulinic acid at the position C-30 results in 30-oxobetulinic acid that had IC_{50} values in the low micromolar range against multiple cancer cell lines. However, this molecule suffered from the general toxicity caused by the presence of the Michael acceptor in its structure, resulting in low selectivity. In this Ph.D. thesis, three different synthetic approaches to modify the 30-oxobetulinic acid at the position C-30 are described in order to overcome its low selectivity and to improve other properties such as low solubility in water. An isosteric replacement was chosen in the first two projects, where the –CH=O formation of the parent compound was replaced with the –CH=N– or –CH=CH– to mask the Michael acceptor. Within the first project, a small series of triterpenic azines was prepared by condensation of variously substituted aromatic hydrazones and 30-oxobetulinic acid. Triterpenic azines had anticancer activities against the lymphoblastic leukemia cell line comparable to 30-oxobetulinic acid, however, selectivity was significantly improved. On the other hand, they were unstable and hard to prepare in higher yields. The Wittig reaction was employed in the second project, where both problems were successfully resolved, and a large group of forty new dienes was prepared. Two representatives were selected due to their high cytotoxicity to study their pharmacological parameters and mechanism of action. Both disrupt mitochondrial permeability and activate selective apoptosis *via* an intrinsic pathway. One derivative was chosen as a potential candidate for further preclinical development. In the third project, 30-oxobetulinic acid was further oxidized to carboxylic acid and transformed to propargyl ester that was able to react in the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC). The connection of the terpene with a rather polar triazole ring may improve the solubility of the molecule in water-based media and the bioavailability. The Heck reaction was optimized for use at the position C-3 in the fourth project. Throughout the work, the effect of double bonds in different parts of the triterpene structure was studied and reviewed in the theoretical part of this thesis.

Abstrakt

Pentacyklické triterpenoidy představují velkou skupinou přírodních látek se širokou paletou biologických aktivit. Mnoho výzkumných skupin se zajímá o jejich syntetické modifikace s cílem využít jejich slibný potenciál při vývoji léčiv. Jednou z nejznámějších sloučenin ze skupiny triterpenoidů je kyselina betulinová, známá svými silnými protinádorovými účinky. Oxidací kyseliny betulinové v poloze C-30 lze připravit kyselinu 30-oxobetulinovou, která vykazuje hodnoty IC_{50} v nízkém mikromolárním rozmezí proti řadě nádorových buněčných linií. Její nevýhodou je však obecně vysoká toxicita, způsobená přítomností Michaelova akceptoru ve struktuře, což má za následek nízkou selektivitu této sloučeniny. V předložené disertační práci jsou popsány tři různé syntetické přístupy modifikace kyseliny 30-oxobetulinové v poloze C-30 s cílem překonat její nízkou selektivitu a zlepšit další vlastnosti, jako je např. nízká rozpustnost ve vodě. U prvních dvou projektů byla zvolena cesta isosterické záměny, při níž byla formace –CH=O výchozí sloučeniny nahrazena za –CH=N– nebo –CH=CH– za účelem zamaskování Michaelova akceptoru. V rámci prvního projektu tak byla připravena malá série triterpenických azinů kondenzací různě substituovaných aromatických hydrazonů a kyseliny 30-oxobetulinové. Připravené aziny vykázaly protirakovinnou aktivitu vůči buněčné linii lymfoblastické leukemie CCRF-CEM srovnatelnou s kyselinou 30-oxobetulinovou, selektivita však byla výrazně zvýšena. Tyto látky byly bohužel nestabilní a obtížně se připravovaly ve vyšších výtěžcích. Wittigova reakce byla použita ve druhém projektu, kde se podařilo oba problémy minimalizovat a připravit knihovnu čtyřiceti nových dienů s konjugovanými vazbami. Dva nejaktivnější zástupci byli podrobeni studiu farmakologických parametrů a mechanismu účinku. Obě látky narušují mitochondriální permeabilitu a aktivují selektivní apoptózu mitochondriální dráhou. Jeden derivát byl vybrán jako potenciální kandidát pro další preklinický vývoj. Ve třetím projektu byla kyselina 30-oxobetulinová dále oxidována v poloze C-30 na kyselinu a alkylována na propargylester, který byl schopen reagovat v mědí(I) katalyzované azido-alkynové cykloadici (CuAAC). Spojením terpenu s poměrně polárním triazolovým kruhem lze zlepšit rozpustnost molekuly ve vodném prostředí a biologickou dostupnost. Heckova reakce byla optimalizována pro použití v poloze C-3 ve čtvrtém projektu. V průběhu celé práce byl studován vliv dvojných vazeb v různých částech struktury triterpenu na biologickou aktivitu, který byl shrnut v teoretické části této práce.

Contents

1 Introduction

Terpenes are one of the largest classes of natural compounds with tens of thousands of representatives to date.[1,2] To be more precise in the nomenclature, terpenes are simple hydrocarbons, while terpenoids contain other elements such as oxygen or nitrogen in their structure and can be further structurally modified. Terpenes, terpenoids, steroids, and other natural substances belong to the group of isoprenoids, however, the names are often used interchangeably.[3]

Terpenes are formally oligomers or polymers containing two or more molecules of isoprene unit. Isoprene or 2-methylbuta-1,3-diene (Fig. 1) is an unsaturated organic compound with the molecular formula C_5H_8. Thus, we can write the general formula of terpenes as $(C_5H_8)_n$, where n represents the number of isoprene units of which they are composed. Consequently, there are hemiterpenoids, consisting only of one unit = five carbon atoms (C_5), monoterpenoids (two units, C_{10}, historically considered the smallest triterpenic molecules, hence their name[4]), sesquiterpenoids (three units, C_{15}), diterpenoids (four units, C_{20}), sesterterpenoids (C_{25}), triterpenoids (C_{30}), tetraterpenoids (C_{40}), and polyterpenoids. Like the majority of natural processes, the biosynthesis of terpenoids is relatively complex – these natural compounds are not formed by a simple polymerization of isoprene units.[2]

Fig. 1: The structure of isoprene.

Terpenoids are probably the largest group of natural substances, and this is allowed by the fact that they vary greatly in size, shape, and structure – they can be acyclic, but they may also contain various numbers of cycles and functional groups. In addition, they may be found all around us and play very diverse roles in living organisms. For example: Menthol (**I**, Fig. 2, MW = 156 $g \cdot mol^{-1}$) and limonene (**II**, MW = 136 $g \cdot mol^{-1}$) are monocyclic monoterpenoids (C_{10}), the former is responsible for the spearmint smell and has the typical "cold flavor", the latter is known for its typical smell of lemons. Retinol (**III**, MW = 286 $g \cdot mol^{-1}$) is one of the diterpenes, better known as a representative of the vitamin A family. Squalene (**IV**, MW = 411 $g \cdot mol^{-1}$) is the basic acyclic triterpenic compound with a long unsaturated chain and molecular formula $C_{30}H_{50}$. Although not very well known to

the public by its name, it is an important precursor for the biosynthesis of other triterpenoids, steroids, and sterols, including cholesterol and steroid hormones (e.g. estrogens) in the human body.[5] Finally, natural rubber is a typical polyterpene consisting of linked isoprene units. This macromolecular biopolymer, *cis*-1,4-polyisoprene (**V**), has unique properties such as high elasticity.[6] In conclusion, terpenes are an inseparable part of everyday life, some of them are being even economically important, for example, essential oils in cosmetics, or rubber in the industry of plastics.[4]

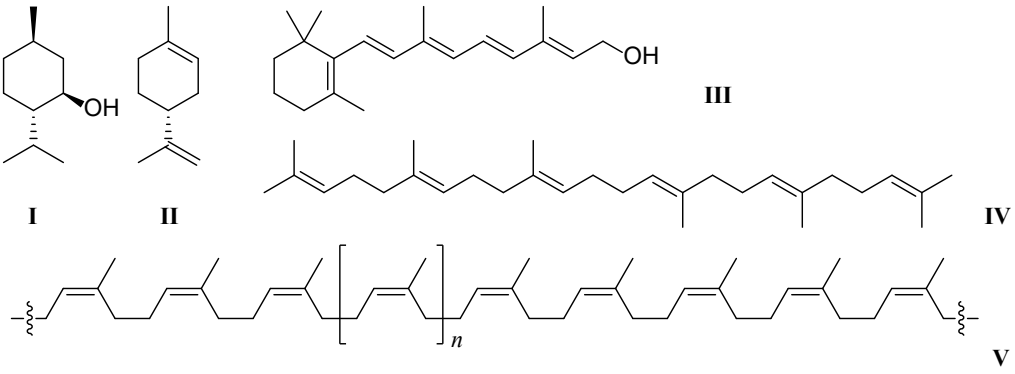


Fig. 2: Structures of monoterpenes menthol (**I**), limonene (**II**), diterpene retinol (**III**), triterpene squalene (**IV**), and polyterpene *cis*-1,4-polyisoprene (**V**).

Triterpenes are a large subgroup of terpenes, in 2006 approximately 5,000 naturally occurring representatives with more than a hundred different skeleton types were known,[7] and this number is still growing. They may be acyclic (squalene) as well as they can consist of one to five cycles and these cycles are usually five- or six-membered (see Fig. 3).[8] According to the number of cycles, they can be further divided into groups such as pentacyclic triterpenes, in which lupane, oleanane, ursane, or hopane can be found.[9] However, all triterpenes have one thing in common – their basic skeleton consists of thirty carbon atoms (six isoprene units) due to their biosynthesis from the essential intermediate – squalene.[10]

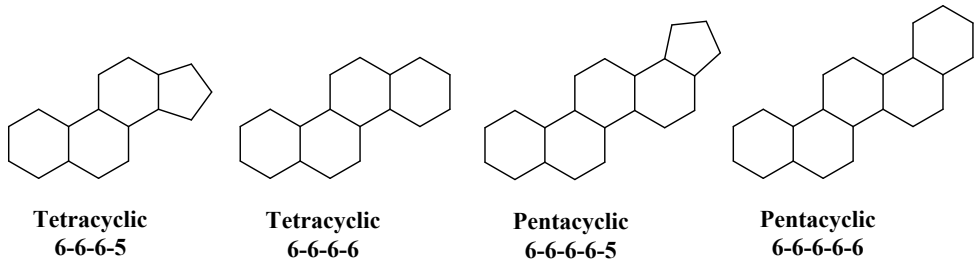


Fig. 3: Examples of basic skeletons of tetracyclic and pentacyclic triterpenes. Sidechains are not shown.

Triterpenes may occur in nature as free, unsubstituted molecules, as well as bound in esters or glycosides (saponins).[11] They are widespread probably in all

living organisms – they were found in both prokaryotes and eukaryotes – in bacteria,[12,13] animals,[14] humans,[15] plants,[16–18] algae[19–21], and fungi.[22–26] Every year, new triterpenes are discovered, isolated, and described.[27–30] Albeit some terpenoids serve important primary functions as photosynthetic pigments (carotenoids), growth and development regulators (plant hormones – gibberellins, strigolactones, cytokinins; animal hormones – steroids),[31] or as elements of membrane structure and function (sterols – cholesterol, Fig. 4, forms part of the cellular membrane in animals),[2] *pentacyclic* triterpenoids serve primarily as a plant defense against pathogens, herbivores, pests,[32] and in many cases, their function still remains unknown. Since there are examples of terpenoids that are essential for their producer while other terpenoids are not, these important terpenoids are considered as primary metabolites and the rest of them as secondary metabolites. This means that they are not essential for the growth, development, and reproduction of an organism.[33]

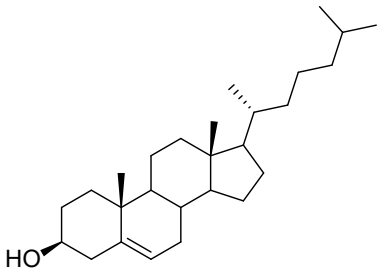


Fig. 4: The structure of cholesterol – tetracyclic triterpenoid.

Pentacyclic triterpenoids represent a highly interesting group of terpenes in terms of their biological activities.[34] Among them, the lupane (**VI**, Fig. 5), oleanane (**VII**), or ursane (**VIII**) analogs are the most important. They can be found mainly in peels, leaves, stems, and bark of numerous plants (e.g. birch and sycamore bark, olive leaves and pomace, fruit peels, etc.).[34] It was found that they have antiviral,[35–37] antibacterial,[38–40] antifungal,[41,42] antiulcer,[43] anti-inflammatory,[44–46] and antidiabetic[47–49] activities, therefore, some species have been used for centuries in folk or traditional Chinese medicine.[50] Furthermore, they exhibit antioxidative,[51–53] hepatoprotective,[54] cardioprotective,[55] neuroprotective,[56] antimalarial,[57–59] anti-HIV,[60–63] and anticancer[64–72] activity. It is more than obvious that due to such a palette of biological activities and structures, triterpenes are extremely interesting for detailed research.

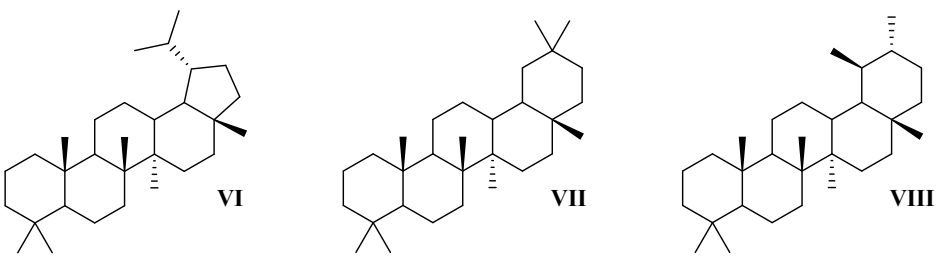


Fig. 5: Structures of lupane (**VI**), oleanane (**VII**), and ursane (**VIII**).

Not to mention only the good properties, pentacyclic triterpenes also have some disadvantages. It results from the nature of their structures that they are not readily soluble in water. This makes it difficult to ensure their delivery to the desired targets in living organisms and specific cells, which affects their bioavailability.[73,74] Furthermore, the effective concentration of the drug is often not low enough to be useful as a therapeutic, sometimes their selectivity is low. Finally, triterpenes have many mechanisms of action,[75–77] some of them still remain unknown, making their studies complicated. Hence, many scientists focus their forces on the deep exploration, development, and improvement of these unfavorable triterpenoid properties. They are looking for possible solutions to the above-mentioned problems by modifications and derivatization of the parent active structures. Every year, hundreds of new triterpenes are synthesized/isolated from natural sources and tested for various biological activities; all studies are periodically summarized in the literature.[78–81] Appropriately modified pentacyclic triterpenes can undoubtedly be a useful tool in the fight against diseases of civilization, such as cancer.

2 State of the art

The theoretical part will be focused on the lupane-type triterpenoids only. The lupane structure (**VI**, Fig. 6, see the numbering) is composed of four six-membered rings A–D and one five-membered ring E, bearing the isopropyl group. Six methyl groups (numbered 23–28) are part of the basic skeleton. All other compounds of this type are derived from the structure below.

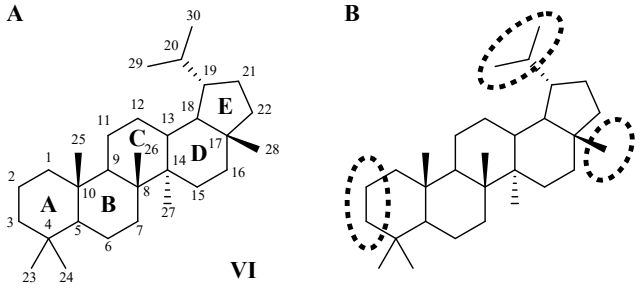


Fig. 6: A) Numbering of the lupane (**VI**) structure and cycles;
B) typical positions for modification.

The most important lupane-type triterpenoids, which are naturally abundant (and can be easily isolated from plants), are lupeol (**IX**, Fig. 7), betulin (**X**), and betulinic acid (BA, **XI**). Lupeol (**IX**) can be found in vegetables or fruit (e.g. mango, apple)[82] and it differs from lupane (**VI**) by the presence of the β-OH group attached to the C-3 carbon. Lupeol (**IX**) is known for an extensive range of biological activities, especially anti-inflammatory, antioxidant, anticancer, antimicrobial, and other pharmacological effects.[83] Betulin (**X**) is present in an external (white) layer of bark of *Betula pendula* in more than 20 %.[84] This compound is responsible for the white color of birch bark, and thanks to such a high content of hydrophobic

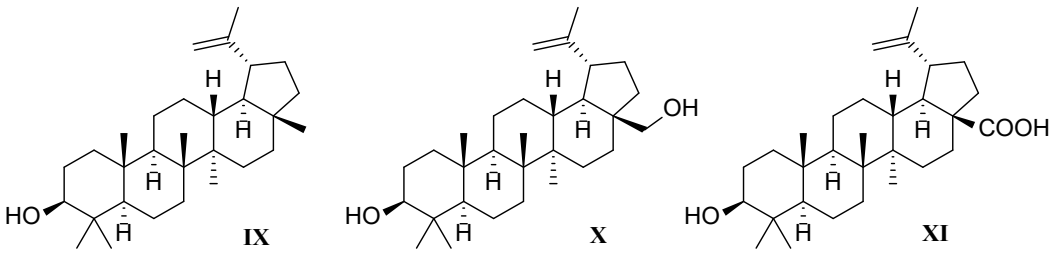


Fig. 7: Structures of lupeol (**IX**), betulin (**X**), and betulinic acid (**XI**).

betulin (**X**) with antifungal properties, the bark molds or rots slower than other parts of the dead birch trunk. Betulin (**X**) contains an additional -OH group attached to the carbon C-28 in comparison with lupeol (**IX**). It is known for its antioxidant, anti-inflammatory, anticancer, and hepatoprotective properties.[85] Betulinic acid (**XI**) is present in the bark of birches and sycamores, and it has a carboxyl at the position C-17. BA can be industrially prepared from abundant betulin (**X**) by the oxidation of its primary 28-CH_2-OH group.[86] The acid **XI** is widely known for its anti-HIV, antibacterial, antimalarial, anti-inflammatory, and mainly anticancer activities.[87] It is worth mentioning that an important derivative of BA is bevirimat (Fig. 8), known for its strong anti-HIV effect.[88] Notice that all three compounds **IX**–**XI** have a double bond C-20(29) unlike the parent lupane (**VI**). Thus, all compounds **IX**–**XI** are unsaturated, all have the polar β-OH group at the A-ring and the -CH_3/-CH_2OH/-COOH group at the position C-17, respectively. Furthermore, they all have significant anticancer properties[89] and this makes lupane derivatives highly interesting for the potential anticancer drug development and SAR studies.[90]

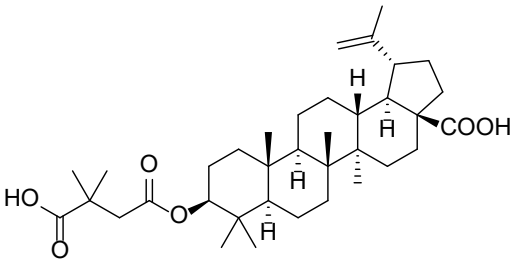


Fig. 8: Bevirimat, an anti-HIV drug derived from betulinic acid (**XI**).

It is obvious that lupane triterpenoids can be synthetically modified in a simple manner only at a limited number of positions (see Fig. 6). Firstly, the A-ring is capable of various types of modification thanks to the presence of the 3β-OH group. This group can be further oxidized to a keto group,[91] then the hyperconjugated protons in the α-position (C-2) open the door to a number of other modifications not only at the C-2 position;[92,93] even a new cycle/heterocycle[94–96] can be attached to the A-ring. The C-28 position is usually modified with respect to the functional group present. The isopropenyl unit is derivatized often at the position C-30 due to the elevated reactivity of the allyl position, or at the double bond C-20(29).

Looking at the three basic lupane representatives **IX**–**XI** (Fig. 7) in terms of double bonds, there is only one double bond present in each of them. From the synthetic point of view, it would be desirable to add another double bond into the structure of lupane triterpenoids in order to expand the possible options for synthetic modifications. More detailed information about the possibilities of adding more double bonds to the neighborhood of the isopropenyl group will be discussed in Chapter 2.1. Regarding the A-ring, there are several options where a new double bond can be introduced. This will be discussed in Chapter 2.2. Fig. 9 summarizes

the possible positions of double bonds in the lupane skeleton, which this work deals with – at the A-ring and at the isopropenyl unit.

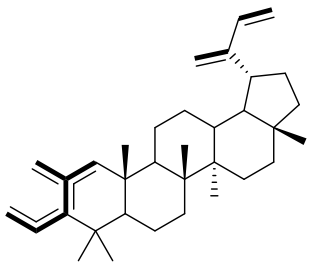
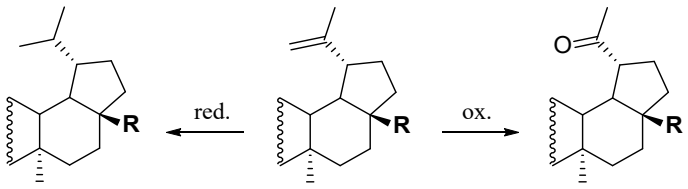


Fig. 9: Theoretical positions of double bonds in the modified lupane-type compounds, discussed in this thesis. The illustration summarizes all the possibilities at the A-ring and at the isopropenyl unit, and so does not respect the valency of carbon atoms.

2.1 Modifications at the isopropenyl moiety of lup-20(29)-ene triterpenoids with the main focus on double bonds

This chapter is focused on the methods for possible modification of the double bond C-20(29) at the isopropenyl part of lupene and other possibilities for introducing a new double bond at the position C-30 in the context of biological, in particular cytotoxic activity. Cyclizations of the whole isopropenyl unit considering the double bond are also discussed. Detailed literature research of the modifications at this position was performed since the vast majority of the practical work of this Ph.D. thesis is devoted to the modification of the C-30 position.

First of all, the effect of the double bond 20(29) itself will be discussed in the context of what happens if the double bond is replaced by a single bond (reduction), or oxidatively cleaved to form a ketone (Scheme 1).



Scheme 1: How does the double bond 20,29 affect biological activity?

Yasukawa et al.[97] tested a set of lupane-type triterpenoids for their ability to inhibit TPA-induced inflammation and tumor promotion in mice. Triterpene samples were applied topically on mouse ears and skin 30 min before each TPA treatment, then the thickness of the ears or the number and diameter of the skin tumors were measured periodically. Results showed a strong inhibition ratio for lupeol (**IX**,

Fig. 7, 87 %) and lupanol (**XII**, Fig. 10, 88 %); there was no significant difference in terms of the double bond C-20(29).

Salin et al.[98] tested betulin (**X**) and betulinic acid (**XI**) derivatives for their antibacterial activity against *Chlamydia pneumoniae*. Among many compounds, betulin (**X**), 20,29-dihydrobetulin (**XIII**, Fig. 10), betulonic acid (**XV**), and 20,29-dihydrobetulonic acid (**XVI**) were evaluated. Hydrogenation of the double bond 20(29) resulted in unchanged activity for betulin (**X**), where inhibition of *Ch. pneumoniae* growth reached 53 % for **X**, and 44 % for its dihydro analog **XIII** at the concentration of 1 $\mu mol \cdot L^{-1}$, while viability remained 99 % for both compounds. On the other hand, in the case of betulonic acid (**XV**), 53% inhibition activity was observed, whereas reduction of the double bond led to 100% inhibition at the same concentration. In addition, the viability increased from 56 % for betulonic acid (**XV**) to 95 % for its dihydro analog **XVI**. In this case, the activity of the hydrogenated derivative **XVI** exceeded the parent compound **XV** significantly.

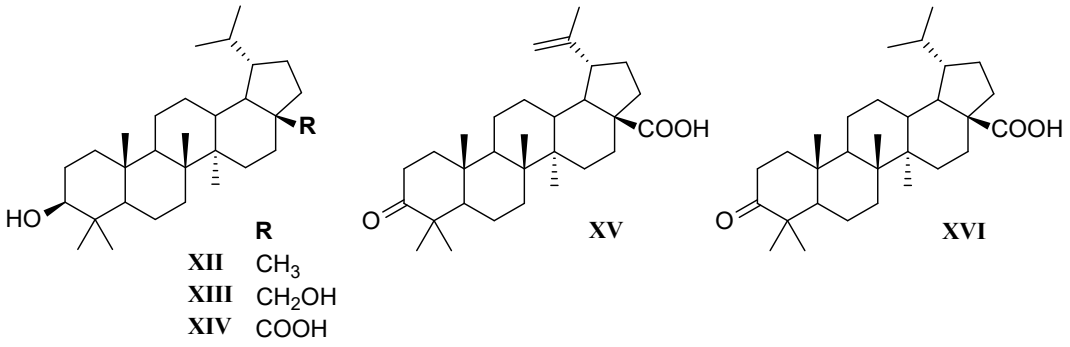


Fig. 10: Structures of betulonic acid (**XV**) and 20,29-dihydrolupanes – lupanol (**XII**), 20,29-dihydrobetulin (**XIII**), 20,29-dihydrobetulinic acid (**XIV**), and 20,29-dihydrobetulonic acid (**XVI**).

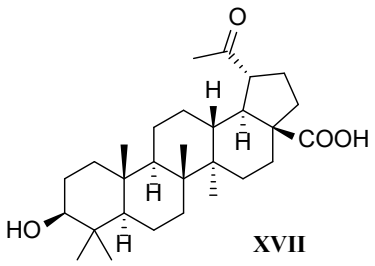
Pohjala et al.[99] tested a set of derivatives of betulin (**X**) for the inhibition activity of *Alphavirus* (Semliki Forest virus, SFV) replication. Betulin (**X**) inhibited replication of SFV with $IC_{50} = 45.5\ \mu mol \cdot L^{-1}$. On the contrary, 20,29-dihydrobetulin (**XIII**) did not have any antiviral activity.

In the literature,[96,100] new derivatives of betulinic acid (**XI**) were synthetized and tested for their cytotoxic activity against the CCRF-CEM line. During the synthesis, 20,29-dihydrobetulinic acid (**XIV**, Fig. 10) was prepared as a precursor and tested together with the starting and final compounds. The IC_{50} value of BA (**XI**) was 30 $\mu mol \cdot L^{-1}$, while for 20,29-dihydrobetulinic acid (**XIV**) the IC_{50} value was in the range of 7–9 $\mu mol \cdot L^{-1}$. This means that for the CCRF-CEM line, the activity of hydrogenated acid **XIV** is higher.

Mukherjee et al.[101] synthesized 20,29-dibromoderivatives of BA (**XI**) and tested them for cytotoxic activity against nine cancer cell lines (MOLT-4, JurkatE6.1, CEM.CM3, BRISTOL8, U937, DU145, PA-1, A549, L132). The precursor, 20,29-dihydrobetulinic acid (**XIV**), showed twofold higher potency on MOLT-4 cell line

(ED_{50} = 0.6 μg · mL^{-1}) than BA (**XI**, ED_{50} = 1.2 μg · mL^{-1}), and comparable activities on CEM.CM3, U937, DU145, and L132. For PA-1 and A549 cell lines, betulinic acid (**XI**) was considered inactive, while 20,29-dihydrobetulinic acid (**XIV**) showed highly potent cytotoxic activity in the range of 0.7–2.2 μg · mL^{-1}.

Another effect can be observed when the double bond 20(29) is cleaved by ozonolysis, which results in platanic acid (**XVII**, Fig. 11). This compound also occurs in nature, albeit in rather small amounts. Platanic acid (**XVII**) is known for moderate anticancer activity.[102]



XVII

Fig. 11: The structure of platanic acid (**XVII**).

In the literature,[103] platanic (**XVII**) and betulinic (**XI**) acids were tested against three cancer cell lines (MCF-7, PC-3, SCC2095). It was found out that acid **XVII** is more active in MCF-7 (IC_{50} = 15 μmol · L^{-1}) and PC-3 (IC_{50} = 14 μmol · L^{-1}) cancer cell lines than BA (**XI**, IC_{50} = 22 μmol · L^{-1} in MCF-7, and IC_{50} = 15 μmol · L^{-1} in PC-3, respectively). For the SCC2095 cancer cell line, IC_{50} = 18 μmol · L^{-1} for BA (**XI**), while for platanic acid (**XVII**) the IC_{50} value was higher than 30 μmol · L^{-1}.

In another article,[104] platanic (**XVII**) and betulinic (**XI**) acids were tested against seven cancer cell lines (518A2, A2780, HT29, MCF-7, A549, 8505C, NIH 3T3). Results showed that BA (**XI**) was moderately active (IC_{50} in the range of 9–17 μmol · L^{-1}) in contrast with platanic acid (**XVII**), where IC_{50} > 30 μmol · L^{-1} for all tested lines. From the above results, we can state that the anticancer activity of platanic acid (**XVII**) is generally lower, but it depends on the respective cell line.

Similar results were observed by Baratto et al.[105] They tested betulinic (**XI**) and platanic (**XVII**) acids against five cancer cell lines (A2780, 8505c, 518A2, MCF-7, A549); cytotoxic activity of BA (**XI**) was in the range of 1.8–2.5 μmol · L^{-1}, while for platanic acid (**XVII**) the IC_{50} value ranged from 63.2 to 97.5 μmol · L^{-1}, indicating lower activity.

Genet et al.[106] tested a set of natural triterpenoids in a SAR study for its TGR5 agonist activity. They found out that BA (**XI**) has one of the highest activities with the EC_{50} = 1.04 μmol · L^{-1} and the efficacy of 83 % compared with 20,29-dihydrobetulinic acid (**XIV**, EC_{50} = 2.17 μmol · L^{-1}, efficacy 60 %), and platanic acid (**XVII**, EC_{50} = 3.11 μmol · L^{-1}, efficacy 156 %). They stated that the replacement of alkene by alkane or ketone in the isopropenyl moiety slightly lowered the

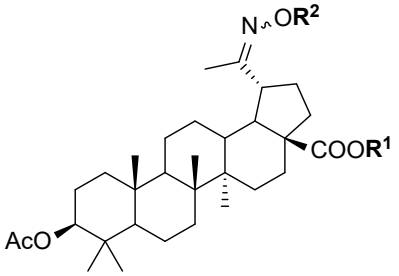
activity, but the effect was weak and appeared compatible with TGR5 activation.

It is clear from the above paragraphs that the hydrogenation of the double bond 20(29) in the isopropenyl moiety to form a single bond or cleavage of the double bond that affords a keto group may significantly change the biological activities of the modified compounds. Nevertheless, it is not possible to draw a general conclusion about the effect. The reduction of the double bond can lead to more cytotoxic compounds, on the other hand, antiviral properties of related analogs may be reduced. Similarly, the oxidation of the double bond leading to platanic acid (**XVII**) can reduce the cytotoxic activity, but it strongly depends on the respective cancer cell line.

2.1.1 Modifications of the double bond C-20(29)

In this section, various modifications of the C-20(29) double bond will be discussed. It includes vinylic substitution reactions at the position C-29 along with reactions of the keto group of platanic acid (**XVII**) leading to substituted hydrazone or oxime derivatives as formal isosteres for the C=C bond. Oxidation, reduction, and deuteration of the double bond are also briefly mentioned at the end of this chapter; however, these reactions often lead to the removal of the double bond.

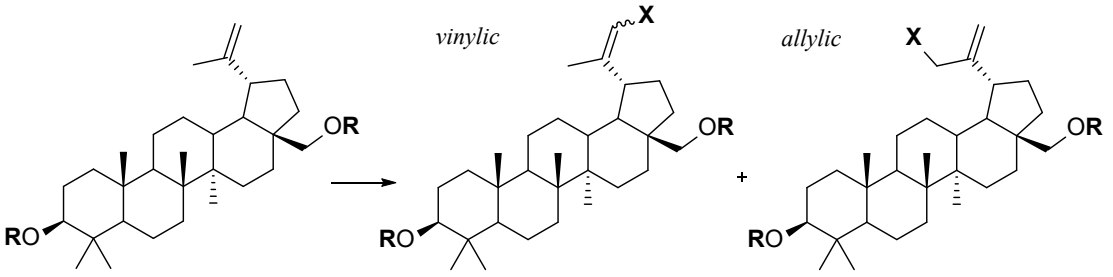
Kahnt et al.[102] continued the work of Heller et al.[107] and prepared thirty-seven new derivatives from platanic acid (**XVII**), seven of them preserving the double bond at C-20 (although the C=O was replaced with C=N as an oxime, see Fig. 12). Hydroxylamine hydrochloride in pyridine was used to prepare compounds **XVIII**–**XIX** (Fig. 12) from un/protected **XVII**, followed by esterification of the -OH group of compound **XIXb** by Ac_2O, propionyl chloride, or benzoyl chloride, respectively (**XX**–**XXII**). All new compounds were tested for the cytotoxic activity in several human tumor cell lines (518A2, A2780, HT29, MCF-7, A549, NIH 3T3) using the SRB assay. Modification at the position C-29 (compounds **XVIII**–**XIX**) resulted in moderate antitumor activity in the range of EC_{50} 6.1–16.5 $\mu mol \cdot L^{-1}$ (parent compound 34.2–47.3 $\mu mol \cdot L^{-1}$), while the *O*-alkyl substituted oximes **XX** and **XXI** had higher EC_{50} values of 13.1–28.7 $\mu mol \cdot L^{-1}$. Compound **XXII** was not soluble enough in solvents compatible with the SRB assay, thus could not be tested.



	R^1	R^2		
XVIIIa	Bn	H	*Z*-oxime	(10%)
XVIIIb	Bn	H	*E*-oxime	(80%)
XIXa	Me	H	*Z*-oxime	(13%)
XIXb	Me	H	*E*-oxime	(72%)
XX	Me	Ac	*E*-oxime	(84%)
XXI	Me	Propionyl	*E*-oxime	(64%)
XXII	Me	Bz	*E*-oxime	(99%)

Fig. 12: Oximes **XVIII**–**XXII** prepared by Kahnt et al.

Bodrikov et al.[108,109] described vinylic and allylic chlorination of betulin (**X**) and betulin diacetate (**XXIII**) using *tert*-butyl hypochlorite at low temperatures (7–10 °C) in acetonitrile, giving predominantly a mixture of C-29 *E* and *Z* isomers **XXIVa** (Scheme 2) in the ratio of nearly 1 : 1 and the overall yield of 72 %. Later, they prepared a small set of C-29 halogenated derivatives **XXIVb**–**XXIVf** in quantitative–low yields using *N*-chloro- (NCS), *N*-bromo- (NBS), and *N*-iodosuccinimides (NIS) in acetic acid at 15–50 °C; in addition to that, Br_2 and I_2 were also used. The formation of the products substituted at their vinylic (**XXIV**) and allylic (**XXV**) positions was studied, the final C-29 *E* and *Z* isomers **XXIVa**–**XXIVf** were identified by X-ray analysis. Recrystallization of the product mixture was successfully employed to enrich the amount of a single isomer. The yields of vinyl-substituted products decrease in the series in the order Cl > Br > I while arising the allyl-substituted products. No biological testing was reported.



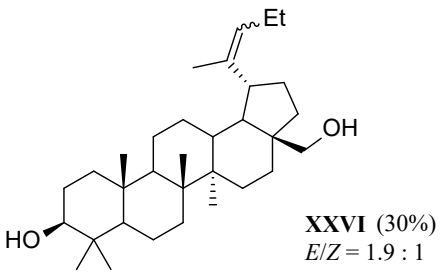
	R
X	H
XXIII	Ac

	R	X	*E*	*Z*
XXIVa	OH	Cl	40%	32%
XXIVb	OAc	Cl	39%	31%
XXIVc	OH	Br	33%	21%
XXIVd	OAc	Br	30%	19%
XXIVe	OH	I	6%	3%
XXIVf	OAc	I	6%	2%

	R	X	
XXVa	OH	Cl	28%
XXVb	OAc	Cl	30%
XXVc	OH	Br	46%
XXVd	OAc	Br	51%
XXVe	OH	I	91%
XXVf	OAc	I	92%

Scheme 2: Vinylic (**XXIV**) and allylic (**XXV**) halogenation of betulin (**X**) and betulin diacetate (**XXIII**) described by Bodrikov et al. Reagents and conditions: NCS, NBS or NIS, acetic acid, 15–50 °C.

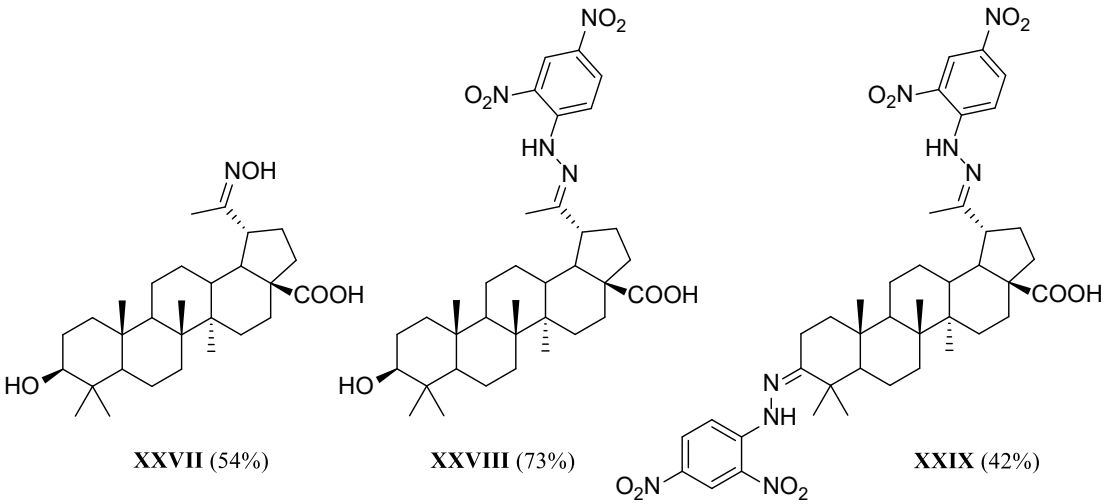
Zhu et al.[110] published an efficient and selective method for iron-catalyzed short-chain alkylation of vinyl arenes, where they also tried to use the developed method for ethylation of the non-conjugated double bond C-20(29) in betulin (**X**). They successfully used $Fe(OTf)_3$ as a catalyst (2.5–5.0 mol %), and various monopercarbonates or peresters as alkylation agents under mild conditions with high yields and exclusive *E* selectivity. However, the reaction of betulin (**X**) did not reach full conversion, leading to a mixture of *E*/*Z* isomers in a ratio of 1.9 : 1 as ethyl Heck product **XXVI** (Fig. 13) in 30% yield. They stated that the developed method is less ideal for non-conjugated olefins, so other simple olefins suffer from poor conversion and selectivity problems.



XXVI (30%)
E/*Z* = 1.9 : 1

Fig. 13: The Heck-type derivative **XXVI** of betulin (**X**) prepared by Zhu et al.

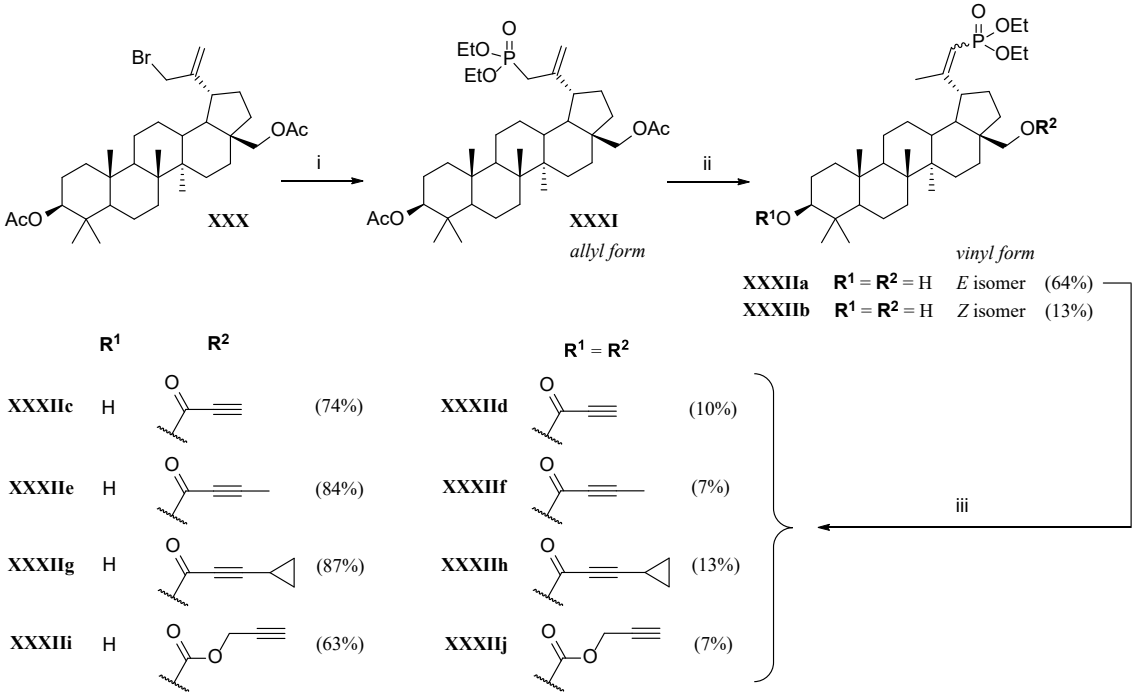
Baratto et al.[105] prepared three derivatives of betulinic acid (**XI**), containing an oxime or hydrazone at the position C-29 (compounds **XXVII**–**XXIX**, Fig. 14). They tested them for cytotoxic activity against five human cancer cell lines (A2780, 8505c, 518A2, MCF-7, A549). The most active derivative **XXVIII** had IC_{50} values in the range of 6.8–11.3 µmol · L^{-1}, similarly as the parent BA (**XI**, IC_{50} 8.8–14.8 µmol · L^{-1}), while the platanic acid (**XVII**), from which derivatives **XXVII** and **XXVIII** were directly prepared, was considered inactive (IC_{50} > 50 µmol · L^{-1}). The remaining two derivatives **XXVII** and **XXIX** had IC_{50} > 50 µmol · L^{-1} as well. Compound **XXVIII** was then tested for selectivity with CCD-18Co normal colon human fibroblasts, the selectivity index SI reached 56 ± 3 (calculated by the IC_{50} value in fibroblasts divided by the IC_{50} value in cancer cell lines). Later, Ullah et al.[111] prepared and tested compounds **XXVIII**, **XXIX** *in vitro* for antitrypanosomal (*Trypanosoma cruzi*) and antimalarial (*Plasmodium falciparum* W2 chloroquine-resistant) activities. Compound **XXVIII** showed higher activity against *P. falciparum* with the IC_{50} value of 6.5 µg · mL^{-1} in comparison to BA (**XI**) with moderate activity IC_{50} = 17.7 µg · mL^{-1}.



XXVII (54%) **XXVIII** (73%) **XXIX** (42%)

Fig. 14: Oxime (**XXVII**) and hydrazone (**XXVIII**, **XXIX**) derivatives of betulinic acid (**XI**) prepared by Baratto et al. and Ullah et al.

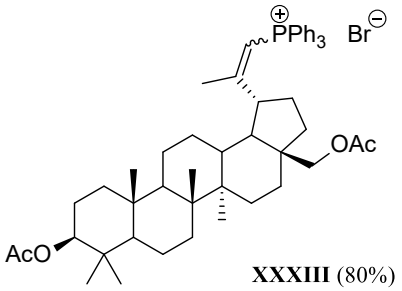
Chrobak et al.[112] described allyl-vinyl isomerization of newly synthesized betulin (**X**) derivatives, containing a phosphonate group at the position C-29. The Michaelis-Arbuzov reaction of 30-bromodiacetyl betulin (**XXX**, Scheme 3) with triethyl phosphite provided allylic betulin phosphonate at C-30 (**XXXI**), which was subjected to hydrolysis of acetylated -OH groups at C-3 and C-28. After this deprotection step that was performed in the presence of KOH in refluxing ethanol for two hours, they observed a mixture of two vinylic isomers **XXXIIa**, **XXXIIb** at C-29 in the ratio of 1 : 0.2 (*E*/*Z*). Obtained *E* isomer **XXXIIa** (64%) was subsequently used for the preparation of C-28 acetylenic derivatives **XXXIIc**, **XXXIIe**, **XXXIIg** by the reaction with acetylenic, propiolic, 2-butynoic, and 3-cyclopropyl-2-propiolic acid in DCM in the presence of DCC and DMAP, or with propargyl chloroformate in benzene and pyridine for **XXXIIi**. The series of compounds **XXXIId**, **XXXIIf**, **XXXIIh**, **XXXIIj** was formed as a 3,28-diester sideproduct of the reaction in small yields 7–13 %. All prepared compounds were tested for their antiproliferative activity against three human cancer cell lines (T47D, SNB-19, C32) using WST-1 assay. Results showed a significant increase of activity for all tested lines in the case of derivatives bearing the phosphonate group at the C-29 position in comparison with betulin (**X**). Additionally, the ester derivatives **XXXIIc**–**XXXIIj** were the most active towards melanoma cell line C32 with IC_{50} in the range of 0.38–9.44 $\mu g \cdot mL^{-1}$. Acetylenic derivative **XXXIIc** containing a small alkynyl group with the terminal C≡C bond had the highest activity for all



Scheme 3: The allyl-vinyl isomerization of betulin (**X**) derivatives. Reagents and conditions: i) $(EtO)_3P$; ii) KOH, EtOH, reflux, 2 h; iii) acetylenic acid, DCC, DMAP, DCM (**XXXIIc**–**XXXIIh**), or propargyl chloroformate, benzene, pyridine (**XXXIIi**–**XXXIIj**).

tested lines in the IC_{50} range of 0.27–0.44 μg · mL^{-1}. Similar betulin phosphonates at the isopropenyl unit were also patented by this research group in 2017.[113]

Tsepaeva et al.[114] introduced a triphenylphosphonium fragment into the betulin diacetate (**XXIII**) to enhance its antitumor activity *via* better ability to pass through the hydrophobic barriers of mitochondria. They described a reaction of 30-bromodiacetyl betulin (**XXX**) with triphenylphosphine, which was expected to give an allylic 30-phosphonium salt product. However, they obtained a mixture of *E*/*Z* isomers of vinylic C-29 derivative **XXXIII** (Fig. 15) in the 80% yield. To confirm the structure, they did advanced NMR experiments. No biological testing was reported.



XXXIII (80%)

Fig. 15: The triphenylphosphonium salt of betulin diacetate (**XXXIII**) prepared by Tsepaeva et al.

In 1992, Roy et al.[115] published a regioselective Vilsmeier-Haack formylation for introducing an aldehyde group at the C-29 position of lupeol (**IX**). He used *N*,*N*-dimethylformamide and phosphorus oxychloride, followed by hydrolysis of the formylated C-3 OH group using disodium carbonate in methanol; the product **XXXIVa** (Fig. 16) was exclusively in *E* configuration (overall yield of 39 %). The preparation of products **XXXIVb** and **XXXIVf** was also reported. This synthetic method was used later by Anand et al.[116], they prepared compounds **XXXIVa**–**XXXIVj** that were subsequently tested for possible antiurolithiatic activity against hydroxyproline-induced hyperoxaluria and calciuria in rats. Unsaturated amines **XXXIVg**–**XXXIVj** were prepared by the reduction of compounds **XXXIVc**–**XXXIVf** using sodium borohydride. The most promising candidate was compound **XXXIVi** which showed higher antihypercalciuric activity and similar antihyperoxaluric activity in comparison with lupeol (**IX**).

Vlk et al.[117] synthesized eleven selectively deuterated and tritiated lupane derivatives with cytotoxic activity – they used the Wittig reaction to introduce deuterium into the position C-29. Firstly, they prepared C-28 protected benzyl platanate (**XXXV**, Scheme 4) from lupeol (**IX**), then, Wittig reaction of the ketone group was performed using deuteromethyltriphenylphosphonium iodide $C(^2H)_3(Ph_3P)I$ and *t*-BuOK at 85 °C. After this reaction, they obtained the desired compound **XXXVI** with deuterated C-29 methylene in the 80% yield.

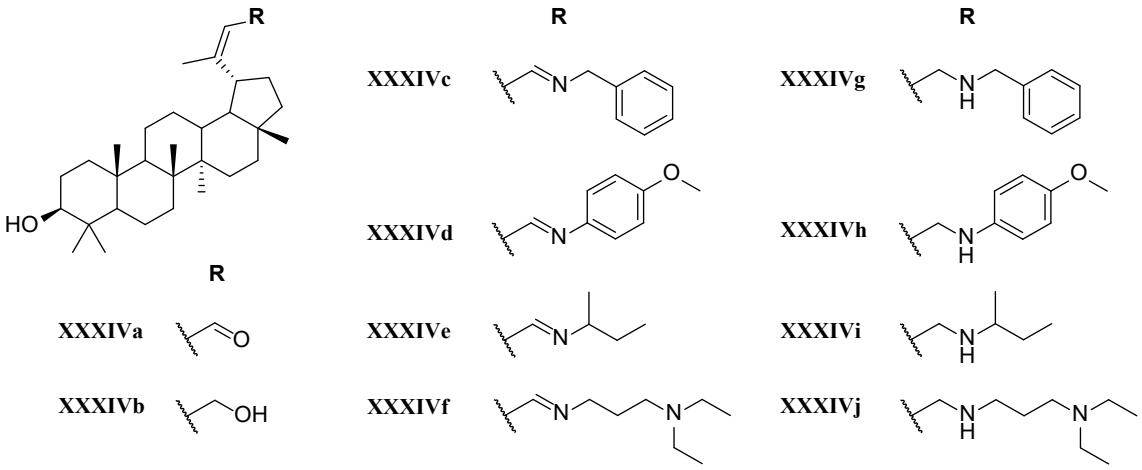
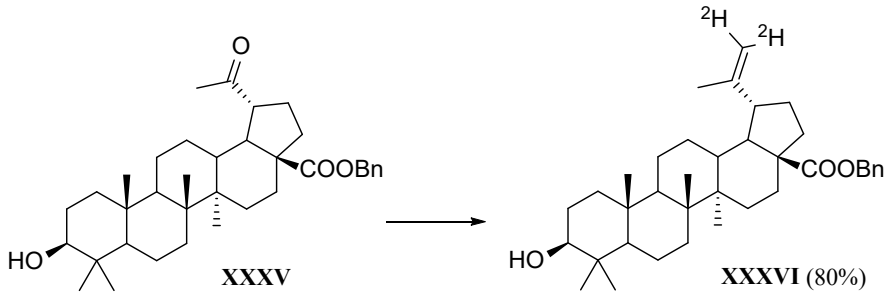


Fig. 16: Lupeol derivatives **XXXIVa**–**XXXIVb** prepared by Roy et al. and Anand et al.

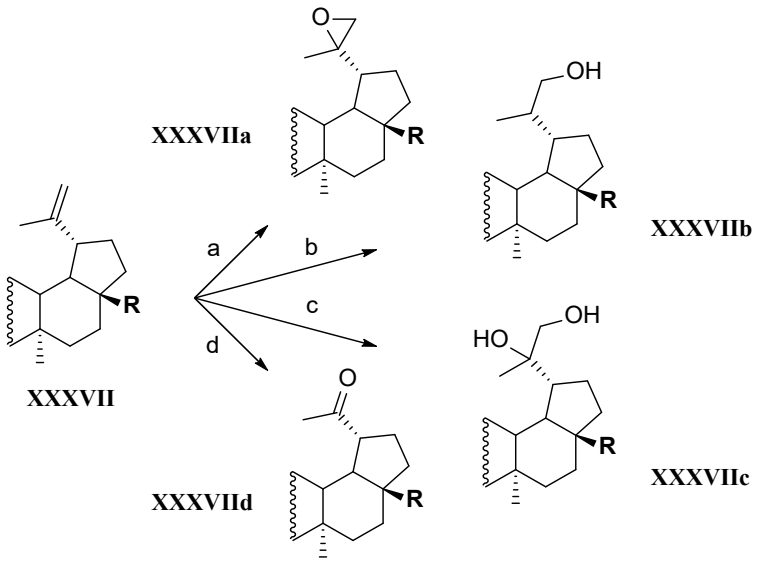


Scheme 4: Deuteration of the C-29 position by Vlk et al. Reagents and conditions: i) $C(^2H)_3I$, PPh_3, THF, −30 °C; ii) *t*-BuOK, THF, 85 °C; iii) EtOH.

Oxidation of the double bond C-20(29) can provide various types of molecules. First of all, the treatment of lup-20(29)-ene derivatives (**XXXVII**, Scheme 5) with *meta*-chloroperoxybenzoic acid yields an epoxy cycle (**XXXVIIa**) in high yields of about 85 %;[118] this type of modification is quite common in the literature, almost eighty compounds have been described.[106,119–123] 30-Hydroxyderivative (**XXXVIIb**) can be prepared by hydroboration, followed by oxidative workup,[106,119] while the 20,29-dihydroxy derivative (**XXXVIIc**) is generated by the reaction of **XXXVII** with OsO_4 in a moderate yield.[120] Subsequent oxidation of **XXXVIIc** with $NaIO_4/KIO_4$,[105,120,124] or the use of ozone and Me_2S is a convenient method for the introduction of the keto group (**XXXVIId**).[106,117,119,125] Instead of OsO_4, RuO_4 can be employed as well in the biphasic solvent system EtOAc–H_2O.[126,127]

Reduction of the double bond C-20(29), which could be easily done by H_2, Pd/C, MeOH at r. t. at elevated pressure 0.5 MPa of H_2, is used to form 20(29)-dihydroderivatives in yields of about 85 %.[106,119]

Some lupane-type triterpenoids modified at the isopropenyl unit have been patented by Swidorski et al.[128] in 2014 for the use as HIV maturation inhibitors, and by Yager et al.[129] in 2008 for the potent antiviral and anticancer usage.



Scheme 5: Oxidation of the double bond C-20(29). Reagents and conditions: a) *meta*-chloroperoxybenzoic acid, DCM, 0 °C to r. t., 1 h; b) BH_3, THF, 0 °C to r. t., 17 h; c) OsO_4, *N*-methylmorpholine-*N*-oxide, acetone/H_2O, r. t., 48 h; d) OsO_4/$NaIO_4$, MeOH/H_2O, r. t., 20 h, or O_3/Me_2S, DCM, −78 °C, 1 min.

2.1.2 Cyclization on the isopropenyl unit

Since an appropriately substituted isopropenyl moiety is suitable for various cyclization reactions, a number of new compounds with many biological activities containing a carbocycle or a heterocycle at the C-20 position have been described. Nowadays, more than four hundred such compounds can be found in the SciFinder database. Two kinds of cyclization products may be obtained: first, a reaction forms a new cycle consisting of the entire isopropenyl unit (**A**, Fig. 17), second, a reaction alternatively forms a three-membered (**B**) or even a five-membered ring attached to the C-20 carbon. The first group of compounds most often contains an aromatic or heterocyclic substituent which may be further substituted, such as examples in the literature.[125,130–132] The second group is represented by an epoxide ring (see the oxidation reactions of the double bond in Scheme 5, Chapter 2.1.1 above), or by a cyclopropane ring, which can be formed by a cyclopropanation reaction of the double bond with carbene.[133,134] There are also examples of five-membered rings as described below (see Scheme 8).

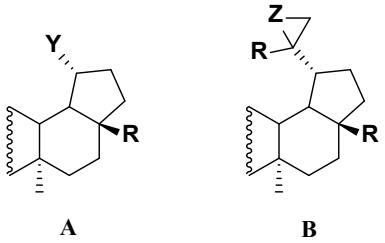
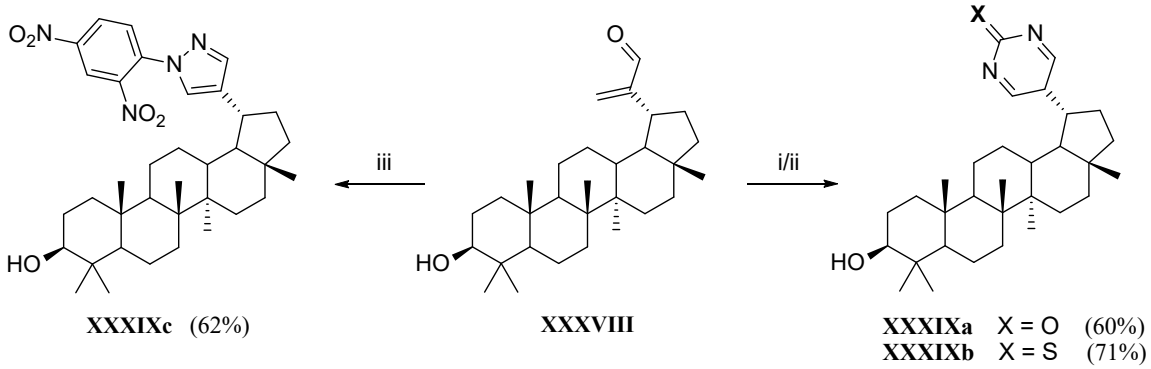


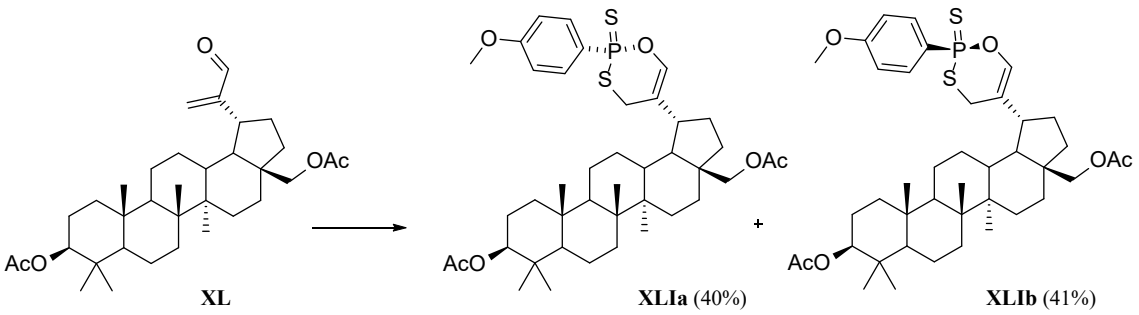
Fig. 17: The most common possible types of cyclization products on the isopropenyl unit. Y = Aromatic ring or heterocycle; Z = O, -CH_2-, or -CX_2-.

Saini et al.[135] designed and synthesized fourteen new compounds from lupeol (**IX**) modified at the C-3 and C-30 positions, and tested them *in vitro* for antitumor activities against three cancer cell lines (MDA MB-231, HeLa, A549). The structure-activity relationship was also described. Three compounds **XXXIXa**–**XXXIXc** (Scheme 6), containing a cycle at the position C-20, were prepared from 30-oxolupeol (**XXXVIII**) by the cyclization of α, β-unsaturated aldehyde moiety using urea/thiourea in EtOH and a catalytic amount of HCl under reflux in 5 h (for compounds **XXXIXa** and **XXXIXb**), or 2,4-dinitrophenylhydrazine (for **XXXIXc**) under the same conditions. Of all tested compounds, derivatives **XXXIXb** and **XXXIXc** showed a significant inhibitory effect against the three tested cell lines in comparison with the parent lupeol (**IX**), moreover, the compound **XXXIXb** was the most active derivative against MDA MB-231 with IC_{50} = 27.1 μmol · L^{-1}. The SAR study showed that the cyclization of α, β-unsaturated functionality at C-30 with the thione group might lead to stronger antiproliferative activity.



Scheme 6: The preparation of lupane derivatives containing a heterocycle at the position 20 by Saini et al. Reagents and conditions: i) urea, HCl, EtOH, reflux, 5 h; ii) thiourea, HCl, EtOH, reflux, 5 h; iii) 2,4-dinitrophenylhydrazine, HCl, EtOH, reflux, 5 h.

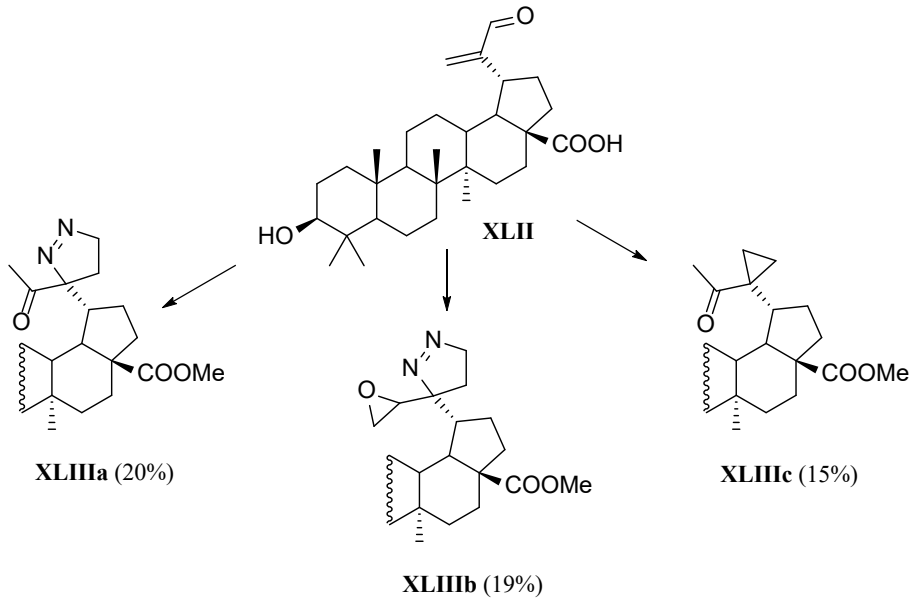
Kvasnica et al.[136] synthesized fifteen new sulfur derivatives of lupane (**VI**) and oleanane (**VII**), one isomeric compound of the series contained oxathiaphosphinine ring at C-20. The reaction of 30-oxobetulin diacetate (**XL**, Scheme 7) with Lawes-



Scheme 7: The reaction of 30-oxobetulin diacetate (**XL**) with Lawesson's reagent by Kvasnica et al. Reagents and conditions: Lawesson's reagent, toluene, reflux, 4 h.

son's reagent in toluene under reflux afforded two isomers **XLIa**, **XLIb** in moderate yields. The absolute configuration of the phosphorus atom was determined by X-ray analysis. All the prepared compounds had no significant cytotoxic activity, tested against highly chemosensitive T-lymphoblastic leukemia CCRF-CEM cells.

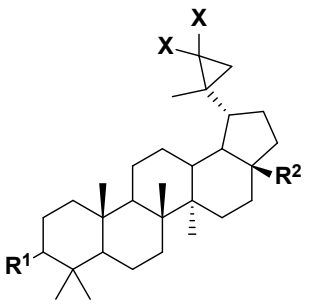
Urban et al.[137] prepared seventeen new compounds, among them, three derivatives of betulinic acid (**XI**) contained variously ring-substituted C-20 position. The methylation reaction of the carboxyl group of 30-oxobetulinic acid (**XLII**, Scheme 8) with diazomethane under standard conditions led to a complex mixture of five products instead of the expected methyl ester, which was formed in 20% yield only. These surprising results were applied on a larger group of activated carbonyl triterpenoids to obtain novel derivatives containing an oxirane or a cyclopropane ring in the molecule. The new compounds were tested *in vitro* for their cytotoxic activity against the CCRF-CEM cell line using MTT assay. Among the compounds **XLIIIa**–**XLIIIc**, all of them were inactive ($IC_{50} > 50$ µmol · L^{-1}) in comparison with the parent aldehyde **XLII** ($IC_{50} = 5$ µmol · L^{-1}).



Scheme 8: The reaction of 30-oxobetulinic acid (**XLII**) with diazomethane. Reagents and conditions: CH_2N_2, Et_2O, r. t., 12 h.

Symon et al.[138] synthesized eleven new cyclopropane derivatives **XLIVa**–**XLIVk** (Fig. 18) of betulin (**X**), betulinic (**XI**), and betulonic acid (**XV**) by the reaction of dichloro- or dibromocarbenes with the double bond of betulin diacetate (**XXIII**). The reactions were performed using sodium trichloroacetate or bromoform as a carbene source, and triethylbenzylammonium chloride as a phase transfer catalyst. After the deprotection of the hydroxyl groups at C-3 and C-28, compounds **XLIVb**, **XLIVf**, and **XLIVi** were oxidized by CrO_3 in acetic acid to obtain betulonic acid derivatives **XLIVc**, **XLIVg**, and **XLIVj**, followed by the reduction of the C-3 keto group by $NaBH_4$ to corresponding betulinic acid derivatives **XLIVd**, **XLIVh**, and

XLIVk. Compound **XLIVi** was prepared from **XLIVf** by the treatment with *t*-BuOLi. All compounds were tested in three human tumor cell lines (Colo 38, Bro, CaOv) using MTT tests. Only the derivative **XLIVh** was shown to be active (ca 10 µmol · L^{-1} for each cell line), comparable with the toxicity of BA (**XI**), however, this compound was more toxic than BA towards the Bro cell line. None of the synthesized compounds demonstrated a cytotoxic activity higher than BA, which could be caused by the very low solubility of the compounds, as the authors suggested.



XLIVa–XLIVk

	R^1	R^2	X	
XLIVa	OAc	CH_2OAc	Br	
XLIVb	OH	CH_2OH	Br	(53%)
XLIVc	O=	COOH	Br	(74%)
XLIVd	OH	COOH	Br	(83%)
XLIVe	OAc	CH_2OAc	Cl	
XLIVf	OH	CH_2OH	Cl	(67%)
XLIVg	O=	COOH	Cl	(61%)
XLIVh	OH	COOH	Cl	(73%)
XLIVi	OH	CH_2OH	H	(72%)
XLIVj	O=	COOH	H	(58%)
XLIVk	OH	COOH	H	(92%)

Fig. 18: Cyclopropane derivatives prepared by Symon et al.

Another way to modify the isopropenyl unit may be a cyclization at the position C-30. Only four lupane-type molecules with a modification at this position are known; the pyrazole (**XLV**, Fig. 19) and isoxazole (**XLVI**) prepared by Nazarov et al.,[139] and pyrazolines **XLVIIa**, **XLVIIb** described in the literature.[140]

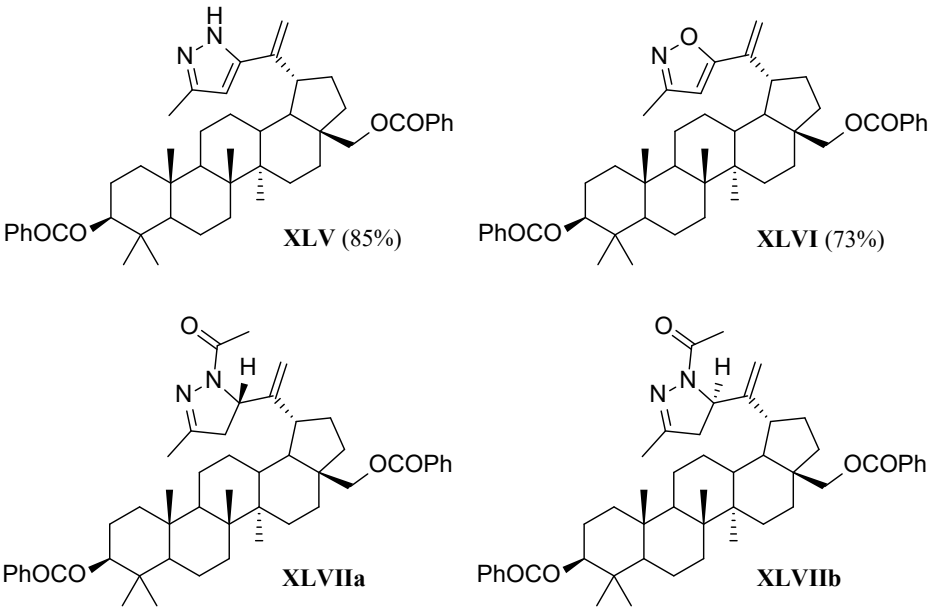
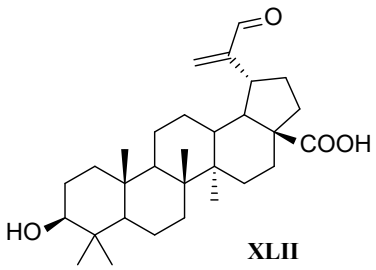


Fig. 19: Pyrazole (**XLV**), pyrazolines (**XLVIIa**–**XLVIIb**) and isoxazole (**XLVI**) of betulin (**X**).

2.1.3 The double bond at the C-30 position

This section will be focused on lupane-type triterpenoids bearing an extra skeletal double bond, which connects the position C-30 with another functionality or is a part of a functional group (for example 30-C=O, 30-COOH, 30-C=NR, 30-C=CHR, etc.), while the double bond C-20(29) remains untouched. Apart from the aldehyde group at the C-30 position, only a few cases of this substituted double bond have been described; in each of them, the C-30 modification is introduced by a reaction of the aldehyde. Unfortunately, no anticancer activity was reported for these compounds.

The most important representative of this group of modifications is 30-oxobetulinic acid (**XLII**, Fig. 20). This compound can be prepared from BA (**XI**) by oxidation of the isopropenyl side chain by SeO_2 in dioxane at r. t. in 24 h,[141] or in 2-methoxyethanol at 140 °C in 4 h.[142] The aldehyde **XLII** was also isolated from aerial parts of O*riganum syriacum* L. and characterized.[143] It is known for a strong non-selective cytotoxic activity in multiple cancer cell lines in the low micromolar range of concentrations (IC_{50} = 1.7 μmol · L^{-1} for the CCRF-CEM line).[137,144] Unfortunately, this compound is toxic for non-malignant cells as well (e.g. BJ, MRC-5), making it unsuitable as an anticancer drug.[145] The toxicity is caused by the presence of the Michael acceptor (α,β-unsaturated aldehyde) which forms nonspecific covalent bonds with unspecified proteins. Despite that, the compound still deserves attention since several triterpenoids containing the Michael acceptor were successful in medicinal chemistry.[146] It should be noted that the structure and synthesis of the aldehyde **XLII** is also protected by several patents.[147,148] It is clear that this compound is popular as a starting point for the preparation of new compounds with similar cytotoxic activity but higher selectivity for tumor cells.[145]

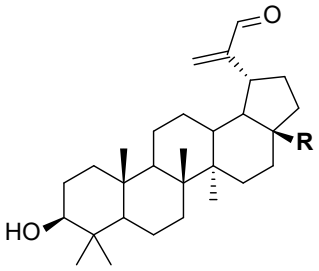


XLII

Fig. 20: 30-Oxobetulinic acid.

Other 30-oxolupane derivatives have also interesting biological activities, not only anticancer. In 2020, Machado et al.[149] prepared and tested 30-oxolupeol (**XXXVIII**, Fig. 21) *in vitro* for the anti-leukemic activity against two cell lines (K562 and Jurkat acute lymphoid leukemia). Results showed that this compound has the most cytotoxic profile among the rest of tested molecules with IC_{50} = 11.7 μmol · L^{-1} in Jurkat cells and IC_{50} = 19.5 μmol · L^{-1} in K562 cells, respectively.

However, the compound significantly reduced the cell viability of healthy human cells, peripheral blood lymphocytes, by 32 % when compared to the control group without treatment. Two years earlier, the same author[150] tested 30-oxolupeol (**XXXVIII**) for its antileishmanial and antitrypanosomal activities. The aldehyde showed the best inhibition potential among the tested compounds – 83 % for *Leishmania amazonensis*, and 98 % for *Trypanosoma cruzi*. The authors stated that the derivatives containing an α,β-unsaturated carbonyl moiety were the most active against both parasites.



	R
XXXVIII	CH_3
XLVIII	CH_2OH

Fig. 21: 30-Oxolupeol (**XXXVIII**) and 30-oxobetulin (**XLVIII**).

Chen et al.[151] tested 30-oxolupeol (**XXXVIII**) for its anticancer effect against lung cancer cell lines (HOP-62, EPLC-272H, COR-L105). The compound showed moderate antiproliferative activity with IC_{50} in the range of 9–17 µmol · L^{-1}.

In the study of Khan et al.,[131] the authors tested the antidiabetic activity of 30-oxolupeol (**XXXVIII**). It was found that the compound **XXXVIII** has the highest potency of glucose uptake stimulatory effect in the L6 skeletal muscle cells (IC_{50} = 4.2 µmol · L^{-1}).

Gutierrez-Nicolas et al.[123] tested 30-oxobetulin (**XLVIII**, Fig. 21) among other compounds for its anti-HIV activity, the method was based on their capacity to perturb X4- and R5-tropic HIV-1-envelope-mediated fusion membrane in the HeLa-based cellular model. 30-Oxobetulin (**XLVIII**) showed low activity (26% inhibition for R5-tropic-Env and 10% inhibition for X4-tropic-Env), while 30-oxolupeol (**XXXVIII**) was inactive.

Ghosh et al.[152] synthesized and tested lupane derivatives for their antileukemic activity against three cell lines (K562, WEHI3, MEL). 30-Oxobetulin (**XLVIII**) showed potent activities against the entire set of cell lines, especially against K562 and WEHI3.

The aldehyde group at the C-30 position can be further oxidized. Selective Pinnick oxidation, which can be done using $NaClO_2$, KH_2PO_4 in the *t*-BuOH/2-methyl-2-butene mixture (1 : 1) at r. t., leads to C-30-carboxy derivatives, e.g. diacid **XLIX** (Fig. 22).[142] No anticancer activities for this compound have been reported yet, however, our preliminary research shows that it is not active.

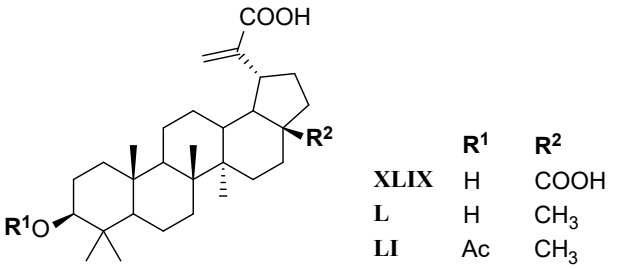


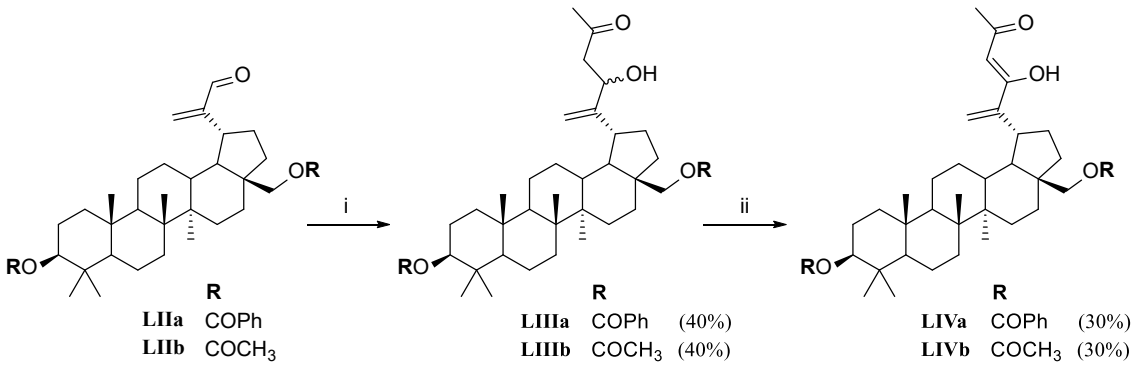
Fig. 22: Derivatives with a carboxyl group at the position C-30.

Zhou et al.[153] tested 3β-hydroxylup-20(29)-en-30-oic acid (**L**, Fig. 22) for its anti-inflammatory activity by its ability to inhibit LPS-activated nitric oxide production in BV2 cell line. This compound showed moderate inhibition of NO production with $IC_{50} = 8.5\ \mu mol \cdot L^{-1}$.

Reutrakul et al.[154] isolated fourteen triterpenoid compounds from *Cratoxylum arborescens* and tested them for the anti-HIV-1 activity using $^{\Delta Tat/Rev}$MC99 and the 1A2 cell line system. The most active compound from the set was acid **L**, which inhibited HIV-1 reverse transcriptase with $IC_{50} = 8.7\ \mu g \cdot mL^{-1}$.

Hata et al.[155] tested 3β-acetoxylup-20(29)-en-30-oic acid (**LI**, Fig. 22) for its effect on B16 2F2 mouse melanoma cell differentiation and proliferation. The compound **LI** markedly inhibited the growth of B16 2F2 cells by the induction of apoptosis with $IC_{50} = 6.8\ \mu mol \cdot L^{-1}$. Reduction of the double bond 20(29) in the molecule **LI** even led to an increase in activity ($IC_{50} = 5.6\ \mu mol \cdot L^{-1}$).

Nazarov et al.[139] described several molecules of lupane (**VI**) and oleanane (**VII**) bearing a pyrazole or isoxazole ring (**XLV**–**XLVI**, Fig. 19 above). During the synthesis, they have prepared two intermediates – enoles **LIVa**, **LIVb** (Scheme 9) with the C-30 double bond. The key step for the preparation of such compounds was an aldol condensation of α, β-unsaturated C-30 aldehyde (**LIIa**, **LIIb**) with acetone, followed by oxidation of the formed β-hydroxyketone (**LIIIa**, **LIIIb**) with PCC in anhydrous chloroform, or by Jones reagent in acetone. Both oxidation methods provided an equal yield of 30 %. Prepared 1,3-diketones **LIVa**, **LIVb** are described as completely enolized at the C-30 atom, the structures were confirmed



Scheme 9: C-30 derivatives prepared by Nazarov et al. Reagents and conditions: i) acetone–benzene 1 : 2, 10% NaOH, r. t.; ii) CrO_3, H_2SO_4, acetone, sodium acetate, r. t., or PCC, DCM, r. t.

by ^{1}H and ^{13}C NMR experiments. A cyclization using hydrazine hydrate in ethanol–acetic acid mixture (1 : 1) provided a C-30 pyrazole derivative in 1.5 h. Similarly, treatment with hydroxylamine hydrochloride in aqueous ethanol in the presence of sodium acetate afforded an isoxazole derivative. No biological testing was done.

Wal et al.[156] designed and synthesized new derivatives of lupeol (**IX**) to evaluate their antipsychotic activity. They prepared a series of molecules, six of them containing a double bond in the imine form (**LVa**–**LVf**, Fig. 23). The preparation consisted of the reaction of 30-oxolupeol (**XXXVIII**) with substituted amines in methanol under reflux for 12 h; yields were not provided. The compounds were subsequently subjected to reduction of the imine double bond to give amine compounds. Some of those amines were found to have the antipsychotic activity and no toxicity.

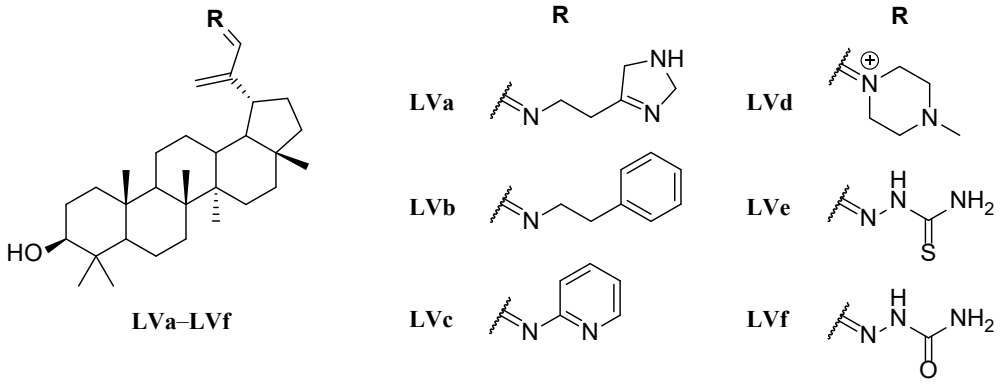


Fig. 23: Imine derivatives **LVa**–**LVf** of lupeol (**IX**) prepared by Wal et al.

The only use of the Wittig reaction on the lupane skeleton at the position C-30 was described by Mishra et al. in 2014.[125] They have prepared a large series of lupeol heterocyclic molecules and evaluated them for antimalarial activity *in vitro* against chloroquine-sensitive 3D7 strain of *Plasmodium falciparum*. Among them, the ethyl ester derivative **LVI** (Fig. 24) was prepared from 30-oxolupeol (**XXXVIII**) by the Wittig reaction with (carbethoxymethylene)triphenylphosphorane in DCM at 40 °C. Antimalarial activity of the compound **LVI** was not increased in comparison with the parent lupeol (**IX**).

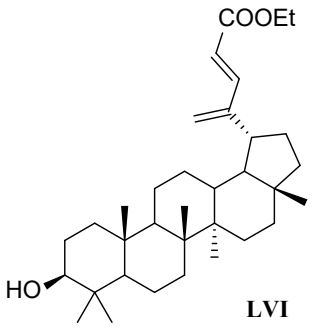
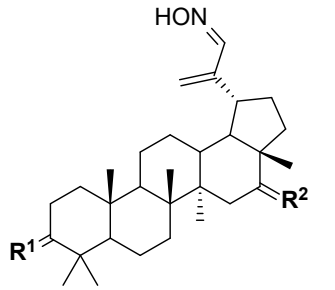


Fig. 24: C-30 derivative **LVI** prepared by Mishra et al. using the Wittig reaction.

Two C-30 oximes were presented by Castro et al.[157] and evaluated *in vitro* as potential butyrylcholinesterase inhibitors using Ellman's assay. The compounds **LVIIa**, **LVIIb** (Fig. 25) were prepared from C-30 aldehyde by a reaction with hydroxylamine hydrochloride in the presence of sodium acetate. None of the molecules had significant inhibition activity.



	R^1	R^2	
LVIIa	NOH	O	(23%)
LVIIb	NOH	NOH	(31%)

Fig. 25: C-30 oxime derivatives **LVIIa**, **LVIIb** prepared by Castro et al.

2.2 Double bonds at the A-ring

In this chapter, a brief overview of possible positions of double bonds at the A-ring of the lupane skeleton will be discussed. A detailed study of the A-ring modifications is beyond the scope of this work as there are hundreds of different variants and the topic was recently reviewed.[158] Generally, there are four options (and their combinations) where a double bond can be present at the A-ring of lupane (see Fig. 26).

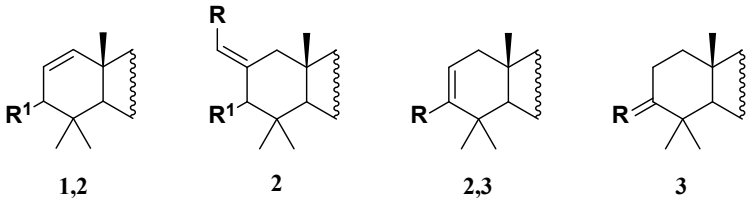
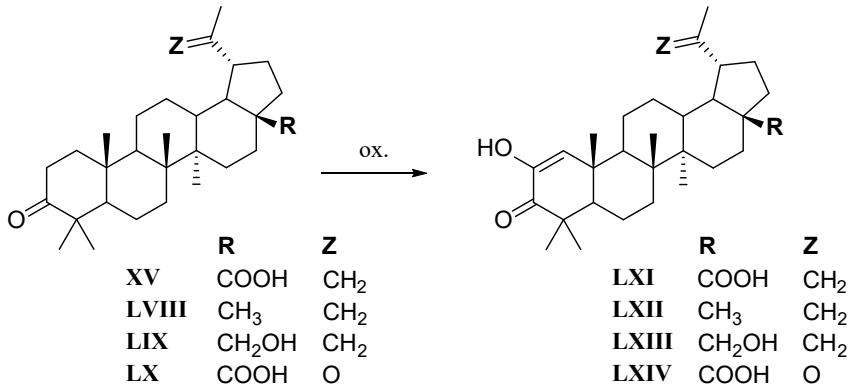


Fig. 26: Possible double bond positions at the A-ring.
R^1 = 3β-OH or =O; R = various substituents.

2.2.1 The double bond C-1(2)

The first option of a double bond between the carbons C-1 and C-2 is also the most common; it is represented by a diosphenol type of compounds (**LXI**–**LXIV**, Scheme 10) that may be synthesized from the 3-oxo precursor, usually betulonic acid (**XV**), lupenone (**LVIII**), or betulone (**LIX**) by the oxidation process using air (or O_2) in basic conditions (*t*-BuOK in *t*-BuOH).[159] The resulting enol form is highly preferred

to its possible tautomer 2,3-diketone. The motif of diosphenol in lupanes is present in many publications.[159–166]



	R	Z
XV	COOH	CH_2
LVIII	CH_3	CH_2
LIX	CH_2OH	CH_2
LX	COOH	O

	R	Z
LXI	COOH	CH_2
LXII	CH_3	CH_2
LXIII	CH_2OH	CH_2
LXIV	COOH	O

Scheme 10: Preparation of diosphenols at the A-ring of lupane compounds.

Moderate cytotoxic activity of betulinic and platanic acid diosphenols **LXI** and **LXIV** (Scheme 10) was described by Hoenke et al.[160] They tested prepared compounds in six cell lines (A375, HT29, MCF7, A2780, FaDu, NIH 3T3). The former representative showed anticancer activity in the IC_{50} range of 10.5–26.1 $\mu mol \cdot L^{-1}$, while diosphenol of platanic acid (**LXIV**) was active only against cancer cell lines MCF7 (IC_{50} = 24.8 $\mu mol \cdot L^{-1}$), and A2780 (IC_{50} = 20.6 $\mu mol \cdot L^{-1}$). Betulinic acid (**XI**) and platanic acid (**XVII**) were inactive against all tested lines.

Ngoc et al.[162] tested diosphenol of BA (**LXI**) against three cancer cell lines (L1210, CEM, HeLa) with the IC_{50} values of 12, 13, and 19 $\mu mol \cdot L^{-1}$, respectively. The compound showed also antiviral activity against HSV-1 and HSV-2 at the EC_{50} of 10–20 $\mu mol \cdot L^{-1}$.

Comparable moderate cytotoxic activity of modified diosphenols of BA **LXVa**–**LXVd** (Fig. 27) was reported by Zhang et al.[161] They tested compounds against five cancer cell lines (HL-60, BEL-7402, SF-763, HeLa, B16). The IC_{50} ranged from 11–30 $\mu mol \cdot L^{-1}$ while the most active derivative **LXVc** had no protection on all functional groups.

Santos et al.[163] synthesized and tested several new derivatives of betulin (**X**) and betulinic acid (**XI**). Compounds **LXVIa**–**LXVIb** (Fig. 27) were prepared and their cytotoxic activity was evaluated against six cell lines (HepG2, Jurkat, HeLa, HT-29, PC-3, BJ). Except for the PC-3 cell line, derivative **LXVIa** was highly active with IC_{50} values in the range of 4.7–9.4 $\mu mol \cdot L^{-1}$, while for non-malignant BJ cells the IC_{50} = 37.2 $\mu mol \cdot L^{-1}$. On the contrary, derivative **LXVIb** showed the highest activity against PC-3 cell line with IC_{50} = 1.8 $\mu mol \cdot L^{-1}$, for the rest of cell lines the IC_{50} values were in the range of 3.3–10.5 $\mu mol \cdot L^{-1}$, and 27.9 $\mu mol \cdot L^{-1}$ for BJ cell line, respectively.

Other articles regarding cytotoxic[159,165,167] or anti-HIV[164] activities of lupane diosphenols were published.

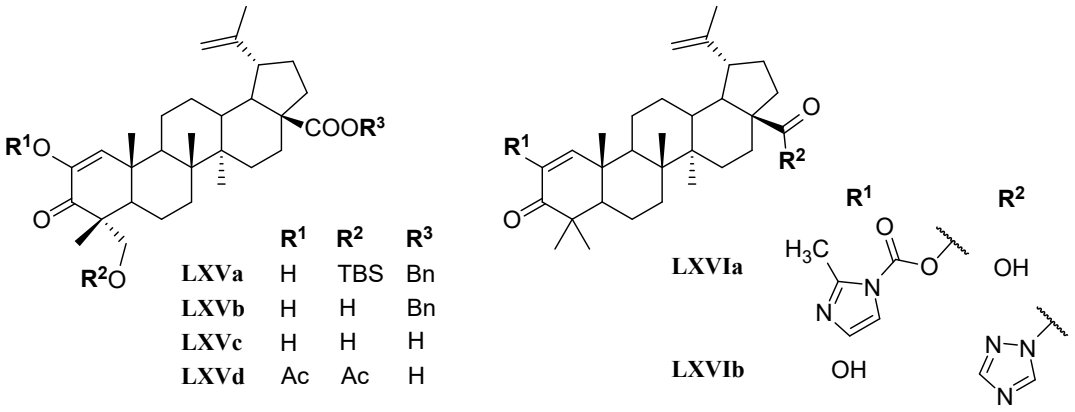


Fig. 27: Diosfenol derivatives **LXVa**–**LXVd** prepared by Zhang et al., and derivatives of betulinic acid diosphenol **LXVIa**, **LXVIb** prepared by Santos et al.

The second way to prepare the C-1(2) double bond is represented by oxidation with DDQ.[165] Resulting enon **LXVII** has the structure shown in Fig. 28.

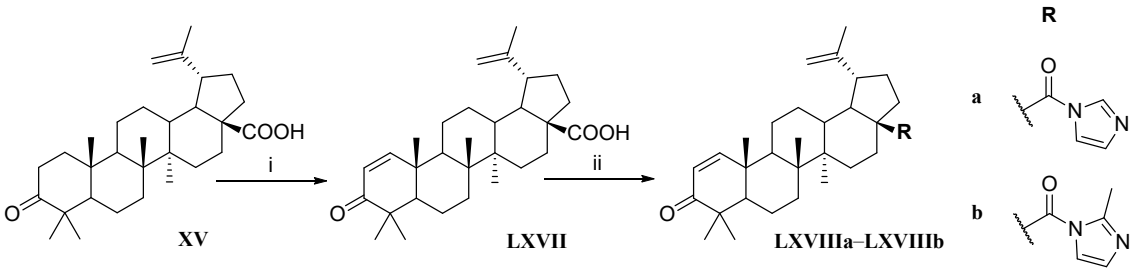


Fig. 28: Enon derivatives of betulonic acid (**XV**). Reagents and conditions: i) DDQ, dioxane, N_2, reflux, 15 h; ii) 1,1'-carbonyldiimidazole (**LXVIIIa**) or 1,10-carbonylbis(20-methylimidazole) (**LXVIIIb**), THF, N_2, reflux, 8–9 h.

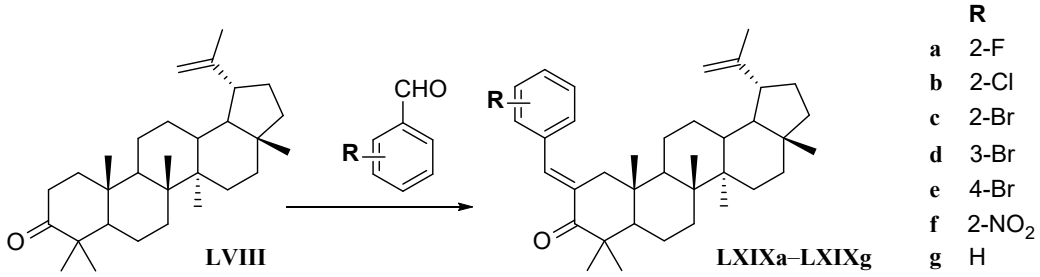
Santos et al.[163,165] tested compounds **LXVIIIa** and **LXVIIIb** (Fig. 28) in the same way as diosphenol derivative **LXVII** above. Compound **LXVIIIa** had high cytotoxic activity against HepG2, Jurkat, and HeLa cell lines in the IC_{50} range of 1.7–3.0 $\mu mol \cdot L^{-1}$. For the second derivative with a methyl group at the imidazole ring (**LXVIIIb**), the IC_{50} ranged from 6.8–12.5 $\mu mol \cdot L^{-1}$.

Other known compounds containing C-1(2) double bond bear cyano,[168–171] carboxy,[169–171] or enamine group,[172] eventually chlorine[171] at the position C-2.

2.2.2 The exocyclic double bond at the C-2 position

The first example of a double bond at the position C-2 is represented by benzylidene and alkylidene derivatives (e.g. structures **LXIXa**–**LXIXg**, Scheme 11) which can be prepared easily by condensation of 3-oxolupanes with aldehydes of choice in basic conditions. Usually, NaOH in EtOH or NaH in dry THF is employed.

Phan et al.[93] prepared seven benzylidene derivatives **LXIXa**–**LXIXg** (Scheme 11) from lupenone (**LVIII**) using aromatic aldehydes substituted in various positions. Compounds were then tested for their α-glucosidase inhibitory and cytotoxic activities. Anticancer activity was tested in the K562 cell line, but the prepared conjugates showed only a weak activity with the best IC_{50} value of 77 µmol · L^{-1} for the derivative **LXIXd**.



Scheme 11: Benzlidene derivatives of lupenone (**LVIII**) prepared by Phan et al. Reagents and conditions: Aldehydes **a**–**g**, NaOH, EtOH, 55 °C, 2 h.

A series of thirty new benzylidene derivatives of betulinic (**XI**) and betulonic (**XV**) acids was prepared by Gupta et al.[173] The synthesis started from BA (**XI**) which was oxidized to betulonic acid (**XV**) by PCC in DCM, followed by condensation reactions using NaH as a base in THF. Finally, benzylidene derivatives **LXXa**–**LXXo** (Fig. 29) were reduced with $NaBH_4$ in MeOH to obtain -OH group at the position C-3 (**LXXIa**–**LXXIo**). All synthesized conjugates were subjected to cytotoxicity screening on a panel of five human cancer cell lines (A-549, PC-3, HCT116, MCF-7, MIA PaCa-2). Results revealed several derivatives with IC_{50} valu-

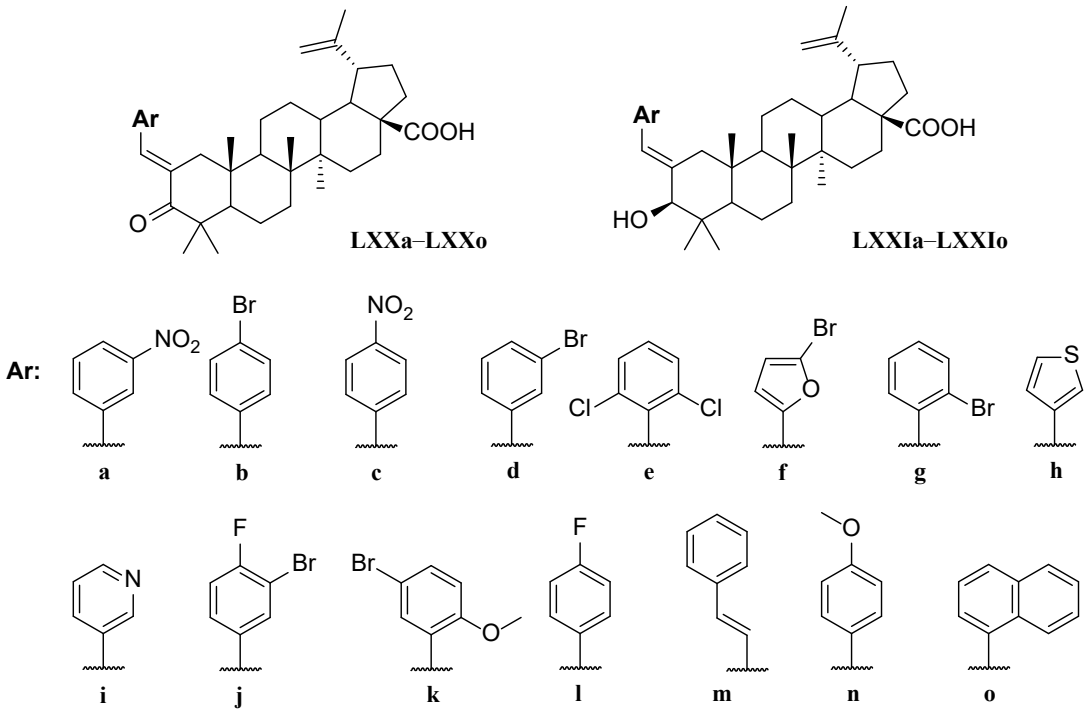


Fig. 29: Benzylidene derivatives **LXX** and **LXXI** prepared by Gupta et al.

es about 1.5 µmol · L^{-1}, the best result was obtained for the derivative **LXXIl** with IC_{50} = 1.18 µmol · L^{-1} for the MCF-7 cell line.

Similarly, ten new benzylidene derivatives **LXXIIa**–**LXXIIj** (Fig. 30) were prepared by Srivastava et al. from lupenone (**LVIII**).[174] The compounds were tested for their antidyslipidemic and antioxidant effects, the most potent derivatives **LXXIId**, **LXXIIh**, and **LXXIIi** reversed the plasma levels of total cholesterol by 24 %, 25 %, and 27 %, phospholipids by 25 %, 26 %, 25 %, and triacylglycerol by 27 %, 24 %, and 24 %, respectively. Moreover, the compounds showed strong *in vitro* antioxidant activity.

Another seven similar new derivatives **LXXIIIa**–**LXXIIIg** (Fig. 30) of methyl betulonate were prepared by Flekhter et al.[175] and tested for hepatoprotective activity. Compounds **LXXIIIb** and **LXXIIIg** showed high bile-expelling activity in the case of hepatitis induced by tetrachloromethane, the compound **LXXIIIg** exceeded the activity of the reference carsil drug.

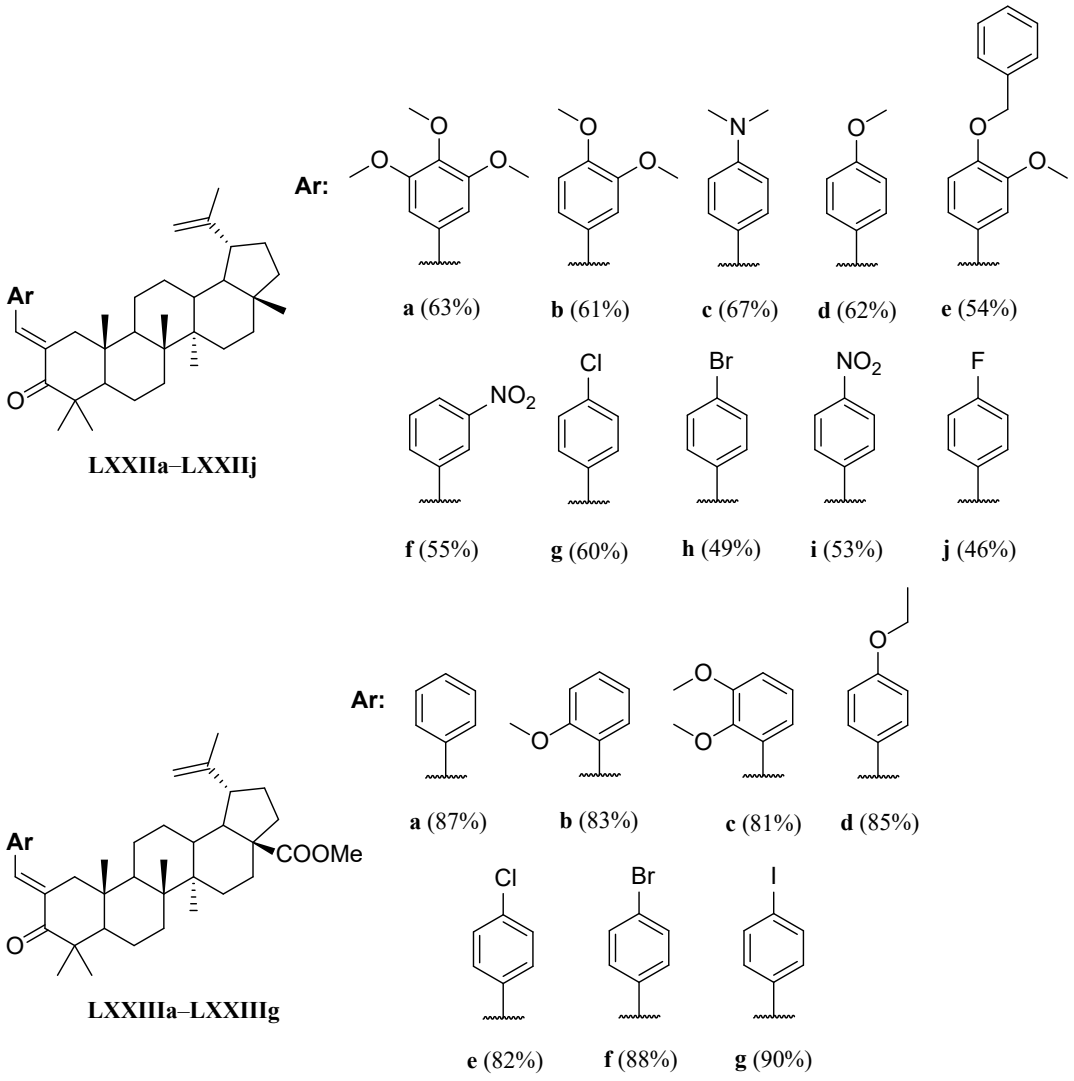


Fig. 30: Benzylidene derivatives **LXXIIa**–**LXXIIj** prepared by Srivastava et al., and **LXXIIIa**–**LXXIIIg** prepared by Flekhter et al.

Two derivatives **LXXIVa**, **LXXIVb** (Fig. 31) containing pyridinylmethylidene substituent at the C-2 position were prepared by Kazakova et al.[176] in the same manner; the antituberculosis activity of the synthesized compounds was evaluated, unfortunately, the compounds did not inhibit the growth of *Mycobacterium tuberculosis*.

Csuk et al.[177] described the high anticancer activity of alkylidene, especially C-2 methylene derivatives. These compounds were synthesized either by the Mannich reaction followed by β-elimination, or by aldol condensation as in the studies above. Compounds **LXXVa**–**LXXVf** (Fig. 31) were tested against a panel of fifteen cancer cell lines (518A2, A431, A253, FADU, A549, A2780, DLD-1, HCT-8, HCT-116, HT-29, SW480, 8505C, SW1736, MCF-7, Lipo) with significantly high cytotoxicity. Derivative **LXXVb**, 2-methylene-betulonic acid, showed IC_{50} values in the range of 0.2–0.6 $\mu mol \cdot L^{-1}$. Derivative **LXXVd** showed similar results in the IC_{50} range of 1.0–1.9 $\mu mol \cdot L^{-1}$. For the alkylidene compounds **LXXVe** and **LXXVf**, cytotoxicity drops significantly. However, all the derivatives were toxic for the non-cancer fibroblasts NiH3T3, indicating low selectivity.

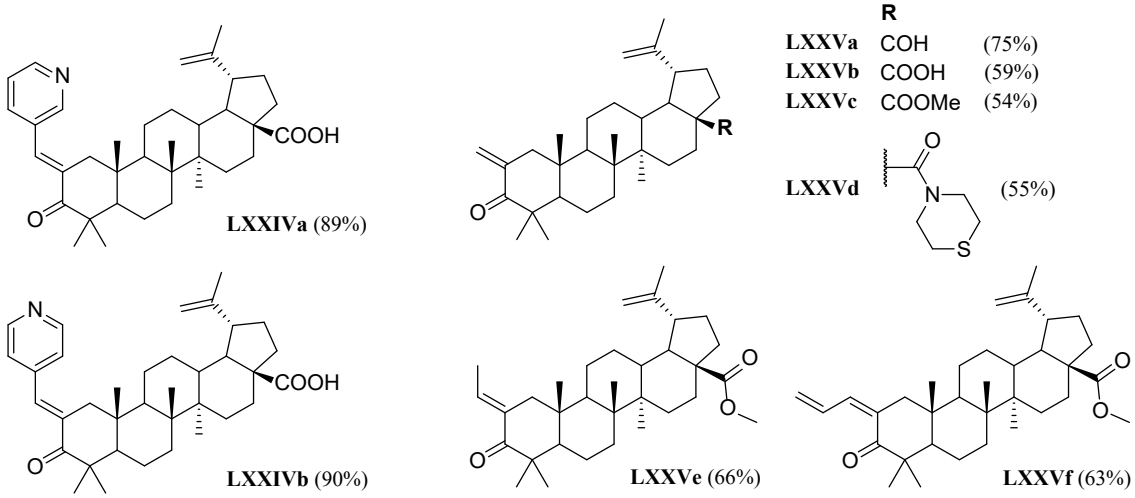


Fig. 31: Pyridinylmethylidene derivatives (**LXXIVa**–**LXXIVb**) prepared by Kazakova et al., and alkylidene derivatives (**LXXVa**–**LXXVf**) prepared by Csuk et al.

Acid **LXXVb** (Fig. 31) was also prepared by Huang et al.[178] and evaluated for its antibacterial and antitumor activities (against K562, A549, and MCF-7 cell lines), however, the results were not promising.

The second group of compounds containing a double bond at the C-2 position are derivatives linked to the nitrogen-containing substituents. 2-Methylidene-ureido- (**LXXVIa**, Fig. 32) and 2-methylidene-thioureido-methylbetulonates (**LXXIVb**) were described in the articles[179–181] and tested for antiviral, cytotoxic, and α-glucosidase inhibitory activities. Compound **LXXIVb** showed cytotoxic ac-

tivity in HCT-116 cancer cell line with $IC_{50} = 5.7\ \mu mol \cdot L^{-1}$; antiviral activity (Influenza A, HSV-1, ECHO-6) was weak. Only compound **LXXVIa** was active in the α-glucosidase inhibition testing.

Similarly, six enamino derivatives of methylbetulonate **LXXVIIa**–**LXXVIIf** (Fig. 32) were synthesized and tested for the immunotropic activity by Tolmacheva et al.[182] Compound **LXXVIIc** showed immunosuppressing influence on humoral and cell-mediated immunity.

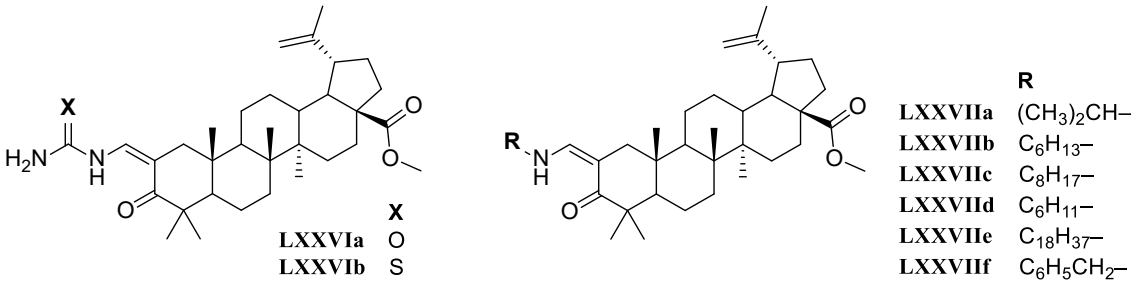


Fig. 32: Derivatives with nitrogen-containing substituents at the C-2 position.

Oximes **LXXVIIIb**, **LXXVIIIc**, **LXXXb**, **LXXXc**, and **LXXXd** (Fig. 33) were prepared and tested for their cytotoxic activity by Konysheva et al.[183,184] Only moderate cytotoxicity was observed for derivatives **LXXXb**, **LXXVIIIc**, and **LXXXc**. Compounds **LXXVIIIa**, **LXXVIIIb**, **LXXIXa**, **LXXIXb**, and **LXXIXe** were described in the articles;[182,185,186] antiviral properties of the prepared derivatives were evaluated.

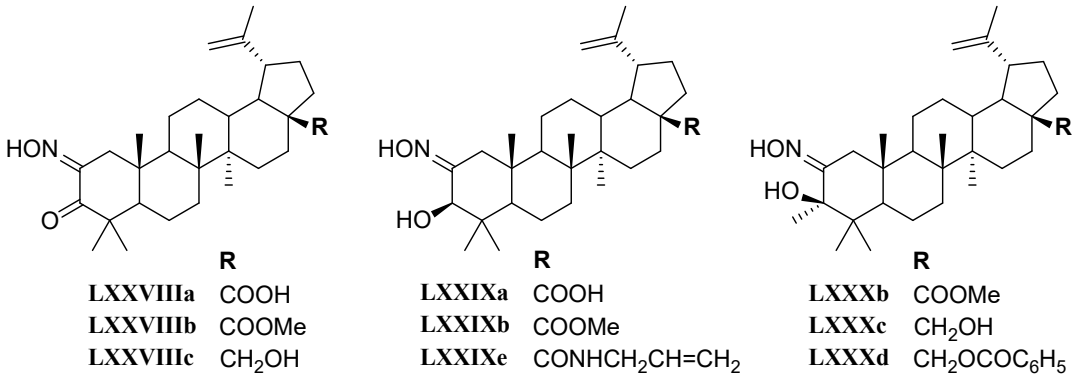
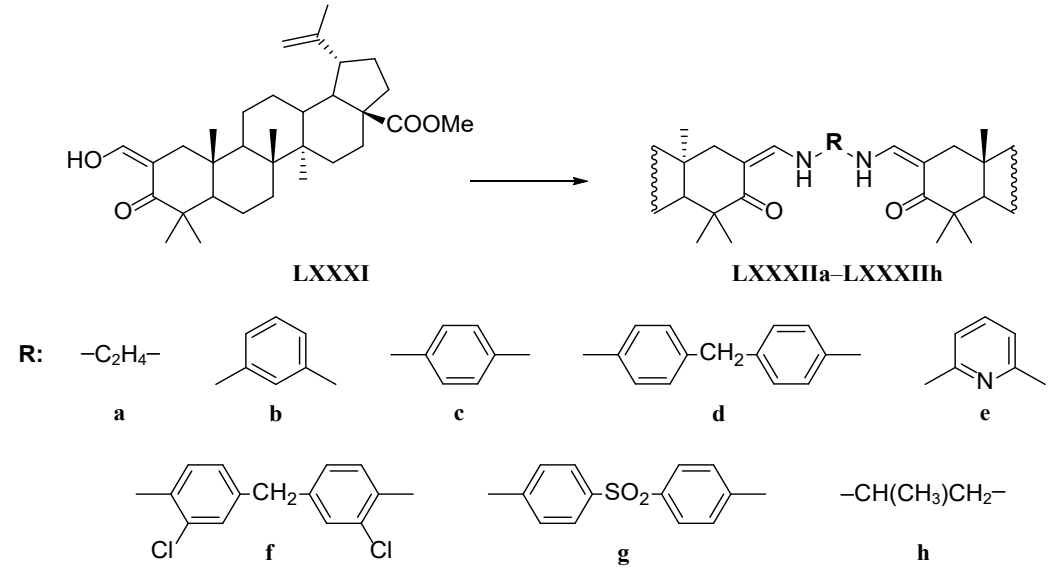


Fig. 33: Oxime derivatives of betulinic acid (**LXXIXa**–**LXXIXe**, **LXXXb**), betulonic acid (**LXXVIIIa**, **LXXVIIIb**), betulin (**LXXXc, LXXXd**), and betulone (**LXXVIIIc**).

Interesting dimers **LXXXIIa**–**LXXXIIh** (Scheme 12), C-2 symmetric bis-enaminones, were prepared from enole **LXXXI** of betulonic acid methyl ester and described by Voronova et al.[187]

The third type of compounds from this group is represented by enoles of the general formula **LXXXIII** (Fig. 34), which can be prepared from 3-oxo derivatives by the reaction with HCOOEt and a base (NaOMe or NaH) in DCM (r. t.) or dioxane (reflux). Such derivatives are described in,[161,171,188] where they were tested

for cytotoxic activities. The compound **LXXXIIIa** was the most active and showed ED_{50} values in the range of 1.7–3.8 μg · mL^{-1} in cancer cell lines SK-MEL-2, A549, and B16-F10, respectively.[171]



Scheme 12: C-2-Bis-enamino dimers prepared by Voronova et al.
Reagents and conditions: i) **R**-diamine, EtOH/benzene 5 : 3, acetic acid.

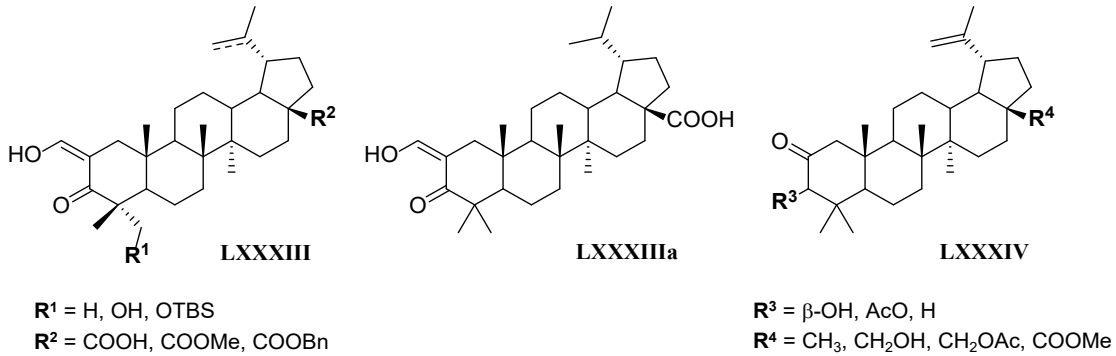


Fig. 34: 2-Enoles (**LXXXIII**) and 2-keto (**LXXXIV**) triterpenoids.

Several cytotoxic derivatives of the general formula **LXXXIV** (Fig. 34) containing a keto group at the C-2 position have been described by Yu et al.[189] and Pettit et al.[190]

2.2.3 The double bond C-2(3)

The double bond between the carbons C-2 and C-3 can be prepared in many different ways, the most interesting and useful ones will be reviewed in this chapter.

The most commonly used method is based on the Suzuki-Miyaura cross-coupling reaction. Starting from the 3-oxo compound, the triflate group is introduced into that position using a strong base – KHMDS, and $PhNTf_2$ in THF at −78 °C in 4 h.

Subsequently, boronic acid of choice, Na_2CO_3, and Pd catalyst react in cross-coupling reaction with high yields, resulting in compounds of the general formula **LXXXV** (Fig. 35) that were described in the literature[191–194] and tested for anti-HIV activities. The most promising derivative **LXXXVa** was in 2018 in the phase IIb clinical trials as a highly potent orally active second-generation HIV-1 maturation inhibitor with $EC_{50} < 15$ nmol · L^{-1}.[191]

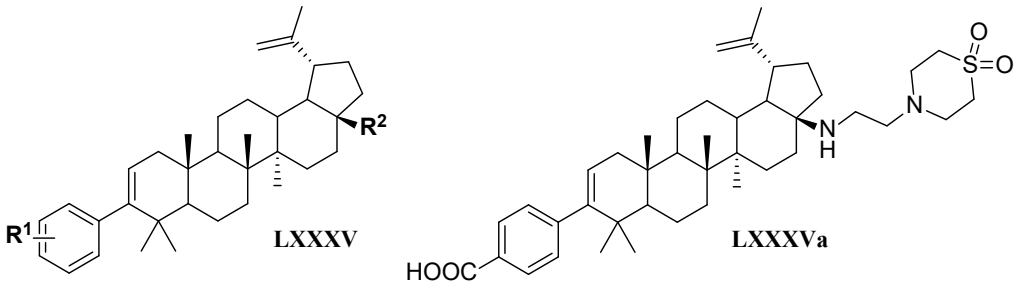


Fig. 35: Lupane derivatives **LXXXV** prepared by Suzuki-Miyaura reaction.

The nitro group can be introduced into the C-2 position along with the formation of the double bond C-2(3) by nitration using conc. HNO_3 in acetic acid at r. t. Such derivative of betulinic acid (**LXXXVI**, Fig. 36) was prepared by Borkova et al.[195] and tested for anticancer activity. The compound showed moderate cytotoxic activity with IC_{50} values in the range of 10–36 µmol · L^{-1} against the CCRF-CEM, CEM-DNR, K562, K562-TAX, and A549 cell lines.

Seven cytotoxic nitrile derivatives **LXXXVIIa**–**LXXXVIIg** (Fig. 36) were prepared by Zhang et al.[196] and tested against five cancer cell lines (HL-60, BEL-7402, SF-763, HeLa, B16). The compounds were prepared by opening the isoxazole ring attached to the A-ring with NaOH or triethylamine. Moderate antiproliferative activity was reported with the best IC_{50} value of 5.9 µmol · L^{-1} for the derivative **LXXXVIIe** (SF-763).

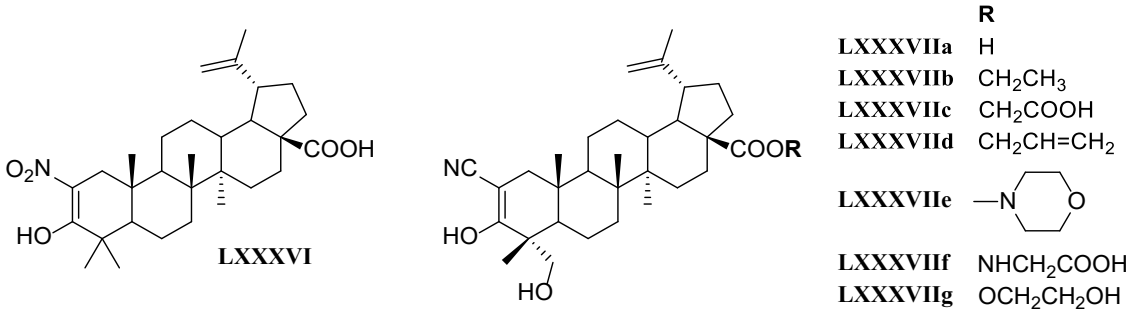
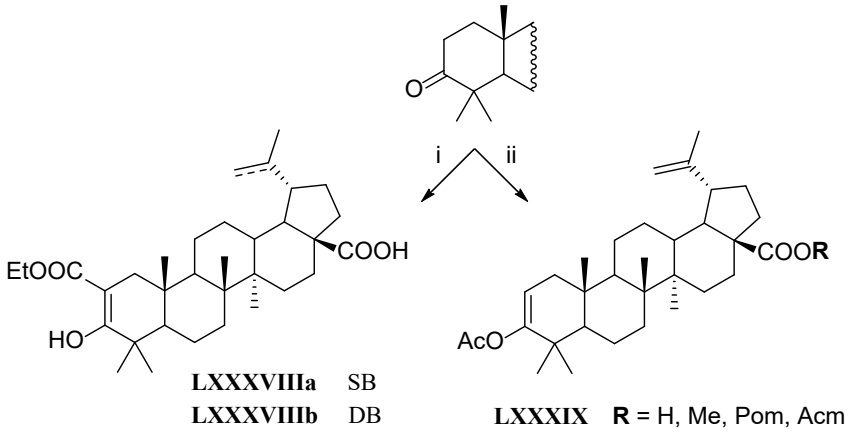


Fig. 36: Derivatives of betulinic acid (**XI**) with a C-2(3) double bond prepared by Borkova et al. (**LXXXVI**) and Zhang et al. (**LXXXVIIa**–**LXXXVIIg**).

Two β-ketoesters **LXXXVIIIa**, **LXXXVIIIb** (Scheme 13) of betulinic acid (**XI**) and 20(29)-dihydrobetulinic acids (**XIV**) were prepared by Urban et al.[188]

from the corresponding 3-oxo parent compound and tested against the CCRF-CEM line; the IC_{50} value was higher than 100 $\mu mol \cdot L^{-1}$.

The same author[167] prepared enolacetates of the general formula **LXXXIX** (Scheme 13) from the 3-oxo compound by its treatment with isopropenyl acetate in the presence of TsOH under reflux. No significant cytotoxic activity was reported similarly to the study mentioned above.



Scheme 13: Various derivatives of betulinic acid (**XI**) with a double bond C-2(3) prepared by Urban et al. Reagents and conditions: i) Et_2CO_3, NaH, dioxane, reflux; ii) isopropenyl acetate, TsOH, reflux.

There are other articles describing different approaches to prepare the double bond C-2(3).[120,136,166,170]

2.2.4 The exocyclic double bond at the C-3 position

The last and as well undoubtedly the largest group of A-ring derivatives contains an exocyclic double bond at the position C-3. The most common type of modification from this group, already mentioned in Chapter 2.1, is a replacement of the 3β-hydroxy group by the keto group, which can be easily obtained by oxidation. Thus, most compounds are derived from lupenone (**LVIII**, Fig. 37), betulone (**LIX**), and betulonic acid (**XV**). Lupenone (**LVIII**) can be prepared from lupeol (**IX**) by oxidation using PCC in DCM at r. t. for 3 h (85% yield).[197] Betulone (**LIX**) can be prepared in the same manner after the protection of the secondary -OH group of betulin (**X**) with e.g. acetate, followed by deprotection. Betulinic acid (**XI**) is oxidized usually by CrO_3, H_2SO_4 in acetone at 0 °C,[198] or using $K_2Cr_2O_7$, acetic acid, and sodium acetate in dioxane at r. t.[188]

Firstly, the influence of the replacement of the 3β-OH group by the keto group on the cytotoxicity of lupane derivatives will be reviewed. The influence of this modification was studied by Kang et al.[199] They tested lupeol (**IX**), lupenone (**LVIII**), betulin (**X**), and betulone (**LIX**) side by side in four human cancer cell lines (A549, SK-OV-3, SK-MEL-2, HCT-15). The results indicate that oxidation of

the C-3 hydroxy group of lupeol (**IX**) to oxo function slightly decreased the IC_{50} value from the IC_{50} range of 10.9–17.1 µmol · L^{-1} to 9.2–15.7 µmol · L^{-1}. For betulone (**LIX**), the activity remained almost the same for the line A549, while for SK-OV-3 the IC_{50} value decreased from 7.5 to 5.1 µmol · L^{-1}, for SK-MEL-2 from 4.1 to 3.3 µmol · L^{-1}, and for HCT-15 from 8.6 to 3.4 µmol · L^{-1}.

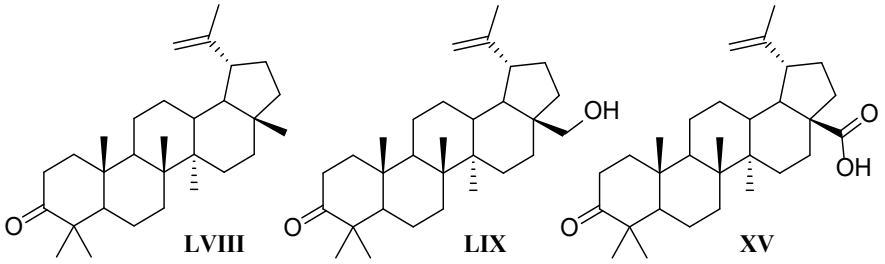


Fig. 37: Structures of lupenone (**LVIII**), betulone (**LIX**), and betulonic acid (**XV**).

In the case of betulonic acid (**XV**), Urban et al.[167] tested betulinic (**XI**) and betulonic (**XV**) acids side by side against five cancer cell lines (A549, DU145, MCF-7, K562, K562-TAX). The results showed a significant increase in activity against all tested lines, approximately 5–9 fold higher. Overall, the oxidation of the C-3 position increases the cytotoxic activity considerably. There are hundreds of lupane-type triterpenoid representatives containing the 3-oxo group with anticancer activities.

Another type of the double bond at the C-3 position is represented by 3-hydroxyimino derivatives of the general formula **XC** (Fig. 38), containing oxime group formed by the reaction of 3-oxo function with hydroxylamine hydrochloride. There are a number of publications concerning testing the cytotoxic activity of variously modified derivatives of this type derived from lupeol (**IX**),[135,197,200,201] betulin (**X**),[201] and betulinic acid (**XI**).[201–206] Antiviral compounds of this type were also reported.[37,207–209] Interesting results of cytotoxicity testing were published by Mukherjee et al.[206] for 3-hydroxyiminobetulinic acid (**XCa**). They tested this compound against nine cancer cell lines (MOLT-4, JurkatE6.1, CEM.CM3, BRISTOL8, U937, DU145, PA-1, A549, and L132) with ED_{50} values in the range of 1.9–8.1 µg · mL^{-1}; for the A549 line the compound was considered inactive.

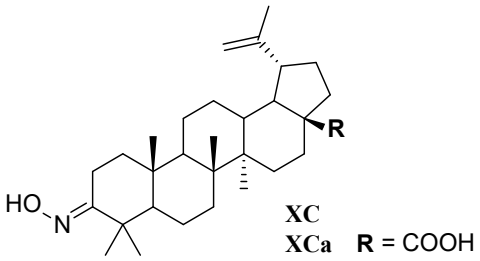


Fig. 38: Oxime derivatives of lupane.

Other substituted oximes, specifically 2-cyanoethoxy imine **XCIa** (Fig. 39), and amidoximes **XCIb**–**XCId** were prepared by Kazakova et al. from methyl betulonate.[210] They tested synthesized compounds for their antiviral and antituberculosis activities, however, no promising results were reported.

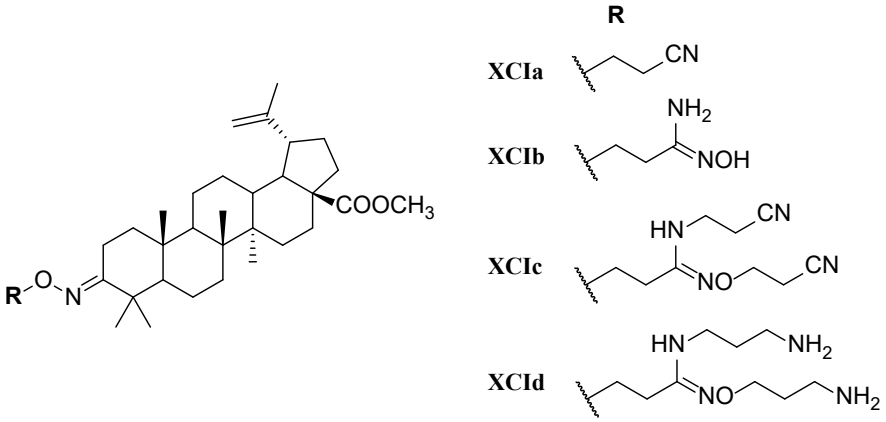


Fig. 39: Substituted oximes **XCIa**–**XCId** prepared by Kazakova et al.

The next group of C-3 nitrogen-containing substituents with a double bond and the structure of the general formula **XCII** (Fig. 40) is formed from the oxime derivatives mentioned above. Such oxime-ester compounds are being synthesized using an acid, bearing a substituent of choice, with DCC, DMAP in dry DMC.[91] Prepared derivatives were tested for their antibacterial, antiproliferative,[197] antidiabetic,[91,106] cytotoxic,[206] and antiviral[211] activities. The derivative **XCIIa** showed cytotoxic activity in the IC_{50} range of 2.2–5.5 $\mu mol \cdot L^{-1}$ against MOLT-4, Jurkat E6.1, CEM.CM3, BRISTOL8, U937, DU145, PA-1, and L132 cancer cell lines.

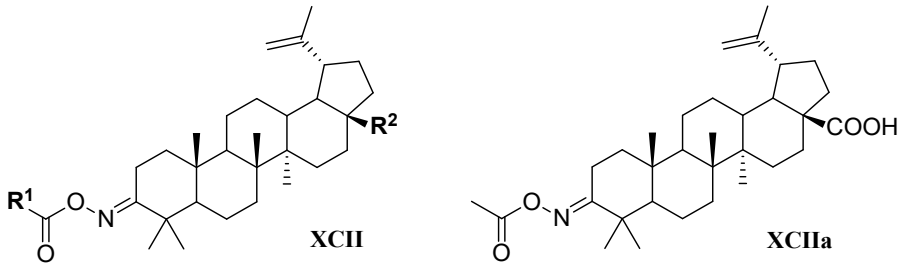


Fig. 40: Oxime-ester derivatives **XCII** of various lupanes (**VI**).

Cytotoxic compounds bearing a hydrazone substituent at the position C-3 of the general formula **XCIII** (Fig. 41) were reported in the literature multiple times.[101,105,111,135,200,205,212,213] Baratto et al.[105] prepared seven derivatives of this type and tested them against five cancer cell lines (A2780, 8505c, 518A2, MCF-7, A549). Compound **XCIIIa** was the best candidate with IC_{50} values in the range of 1.76–2.51 $\mu mol \cdot L^{-1}$ against all tested cell lines.

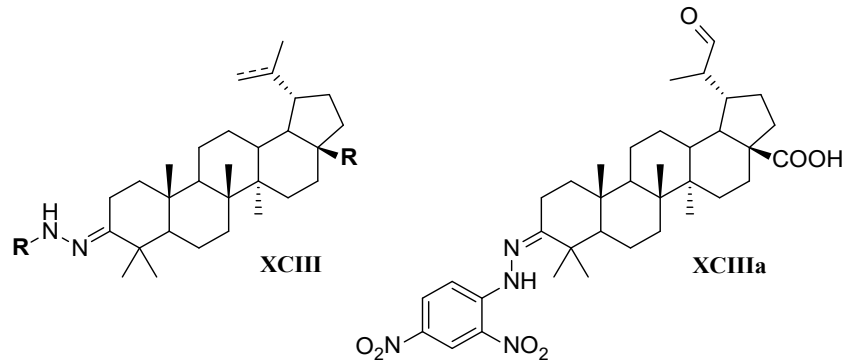


Fig. 41: Hydrazone derivatives **XCIII** of various lupanes (**VI**).

Two cytotoxic C-3 iminoderivatives were described by Ledeti et al.[212] (compound **XCIVa**, Fig. 42) and Rajendran et al.[205] (compound **XCIVb**).

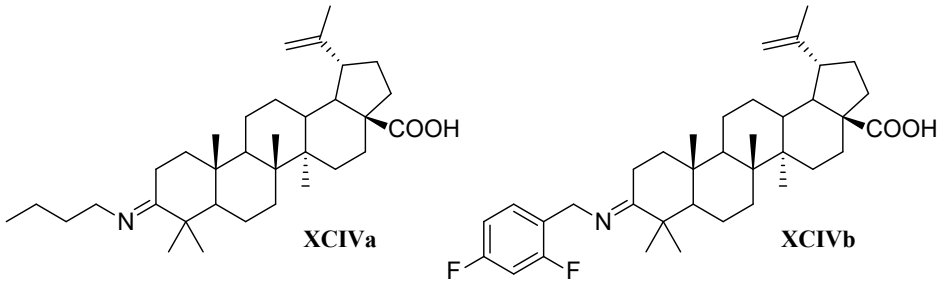
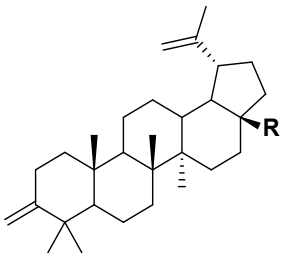


Fig. 42: Iminoderivatives **XCIV** of betulinic acid (**XI**).

Methylidene group at the position C-3 can be prepared by the reaction of the 3-oxo compound with the Wittig reagent, i.e. methyltriphenylphosphonium bromide. Such derivatives of betulinic acid **XCV** (Fig. 43) with anticancer properties are listed in the patent by Ramadoss et al.[214]



XCV R = COOH, COOR, CONR

Fig. 43: C-3-Methylidene derivatives **XCV** of betulinic acid (**XI**).

2.3 Huisgen cycloaddition and CuAAC reaction

The concept of Huisgen 1,3-dipolar cycloaddition, including the azide-alkyne Huisgen 1,3-dipolar cycloaddition, was introduced by Rolf Huisgen in the early 1960s.[215–217] However, the reaction between an azide and a terminal alkyne at a higher temperature providing a mixture of 1,4- and 1,5-triazole regioisomers (**A**, Scheme 14) was described by A. Michael already in 1893.[218] Generally, the 1,3-dipolar cycloaddition is a reaction between a 1,3-dipole (containing 4π electrons, a positive and a negative charge, e.g. azides, diazoalkanes, ozone, etc. – see Fig. 44) and a dipolarophile (2π electrons, a variety of double- and triple-bond systems) leading to a five-membered heterocycle.[219–221] This elegant way for the preparation of isoxazole, pyrrole, pyrazole, triazole, and other similar heterocyclic compounds was not heavily used before Huisgen's reports.[219] However, revealing their potential, these reactions became massively utilized and represent a milestone in the development of click chemistry.[222]

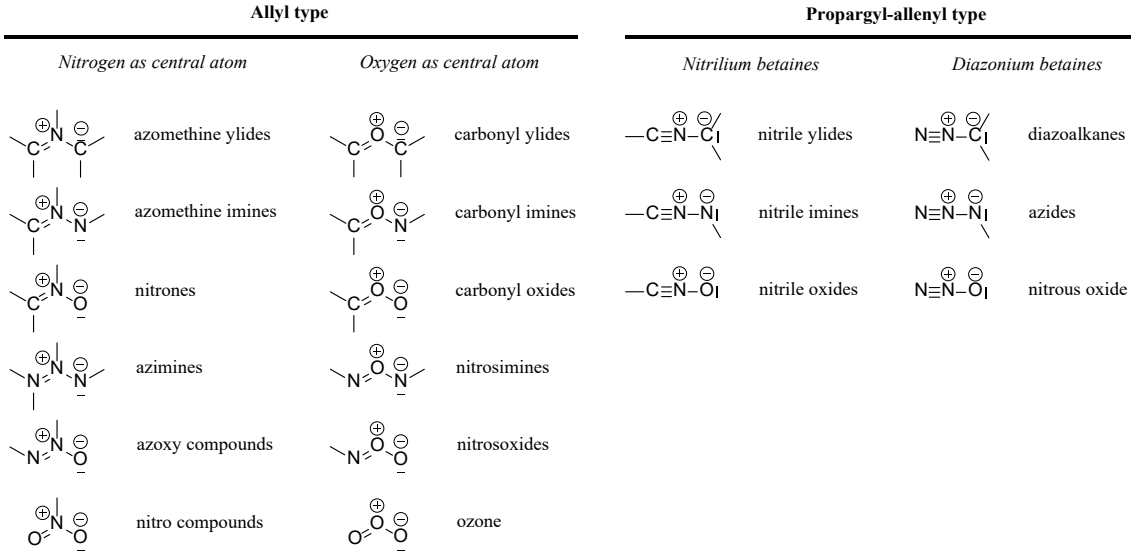
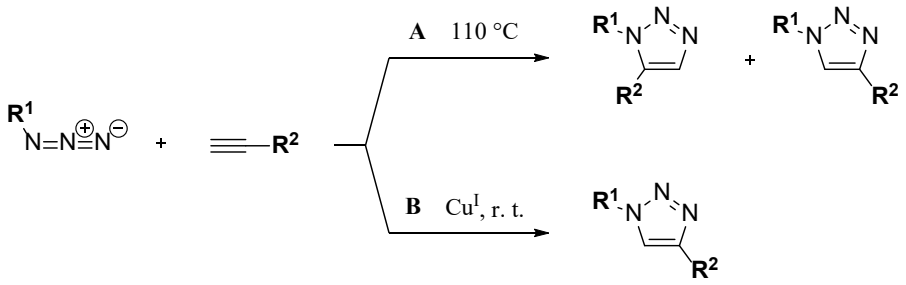


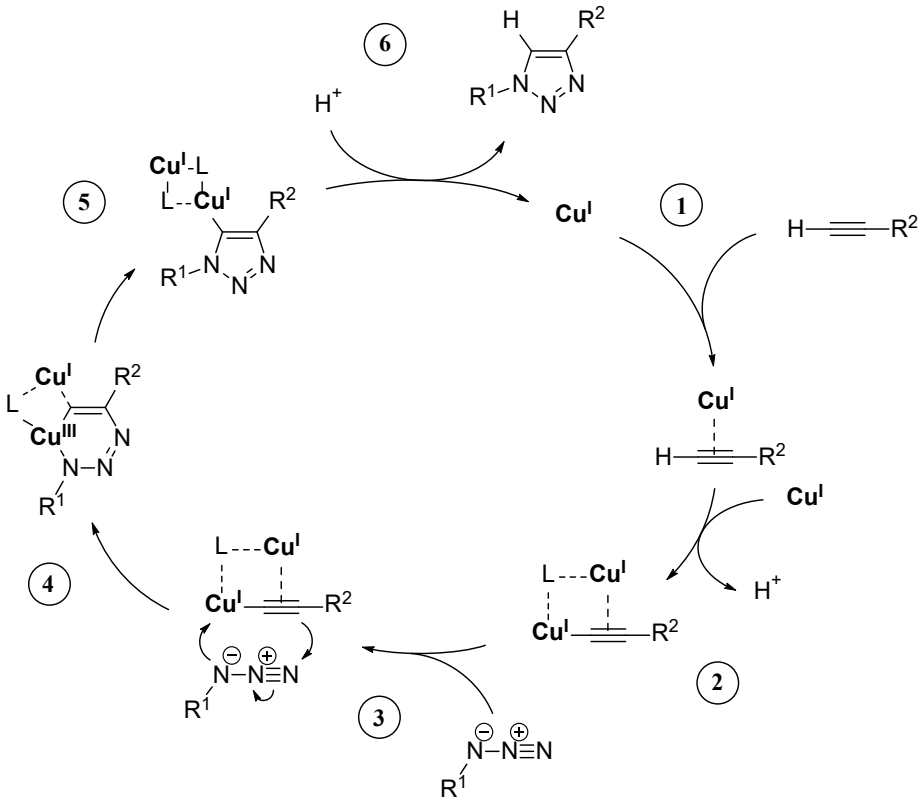
Fig. 44: Classification of 1,3-dipoles from the second period of the periodic table useful for 1,3-dipolar cycloadditions.[219]



Scheme 14: The difference between A) the azide-alkyne Huisgen 1,3-dipolar cycloaddition and B) the Cu-catalyzed azide-alkyne cycloaddition (CuAAC).

In contrast to the classic thermal azide-alkyne Huisgen 1,3-dipolar cycloaddition which is known for more than 60 years, the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) was described only recently in 2002 by two research groups independently – M. Meldal from Denmark, and K. B. Sharpless from California.[223,224] In this metal-catalyzed reaction, only the 1,4-disubstituted regioisomer of the 1,2,3-triazole is formed very rapidly from an azide and a terminal alkyne (**B**, Scheme 14) in a high yield under mild conditions (room temperature, water and oxygen tolerant) compared to the original slow non-catalyzed variant, requiring temperatures about 100 °C.[222] The benefit of Cu catalysis is attributed to overcoming the kinetic barrier in the formation of the triazole ring.[222] Thus, the CuAAC represents the best example of *click* reaction (i.e. "ideal" reaction that provides libraries of compounds as simple and efficient as possible), as it meets all the requirements defined by Sharpless in 2001: "*The reaction must be modular, wide in scope, give very high yields, generate only inoffensive byproducts that can be removed by nonchromatographic methods, and be stereospecific (but not necessarily enantioselective). The required process characteristics include simple reaction conditions (ideally, the process should be insensitive to oxygen and water), readily available starting materials and reagents, the use of no solvent or a solvent that is benign (such as water) or easily removed, and simple product isolation. Purification – if required – must be by nonchromatographic methods, such as crystallization or distillation, and the product must be stable under physiological conditions.*"[225] In short, this is the reason why CuAAC became so popular not only in biorthogonal chemistry, polymer or material science. A number of reviews were published covering these topics.[222,226–231]

The mechanism of the CuAAC reaction is at least dinuclear and starts with a formation of a π-complex from an alkyne and the copper(I) catalyst (**1**, Scheme 15). Then, the copper(I)-acetylide (**2**) is generated even in absence of a base. The process is followed by the formation of a ternary complex with the coordinated azide group (**3**). The nucleophilic attack of azide leads to the formation of a six-membered ring (**4**) making the first C–N covalent bond, while one copper(I) is oxidized to copper(III).[226] The second C–N bond is formed by the reductive ring contraction providing the 1,2,3-triazolide (**5**), which deprotonates the next molecule of the alkyne (**6**) to complete the catalytic cycle, while the desired 1,4-regioisomer is released.[222,226,232]



Scheme 15: The mechanism of the CuAAC reaction.[226]

The copper(I) catalyst is most often generated *in situ* from the Cu(II) pre-catalyst – $CuSO_4 \cdot 5H_2O$ using sodium ascorbate, which acts as a reducing agent.[231] Then, the reaction can be run in a broad variety of solvents, mostly in alcoholic or aqueous solutions (and their combinations) without an inert atmosphere. Alternatively, $Cu(OAc)_2$ may be used without any reducing agents as it is reduced during the reaction or by alcoholic solvent oxidation.[233,234] It is also possible to use copper(I) salts directly, however, then the copper(I) needs to be stabilized against moisture by complexation, and the reaction must be run under inert gas, otherwise the Cu(I) disproportionation to Cu(0) and Cu(II) occurs immediately, terminating the reaction. Bulky substituents (PPh_3, $P(OEt)_3$, BINAP, etc.) can be used for this purpose, however, nitrogen-containing ligands (simple amine bases such as Et_3N, DIPEA, or more sophisticated polydentate pentamethyldiethylenetriamine – PMDETA) can provide much better protection, control, and even accelerate the reaction by promoting the initial copper(I)-acetylide formation.[224,235] *N*-heterocyclic carbene ligands (NHC) provide remarkable stability toward moisture, heat, and oxygen, making CuAAC running in the absence of any other supplements.[236] Thus, the homogeneous variant of the CuAAC reaction (reagents and the catalyst in the same phase) is the most widely used, however, the heterogeneous catalysis[237] has the biggest advantage in the ease of separation of the product from the catalyst, that can be even re-used, making this chemistry more "green". Moreover, the presence of residual copper within the final product is problematic in particular for biological applications

where the copper(I) is highly toxic, creating the reactive oxygen species (ROS), generated by an interaction of Cu(I) and Cu(II) with oxygen.[227] Many different and efficient purification methods were developed to get rid of the Cu traces, but in the case of heterogeneous catalysis, the active copper(I) catalyst is fixed to support materials such as polymeric substrates, carbon materials, or inorganic solids, and can be simply filtrated after the reaction.[222] Even metallic copper can be directly employed in the presence of oxygen environment, often promoted by ultrasonication[238] or microwave radiation.[239]

The copper-free variant of the reaction was also developed to overcome the problem of Cu toxicity in living cells. Strain-promoted azide-alkyne cycloaddition (SPAAC) was described by C. R. Bertozzi in 2004.[240] They used substituted strained cyclooctynes in absence of a Cu catalyst which provided the same desired products as in CuAAC, however, the rate of the reaction was significantly slower and the reaction proceeded as [3+2] cycloaddition in the same manner as classic Huisgen 1,3-dipolar cycloaddition.[241] Interestingly, this synthetic approach was used to enable *in vivo* imaging of developing zebrafish embryos.[242]

Importantly, the ruthenium-catalyzed variant of the 1,3-dipolar azide-alkyne cycloaddition (RuAAC) yielding 1,5-triazole regioisomers was described.[243] Unlike the Cu-catalyzed variant, in which the 1,4-regioisomer is formed, both terminal and internal alkynes can be used.[244]

2.3.1 CuAAC reactions in the chemistry of triterpenes

Although triterpenes have several advantageous properties such as a large palette of biological activities, low toxicity, simple availability from natural sources, and natural biodegradability, their clinical use is rare. Some reasons for that have already been mentioned in the introductory part of this thesis: Triterpenes are very lipophilic molecules and therefore cannot be efficiently adsorbed from the gastrointestinal tract, their therapeutic concentrations are often higher in comparison to currently used drugs, and their multi-targeted properties make it difficult to evaluate the structure-activity relationship. One of the most common possibilities to improve the bioavailability, to reduce some of the abovementioned disadvantages, and to selectively target the natural triterpenoids to cancer cells, is to prepare their semisynthetic conjugates with a modifying small molecule (such as a heterocycle, sugar, PEG, fluorescent molecule, biotin, folic acid) that may be performed with the use of a cycloaddition reaction. The copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) is probably the most popular reaction for conjugation which brings a triazole heterocycle to the molecule. This heterocycle alone may serve as a part of a pharmacophore,[245] altering the mechanism of action of the parent compound,

or as a water solubility enhancer to increase the hydrophilicity of the potential drug.[246]

In 2017, we published a review article focusing on the preparation and evaluation of achieved improvements of conjugates of triterpenes with variously substituted triazoles.[247] The CuAAC reaction is used almost exclusively for this purpose, leading to a connection of the terpene with another molecule *via* a triazole-containing linker. The review contains articles up to 2017, and since then the number of articles is growing rapidly. From 2017, twenty-two new studies,[92,248–268] one review article,[269] and four patents[70,270–272] dealing with lupane-type compounds containing a triazole ring have been published which testifies to the popularity of this synthetic approach.

Our research group is also interested in the study of this type of molecules and their biological properties. One of the four parts of this thesis is a subproject dealing with the CuAAC at the C-30 position. In this thesis, the CuAAC reaction was used for the extensive modification of the position C-30 after the advantage of the elevated reactivity of this site in the neighborhood of the double bond 20(29) was taken. Detailed information will be given in Chapter 4 (Results and discussion). The review article is attached in Appendix I:

Pokorný, J.; Borková, L.; Urban, M., Click Reactions in Chemistry of Triterpenes – Advances Towards Development of Potential Therapeutics, *Current Med. Chem.* **2018**, *25* (5), 636–658.

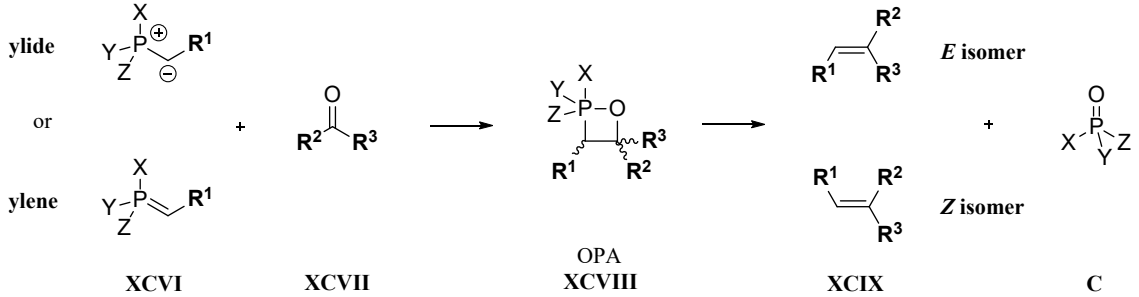
2.4 The most common methods used to introduce a new C=C double bond into the triterpenoid structure

This chapter deals with another two well-known tools of organic synthesis, which were used in this thesis for the preparation of new series of triterpenoids. Both authors of the reactions were awarded the Nobel Prize.

2.4.1 Wittig reaction

The Wittig reaction[273] is probably the most important method for introducing a new double bond into the molecule to form an olefine. Albeit it has been known for almost 70 years, it is still very popular in organic synthesis[274] and its exact mechanism was fully described only recently.[275–277] There are many reviews covering this topic.[278–281] Georg Wittig was awarded the Nobel Prize in 1979 together with Herbert C. Brown for their development and use of phosphorus- and boron-containing compounds in organic synthesis.

In the Wittig reaction, a phosphorus compound called ylide or ylene (**XCVI**, Scheme 16) acts as the "Wittig reagent". The ylide is usually formed *in situ* in the reaction of phosphonium salt, prepared typically by alkylation of triphenyl phosphine, with a base such as butyllithium, $NaNH_2$, LDA, or potassium *tert*-butoxide. Its structure can be represented by fully ionic ylide form or as ylene, however, the P–C bond has a rather ionic character with a contribution to the stabilization of the carbanion by phosphonium.[276] Reacting with a carbonyl compound (**XCVII**, an aldehyde or a ketone), alkene products (**XCIX**) are formed with phosphine oxide (**C**) as the side product.



Scheme 16: The general Wittig reaction of ylide (**XCVI**; X, Y, Z = alkyl/aryl/alkoxy; $\mathbf{R^1}$ = alkyl/aryl/vinyl/EWG) and aldehyde (**XCVII**; $\mathbf{R^2}$ = $\mathbf{R^3}$ = H – formaldehyde; or $\mathbf{R^2}$ = alkyl/aryl, $\mathbf{R^3}$ = H) or ketone ($\mathbf{R^2}$ = alkyl/aryl, $\mathbf{R^3}$ = alkyl/aryl) to form an alkene (**XCIX**) and phosphine oxide (**C**).

The mechanism of the reaction was extensively studied and explained in great details in the literature.[276] First, it must be stated that there is a clear difference between the mechanism in the presence and absence of Li salts – the exact Li-present mechanism still remains unknown. In summary, the Li salt-free Wittig reaction proceeds under kinetic control, and the first formed and the only intermediate is the oxaphosphetane ring (OPA, **XCVIII**, Scheme 16). The formation of OPA is an irreversible [2+2] cycloaddition; the stereochemistry of the resulting olefine is set during the formation of OPA. The OPA then decomposes stereospecifically with respect to the carbon atoms of the new alkene as can be seen in Scheme 16.

Stereoselectivity of the resulting olefine is strongly dependent on the nature of ylide used in the reaction. According to the substituent R^1 attached to the α-carbon (see Scheme 16), ylides may be assigned into three categories (Fig. 45). 1) *Non-stabilized* ylides are those bearing an alkyl R^1 group. They are called *reactive* due to their high ability to react even with ambient moisture; therefore, they are not stable in air and need to be prepared *in situ* under inert gas. Typical non-stabilized ylides derived from triphenyl phosphine are usually *Z* selective and react very rapidly at low temperatures. 2) *Semi-stabilized* ylides bear phenyl or alkenyl R^1 group which stabilize their ionic character by conjugation with the unsaturated group or aromatic ring. Therefore, they are less prone to hydrolysis and so more stable. This group of ylides is probably the most frequently used, they also react in a matter of seconds or minutes; however, the typical benzylidene triphenylphosphoranes are not very selective and provide approximately an equal amount of *E* and *Z* isomers, depending on the reaction conditions. It is worth mentioning that the selectivity could be enhanced by appropriate choice of substituents X, Y, Z, as can be seen in Fig. 45. 3) If the R^1 group is represented by EWG such as carbonyl, ester, nitrile, sulfone, or other similar function, such ylides are referred to as *stabilized*, and they are typically stable in air, and often commercially available as they can be isolated and purified. Some stabilized ylides derived from triphenyl phosphine do not react easily in the Wittig reaction, at least, they often require heating. Generally, this class of ylides usually provides alkenes with high *E* selectivity. An overview of the ylide selectivity depending on the choice of X, Y, Z substituents is shown graphically in Fig. 45.

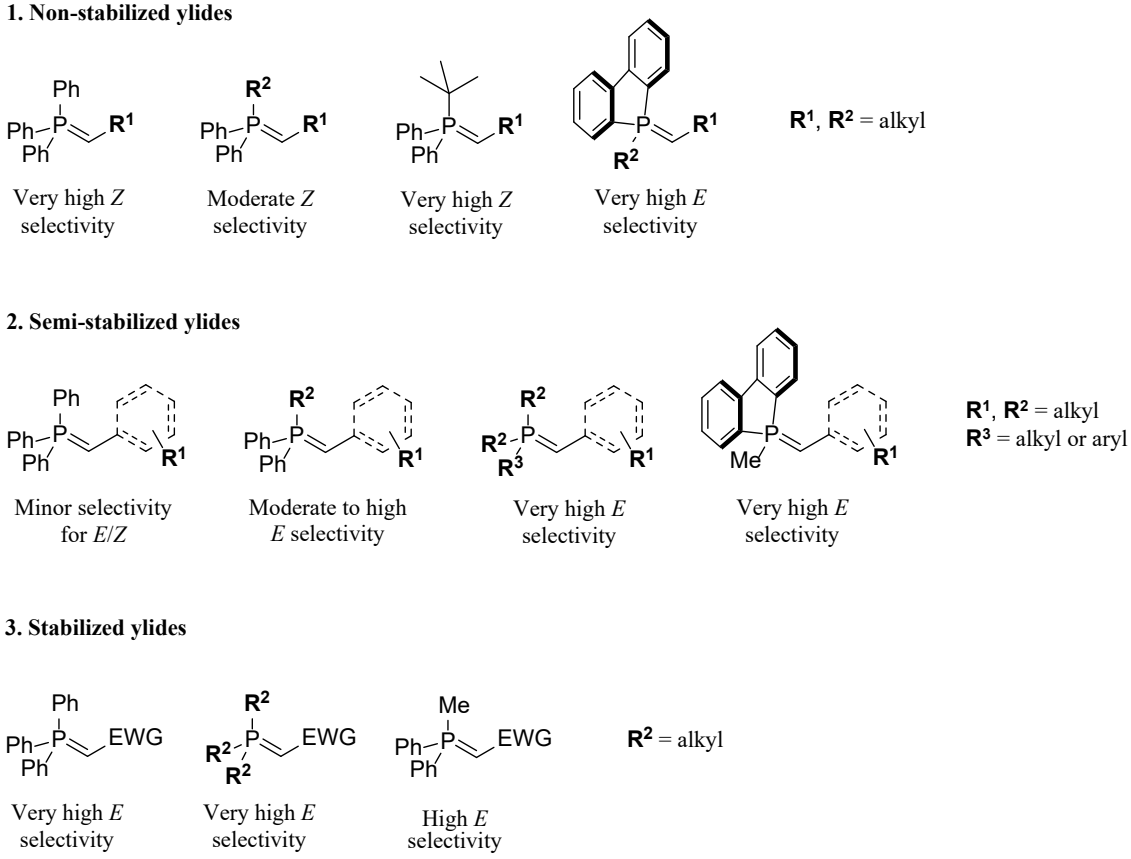


Fig. 45: The selectivity of different ylides used in the Wittig reaction depending on the substituents.

Although the stereoselectivity of the Wittig reaction can be controlled to a certain extent by a choice of different substituents of the ylide itself (**CI**, Fig. 46), other phosphorus-derived active reagents have been discovered and described to improve the scope and selectivity of the original reaction, or to facilitate the purification, as the phosphine oxide side product is sometimes hard to separate from the reaction mixture. The first modification was done by L. Horner et al.[282] in 1958, who employed phosphine oxides (**CII**, Fig. 46) to stabilize the active carbanion nucleophile. Moreover, phosphinate salt, generated in the reaction as the side products, is water-soluble and easy to separate.[283] Later, in 1961, another improvement was described by W. S. Wadsworth and W. D. Emmons.[284] They used phosphonate-stabilized carbanions **CIII** (described also by Horner earlier) to reach almost exclusive *E* selectivity; the phosphate salt originating from the reaction is again water-soluble, making workup easier. This reaction became very popular in organic chemistry and now it is known as the "Horner-Wadsworth-Emmons (HWE) reaction".[285] In 1983, M. Schlosser employed soluble lithium salts in the original Wittig reaction to yield alkenes with very high *E* selectivity.[286] This method is known as "Schlosser modification" of the Wittig reaction. Another update of the HWE reaction was

done by Still and Gennari in 1983.[287] They successfully overcame the stereoselectivity limitation using bis(2,2,2-trifluoromethyl) phosphonates **CIV**; the developed *Z* selective HWE reaction is also widely used, referred to as "Still-Gennari olefination".[285]

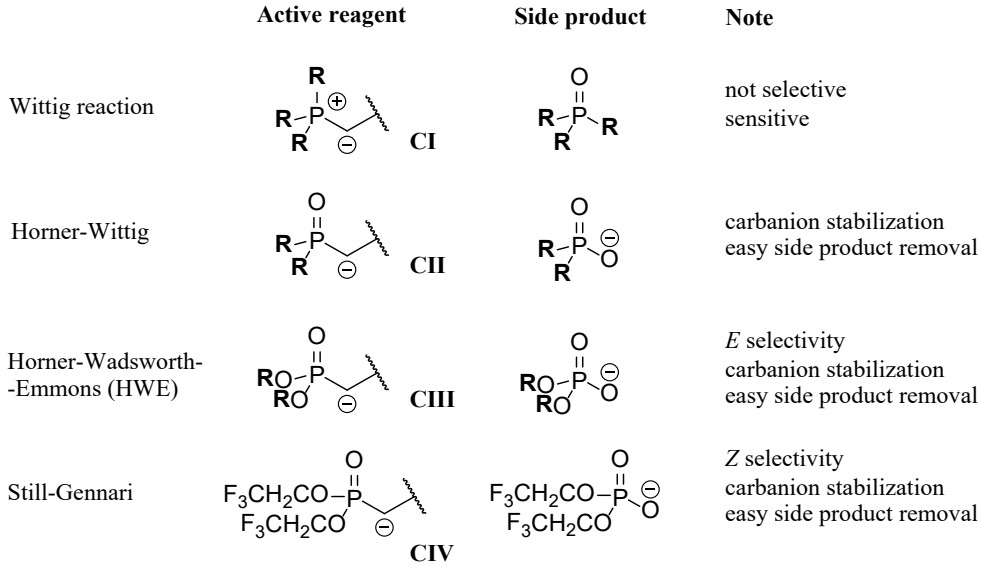


Fig. 46: Modifications of the original Wittig reaction.

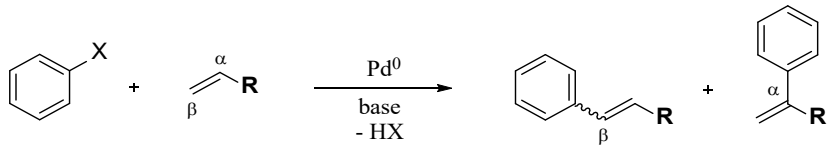
Other olefination methods are also commonly used in organic chemistry, in particular Julia olefination[288] and its modified versions, which use variously substituted sulfone carbanions as the active reagent (Julia-Lythgoe, Julia-Kocienski).[289] The Peterson olefination uses silyl carbanions and it was described in 1968.[290] Alkenes can be also prepared by metathesis reactions.[291] In 2005, Y. Chauvin, R. H. Grubbs, and R. R. Schrock were awarded the Nobel prize for the development of metathesis of olefines in organic synthesis.

The use of the Wittig reaction in triterpenoid chemistry is not rare, it can be found in the literature.[117,292–295] However, in only two publications,[125,296] authors used this method to introduce a new double bond at the isopropenyl moiety, as discussed above.

2.4.2 Heck reaction

The Heck reaction is well known cross-coupling method for the formation of C–C bond, and at the same time for introducing a new double bond into the molecule. It was firstly described by Mizoroki et al.[297] in 1971, and developed by Richard F. Heck in 1972.[298,299] Moreover, R. F. Heck was awarded the 2010 Nobel Prize in chemistry together with Ei-ichi Negishi and Akira Suzuki for palladium-catalyzed cross-coupling reactions in organic synthesis.

In the original reaction, an aryl halide reacts with an alkene to form a C–C bond in the presence of a Pd catalyst, appropriate ligands (not necessary), and a base with a formal loss of hydrogen halide as is shown in Scheme 17. However, the reaction may sometimes lead to two regioisomeric products depending on the olefine properties. With electron-deficient alkenes, such as acrylates or acrylonitriles, linear β-arylated products are formed, while electron-rich alkenes (enamides, acyclic enol ethers) usually provide a mixture of α- and β-arylated products.[300] Several reviews were published focusing on this topic.[301–304]

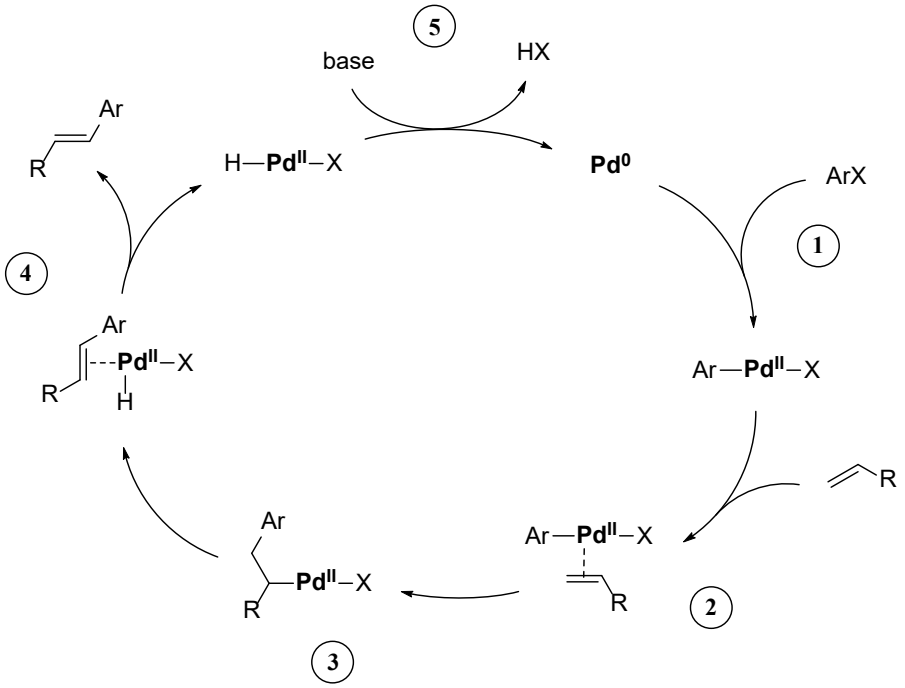


Scheme 17: The general Heck reaction.

The mechanism of the reaction starts with the oxidative addition of Pd(0) into the aryl-halogen bond (**1**, Scheme 18). It should be noted that the Pd(0) catalyst is usually generated *in situ* before the first reaction step from the Pd(II) pre-catalyst using various reductive agents. The most common way is the use of $Pd(OAc)_2$ and triphenylphosphine, however, there is a wide variety of other possible combinations.[301] Then, the π-complex with the alkene is formed (**2**), followed by a migratory insertion of the alkene into the palladium-carbon bond (*syn*-addition step) to form the alkyl-palladium intermediate (**3**). After an appropriate structural reorganization which often occurs, the *syn*-β-hydride elimination leads to the formation of a new Pd-alkene π-complex (**4**). The complex is destroyed while a thermodynamically favored *trans*-olefin is usually released. Then, a base is employed in the last reductive elimination step (**5**) to regenerate the palladium catalyst from Pd(II) to Pd(0).

The extent of the reaction has evolved significantly over the years and now it has a broad range of synthetic applications including intramolecular variants.[305] It is possible to couple iodides, bromides[299], chlorides[306], as well as pseudo-halides – triflates[307], tosylates[308], mesylates[309], and aryl diazonium salts.[310] The coupling of ethene to a variety of haloarenes is an elegant way to prepare substituted styrenes or even stilbenes.[311] Monosubstituted or 1,1-disubstituted alkenes are used most commonly due to their increased reactivity, however, there are even some examples with tetrasubstituted alkenes.[312] As for the Pd catalysts, many palladium salts and complexes are commercially available ($Pd(PPh_3)_4$, $Pd(dba)_3$, $PdCl_2$, $Pd(PPh_3)_2Cl_2$, etc.); the first option is often air-stable and cheap $Pd(OAc)_2$, however, elemental palladium on charcoal, or special nucleophilic carbene Pd complexes can be also employed in specific situations.[313] Equally, there is plenty of ligands, additives, and bases that can be used to control the regioselectivity of the reaction; typically, the appropriate "catalyst cocktail" is essential for the desirable course of the reaction,

where only simple switching from Et_3N to K_2CO_3 can dramatically change the final product.[313,314] From solvents, DMF is used most often, but the reaction proceeds in a variety of other solvents (MeCN, DMSO, dioxane, benzene, toluene, $CHCl_3$, etc.), including water[315,316], or it can be completely solvent-free (the base or the alkene acts as a solvent).[313,317] It is obvious that the Heck reaction has an irreplaceable position in organic synthesis and its application is still evolving – a detailed description is beyond the scope of this chapter.



Scheme 18: The mechanism of the Heck reaction.

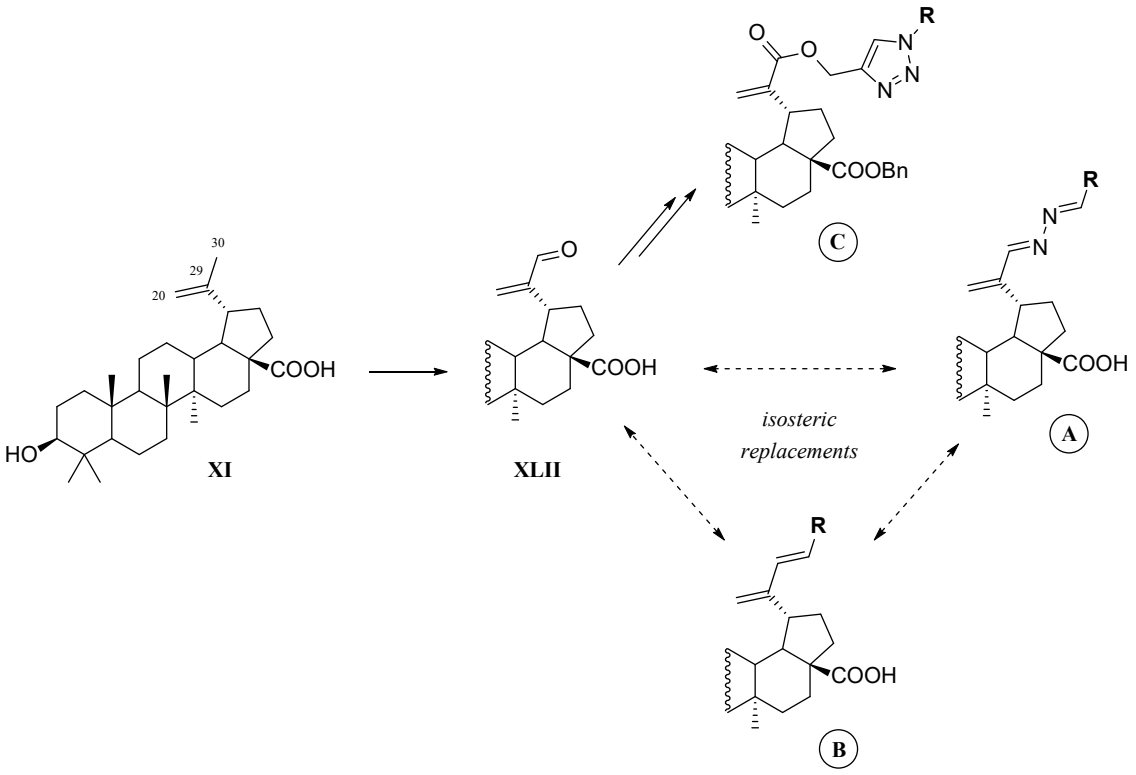
Similarly as the Heck reaction, the Sonogashira coupling reaction is used to form the C–C bond from a terminal alkyne and an aryl or vinyl halide using Pd and Cu catalysts.[318] Thus, instead of a double bond, a triple bond can be introduced into the molecule by this reaction.

In triterpenoid chemistry, only one study was focusing on the preparation of new derivatives using this reaction at the position C-30, however, the results were not optimistic and only small yields were obtained.[110]

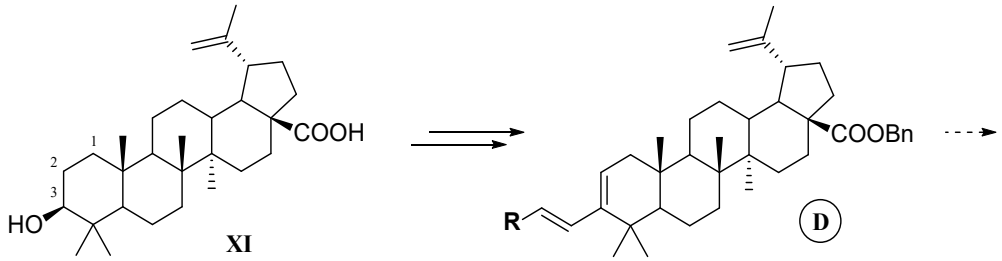
3 Aims of the work

The aims of this Ph.D. thesis are the following:

- theoretical overview focused on modifications of double bonds of lupane-type triterpenoids and their biological activity
- synthesis, purification, and characterization of new betulinic acid (**XI**) derivatives modified at the position C-30 according to Scheme 19
- development of a synthetic route for the preparation of new compounds with a double bond using Heck reaction according to Scheme 20
- cytotoxic testing of the prepared final compounds (performed by the biological department at the Institute of Molecular and Translational Medicine, Faculty of Medicine, Palacký University Olomouc)



Scheme 19: Derivatives of betulinic acid (**XI**) modified at the C-30 position: A) aromatic azines, B) Wittig products, C) triazoles.



Scheme 20: D) Derivatives of betulinic acid (**XI**) modified at the C-3 position using the Heck reaction.

Based on the literature review, it is clear that betulinic acid (**XI**) still has a lot to offer due to its wide scope of biological activities. There are many unexplored options for further modifications and improvement of its undesirable properties. In particular, 30-oxobetulinic acid (**XLII**) with its low IC_{50} values could potentially be a hot candidate for cancer treatment, but the major drawback of this compound is the low selectivity between cancer and healthy cells caused by the presence of the Michael acceptor. Therefore, this thesis aimed to find synthetic approaches leading to new betulinic acid (**XI**) derivatives with anticancer activity modified especially at the C-30 position, which tried to overcome the problem of low selectivity of the parent compound **XLII** with the preservation of the 30–C=X double bond.

Nitrogen-containing substituents in both C-30 and C-3 positions, such as variously substituted imines, hydrazones, or oximes, significantly contributed to the biological activity of the prepared lupane compounds.[156,157,206,210,319] In the first project, analogous approach was applied for masking the aldehyde moiety in 30-oxobetulinic acid (**XLII**), and novel hydrazone conjugates bearing variously substituted aromatic rings were prepared (A, Scheme 19). These derivatives were used to verify whether such modification of **XLII** could help to improve the selectivity while retaining the activity against cancer cells. After this study was finished, the improvement in selectivity was successfully confirmed, however, limited stability of the hydrazone moiety became apparent, which led to a second aim of the thesis – to modify the aldehyde group at the C-30 position using Wittig reactions, which represent a bioisosteric replacement of the azine bridge with a carbon atom. The new double bond 30–C=C was expected to act similarly to the original 30–C=N in living cells, but its stability could be much higher (B, Scheme 19).

The next goal was to prepare new betulinic acid derivatives using Cu(I)-catalyzed azide-alkyne cycloaddition in order to build on the previous research in this area (C, Scheme 19).

Finally, the last target was to optimize the use of the Heck reaction as a modern cross-coupling method for introducing a new double bond attached to the A–ring of the lupane skeleton (D, Scheme 20). The scope of this reaction has not been well explored in the chemistry of triterpenes yet. Therefore, its use was evaluated in terms of double bonds at the A-ring at the position C-3 where similar cross-coupling reactions on triterpenoids were performed previously.

4 Results and discussion

Starting with this chapter, the numbering of molecules is in Arabic form and differs from the previous parts (Roman numerals) to graphically separate the author's work.

4.1 Modifications of betulinic acid at the isopropenyl moiety

4.1.1 Azines

This part was published in the Future Medicinal Chemistry journal in 2018, the original article is attached (Appendix II):

Pokorný, J.; Krajčovičová, S.; Hajdúch, M.; Holoubek, M.; Gurská, S.; Džubák, P.; Volná, T.; Popa, I.; Urban, M., Triterpenic azines, a new class of compounds with selective cytotoxicity to leukemia cells CCRF-CEM, *Future Med. Chem.* **2018**, *10* (5), 483–491.

In order to improve the low selectivity of the 30-oxobetulinic acid (**2**, Scheme 21), condensation reactions with substituted aromatic hydrazones were performed to mask the Michael acceptor present in the parent compound. New derivatives containing fully conjugated azine bridge showed promising anticancer properties with high selectivity towards the CCRF-CEM cancer cell line. The disadvantages were low yields of the desired compounds and limited stability in water. It was sufficient for the *in vitro* tests but would likely be insufficient in anticancer drugs.

Ten new derivatives of betulinic acid (**1**, Scheme 21) were prepared, five as free acids at the position C-28 (**4a**–**4e**), and five with protection by benzyl ester at C-28 carboxyl function (**7a**–**7e**). All compounds were fully characterized and tested for their cytotoxic activities.

The synthesis started from commercially available acid **1**, which was oxidized in allylic position using SeO_2 in 2-methoxyethanol to obtain 30-oxobetulinic acid (**2**)

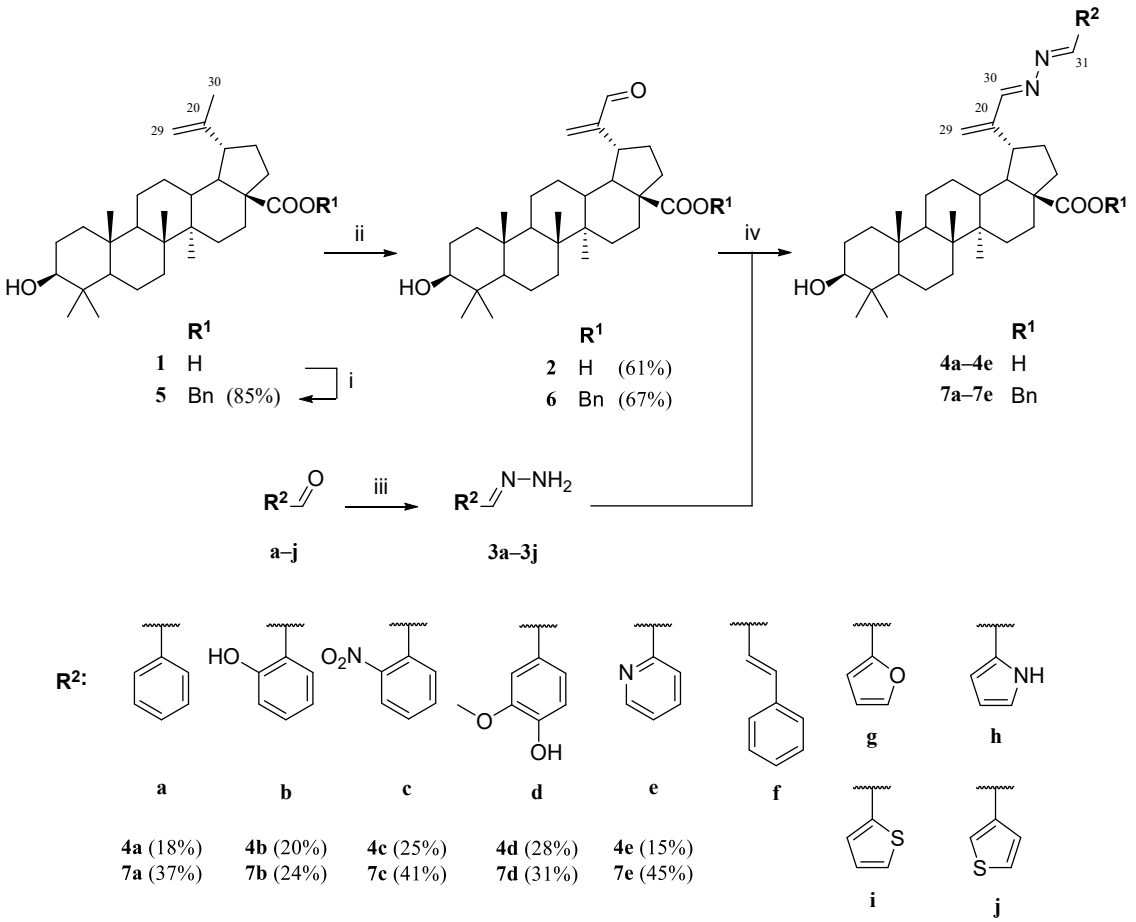
in 61% yield. Aromatic hydrazones **3a**–**3e** were prepared from corresponding aldehydes **a**–**e** by a reaction with hydrazine hydrate in EtOH[320] and were subsequently used in the next reaction step without purification. Reactions with the aldehyde **2** in EtOH under reflux afforded azines **4a**–**4e** in low yields 15–28 %. Optimization of the reaction conditions (temperature, reaction time) as well as the amount of reactants did not lead to higher yields. At room temperature the reaction was not completed, while at prolongated reaction time under reflux the yield was reduced due to sidereactions. A similar situation was observed for the amount of hydrazone (**3**) where at least two equivalents of the reactant were required for full conversion of the starting terpene, while the excess (3 eq. and more) has already caused higher rates of a formation of a mixture of unknown polar sideproducts visible on TLC (hydrazides may be formed from the free carboxyl group), so most of the terpene mass was lost. Moreover, low yields were also caused by difficult purification on column chromatography.

Therefore, the carboxyl function was protected by benzyl ester to avoid the side reactions, and a new series of compounds **7a**–**7e** was prepared in the same manner with higher yields of 24–45 %. Unfortunately, most of the terpene mass was transformed to unknown sideproducts, hence we decided to find out what happened during the reaction. An identification of the reaction mixture *via* analytical HPLC-MS revealed a formation of the triterpenic hydrazone **9** (Fig. 47) and a dimer **11**. The last stated was then isolated in a small amount together with benzyl betulinate **5** – a product of the Wolff-Kishner reduction. These three sideproducts were then observed in each reaction mixture as corresponding spots on TLC, the same situation was found for the reactions with the free acid **2**, where we proved the sideproducts by LC-MS. The rest of the polar mass standing on the TLC start remained unanalyzed.

Having a decent yield improvement and easier purification with protected derivatives, we also tried some heterocyclic aldehydes – substituents **g**–**j**, cinnamaldehyde **f**, and phenylhydrazine. In all cases, these reactions were not successful due to the high instability of the final products. In the case of cinnamaldehyde **f**, the product was prepared as a mixture of isomers and purified, but after a few hours it always decomposed. Even worse situation was observed for the heterocyclic aldehydes **g**–**j** and phenylhydrazine, where the product was visible as a single spot on TLC, but it always rapidly decomposed during the column chromatography. Subsequently, we tried reactions of the aldehydes **6** or **2** with hydrazine hydrate to form hydrazone compound **8** or **9**. Unfortunately, it always led to decomposition during column chromatography as well, thus they were not isolated. It is likely that the conjugation of the azine bridge with a six-membered aromatic ring is essential for stability, hence, only derivatives with variously substituted six-membered aromatic rings (**a**–**d**) or pyridine (**e**) were obtained as final products. Azines **4a**–**4e**

and **7a**–**7e** were identified as *E* isomers at both double bonds (NOESY), and the structures were proved by characteristic signals in ^{1}H NMR spectra:

- five singlets in the range of 0.70–1.00 ppm, all with integrals 3, were assigned to five methyl groups of the lupane skeleton,
- doublet of doublets around 3.15–3.20 ppm with integral 1 and the coupling constants 11.3 Hz and 4.8 Hz was assigned to H-3α,
- multiplet in the range of 3.50–3.65 ppm with integral 1 was assigned to H-19β,
- two doublets around 5.12 ppm and 5.21 ppm with integrals 1 and the coupling constants about 12.3 Hz, and a multiplet in the range of 7.28–7.43 ppm with integral 5 were assigned to the benzyl group for protected derivatives **7a**–**7e**,
- two singlets around 5.5 ppm and 5.7 ppm with integrals 1 were assigned to the protons H-29a and H-29b,
- two singlets in the range of 8.2–8.9 ppm (depending on the aromatic ring substituents) with integrals 1 were assigned to the azine protons H-30 and H-31,
- two multiplets in the ranges of 7.40–7.45 ppm with an integral 3, and 7.77–7.83 ppm with an integral 2 were assigned to the substituent **a**,
- three multiplets in the ranges of 6.89–6.94 ppm with an integral 1, 6.98–7.02 ppm with an integral 1, and 7.29–7.37 ppm with an integral 2 were assigned to the substituent **b** (2-hydroxy),
- four multiplets in the ranges of 7.56–7.62 ppm, 7.66–7.71 ppm, 8.02–8.07 ppm, and 8.17–8.21 ppm, all with an integral 1, were assigned to the substituent **c** (2-nitro),
- three multiplets in the ranges of 6.93–6.98 ppm, 7.16–7.21 ppm, and 7.48–7.51 ppm, all with an integral 1, and a singlet at 3.95 ppm with an integral 3 were assigned to the substituent **d** (4-hydroxy-3-methoxy),
- four multiplets in the ranges of 7.34–7.38 ppm, 7.77–7.82 ppm, 8.05–8.08 ppm, and 8.70–8.73 ppm, all with an integral 1, were assigned to the substituent **e** (pyridine).



Scheme 21: Preparation of the azine derivatives **4a**–**4e** and **7a**–**7e**. Reagents and conditions: i) Benzyl bromide, K_2CO_3, THF, reflux, 4 h; ii) SeO_2, 2-methoxyethanol, 110 °C, 4 h; iii) $N_2H_4 \cdot H_2O$, EtOH, reflux, 15 min; iv) **3a**–**3j** or phenylhydrazine, EtOH, reflux, 2–6 h.

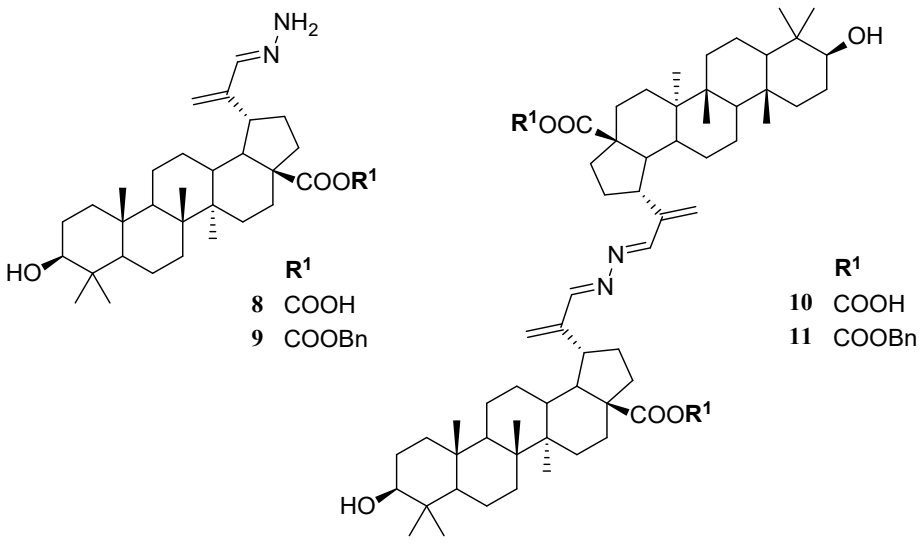


Fig. 47: Sideproducts of the reactions.

Biology

Prepared azines **4a**–**4e** and **7a**–**7e**, together with parent compounds – betulinic acid (**1**) and 30-oxobetulinic acid (**2**) were evaluated for their cytotoxic activity *in vitro* using MTS assay against a panel of eight cancer cell lines (CCRF-CEM, CEM-DNR, HCT116, HCT116 $p53^{-/-}$, K562, K562-TAX, A549, U2OS), and two human normal fibroblasts (BJ, MRC-5). Results of the IC_{50} values are displayed in Tab. 1.

Tab. 1: Cytotoxic activities of prepared azine derivatives **4a**–**4e** and **7a**–**7e** in eight tumor and two normal fibroblast cell lines. The most active derivative **4d** is highlighted.

Comp.	IC_{50} ($\mu mol \cdot L^{-1}$)[a]										
	CCRF-CEM	CEM-DNR	HCT 116	HCT116 $p53^{-/-}$	K562	K562-TAX	A549	U2OS	BJ	MRC-5	SI[b]
1[c]	45.5	45.4	38.0	>50	40.0	43.1	43.4	>50	37.6	32.9	0.7
2[c]	**3.1**	**3.7**	**2.5**	**3.0**	7.6	**3.3**	**3.8**	**3.5**	6.1	**4.1**	1.6
4a	**4.5**	30.9	21.5	16.7	34.9	28.3	25.2	29.9	>50	>50	>11.1
4b	5.6	26.8	29.4	20.8	39.8	26.8	34.2	23.4	>50	40.4	>8.1
4c	8.8	31.9	39.0	36.3	47.8	46.3	33.0	37.1	>50	>50	>5.7
4d	**3.4**	27.1	11.4	11.5	15.6	28.8	29.7	14.1	>50	48.9	>14.7
4e	**3.9**	27.9	9.6	16.6	20.7	28.7	28.1	10.5	45.2	33.1	10.0
7a	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	-
7b	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	-
7c	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	-
7d	21.7	>50	45.9	37.9	34.5	>50	47.0	33.5	32.1	36.9	1.6
7e	18.8	>50	>50	30.3	25.6	>50	48.5	30.5	45.7	45.3	2.4

[a] The lowest concentration that kills 50 % of cells. The maximum standard deviation in cytotoxicity assays is typically 15% of the average value. [b] Selectivity index is calculated for the IC_{50} of the CCRF-CEM line vs. average IC_{50} values of both fibroblasts (BJ and MRC-5). [c] Parent compound is used as a standard.

Results showed that all derivatives **4a**–**4e** with free carboxyl group were active against the CCRF-CEM line in a low micromolar range, compound **4d** was the most active and selective with the selectivity index SI > 14.7, meaning significant improvement in comparison with the parent compound **2** which suffered from general toxicity. Cell cycle analysis and nucleic acid synthesis in CCRF-CEM cell lines were further evaluated to show that compounds trigger selective apoptosis *via* intrinsic pathway; for more detailed information, please see the original article in Appendix II.

4.1.2 Wittig reactions

Results from this project were published in the European Journal of Medicinal Chemistry in 2021, the original article is attached (Appendix III):

Pokorný, J.; Olejníková, D.; Frydrych, I.; Lišková, B.; Gurská, S.; Benická, S.; Šarek, J.; Kotulová, J.; Hajdúch, M.; Džubák, P.; Urban, M., Substituted Dienes Prepared from Betulinic Acid – Synthesis, Cytotoxicity, Mechanism of Action, and Pharmacological Parameters, *Eur. J. Med. Chem.* **2021**, *224*, 113706.

In the previous project, triterpenic azines were proved to be selective against the CCRF-CEM cancer cell line in comparison with the parent compound, 30-oxobetulinic acid (**2**, Scheme 21). However, they also exhibited low stability and their preparation suffered from poor yields. Therefore, it was decided to modify the same substrate using Wittig reactions to obtain isosteric analogs with conjugated double bonds and variously substituted aromatic ring. Subsequent SAR study – cytotoxic activities, cell cycle analyses, and pharmacological parameters of the most active derivatives revealed valuable information. The theoretical background of the Wittig reaction was discussed in Chapter 2.4.1.

In total, forty-nine triphenylphosphonium salts **12.1**–**12.51** (Fig. 48) were prepared and two purchased to react in the Wittig reaction with 30-oxobetulinic acid (**2**). Forty new triterpenoid conjugates **13.1**–**13.49** (Scheme 22) were obtained, fully characterized, and tested for cytotoxic activity in eight tumor cell lines and two noncancer fibroblasts.

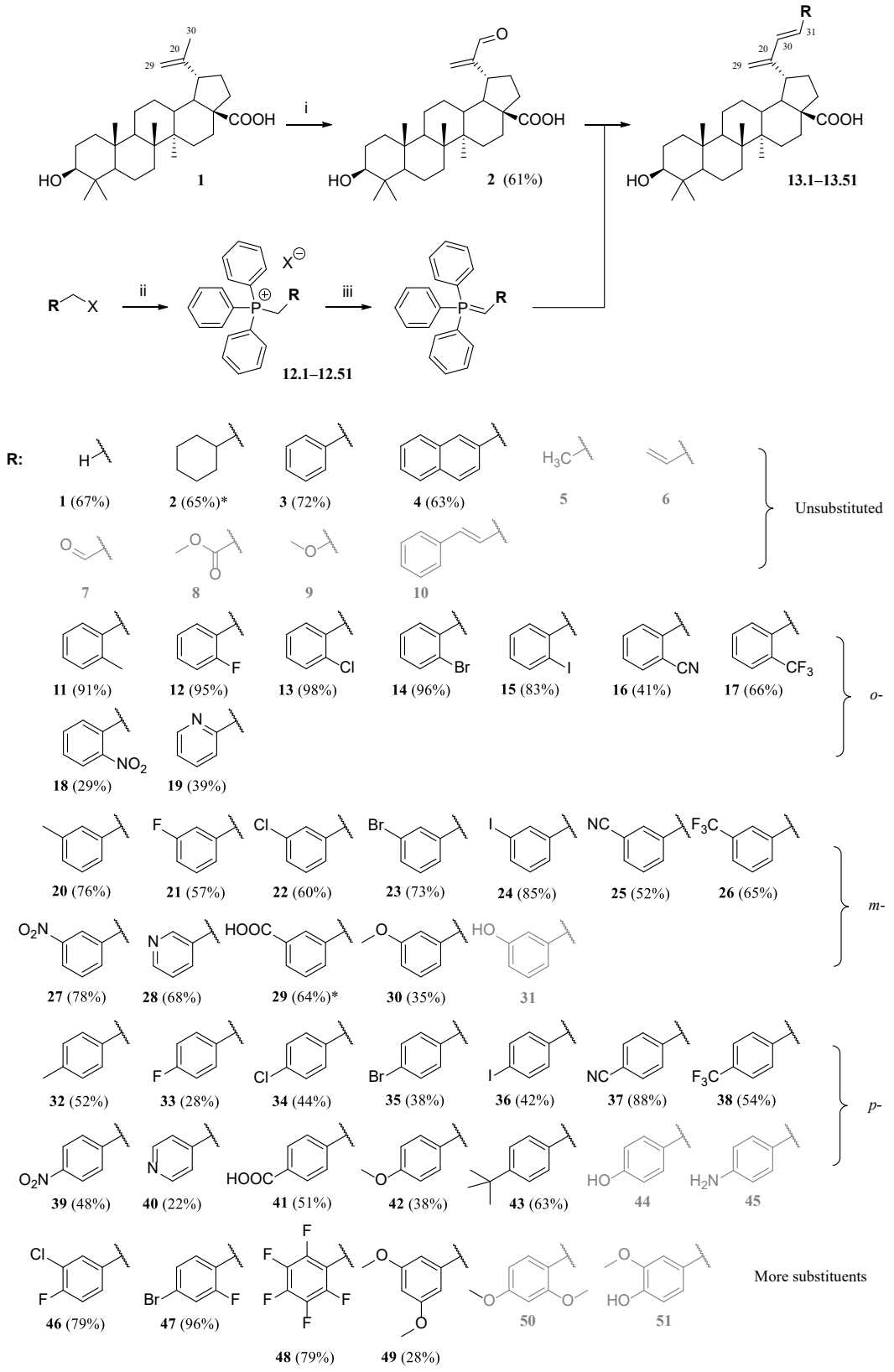
First, betulinic acid (**1**, Scheme 22) was oxidized in allylic position by SeO_2 to 30-oxobetulinic acid (**2**), which served as a substrate in the Wittig reaction. Then, a wide palette of triphenylphosphonium salts **12.1**–**12.51** was prepared (and two purchased) from corresponding commercially available halides. Most of them were bromides, **12.5** and **12.6** were prepared as iodides, and **12.7** was purchased as chloride. Reactions were carried out overnight in toluene or acetonitrile under reflux, usually, the white crystalline precipitate was obtained in quantitative yield without any special conditions. Pyridine salts **12.19**, **12.28**, and **12.40** were prepared from corresponding bromomethylpyridine hydrobromides, which were firstly neutralized using K_2CO_3, and then reacted with triphenyl phosphine to create the salts. Amino salt **12.45** was prepared from 4-nitrobenzyl bromide, which was reduced by H_2, Pd/C, and then, 4-aminobenzyl bromide reacted with triphenylphosphine in toluene under reflux as usual. Salts bearing hydroxy groups attached to the aromatic ring (**12.31**, **12.44**, and **12.51**) were prepared from corresponding benzyl alcohols, which reacted with triphenylphosphine hydrobromide in acetonitrile under reflux. Unfortunately, none of these three salts provided the

desired product in the subsequent Wittig reaction. Dimethoxy salt **12.50** was prepared from 2,4-dimethoxybenzyl alcohol using triphenylphosphine hydrobromide, however, it was found that the salt itself is very unstable as well as the prepared Wittig product **13.50**, which always decomposed during or immediately after the column chromatography.

The Wittig reactions with the aldehyde **2** and salts **12.1**–**12.51** were performed under an argon atmosphere using a vacuum line and dry Schlenk flask to avoid degradation of the ylide prepared *in situ* during the first reaction step using *t*-BuOK as a base. An optimization of used solvent, amount of reactants, and reaction temperature were done to achieve the best course of the reaction. At the beginning, reactions were carried out in toluene under reflux with an excess of the triphenylphosphonium salt and a base according to the literature,[117] but only a partial conversion of the starting aldehyde **2** was observed (about 60–70 %, visual TLC analysis). Therefore, several solvents were tried in parallel test reactions (toluene, THF, DCM, $CHCl_3$, DMF, EtOAc) using methyltriphenylphosphonium bromide salt **12.1** with the best results for anhydrous THF, where full conversion of the starting terpene was achieved hand in hand with sufficient solubility of all reactants. Successful formation of the ylide in the reaction mixture was always characterized by a rapid color change (bright colors from yellow to deep violet); interestingly, the color of the prepared ylide differed for each solvent. Completion of the Wittig reaction was usually visible as decolorization of the reaction mixture when run overnight, however, some reactions were completed in a few hours without color change.

Next, the optimum amount of used salt and the base was examined. It was found that at least 3.0 equivalents of the salt with 4.5 equivalents of *t*-BuOK are needed for the 100% conversion of the aldehyde **2** to the desired product; smaller amounts were already insufficient to complete the reaction. Potassium *tert*-butoxide was considered basic enough for deprotonation of the triphenylphosphonium salt to form the ylide, however, butyllithium can be employed as well. There were only two exceptions in used amounts – reactions with –COOH substituted salts (**12.29**, **12.41**) needed double amount of the base (9.0 eq) to complete the reaction.

During the optimizations, both *E*/*Z* isomers were often found in various ratios in NMR spectra of the resulting Wittig products after purification. From NMR analyses, it was found that the reaction stereochemistry outcome is dependent on the reaction temperature. When the Wittig reaction was performed at a higher temperature, the amount of the *Z* isomer was usually higher (up to 3 : 1 for *E*/*Z* in refluxing toluene, depending on the nature of the ylide used), while at room temperature the ratio was lower but still significant (up to 5 : 1 for *E*/*Z* in toluene, NMR analysis). Unfortunately, the two isomers were not always visible on TLC as two spots, and they were inseparable using standard column chromatography. Finally, the reaction temperature at 0 °C during the addition of the aldehyde **2** into



Scheme 22: Wittig reactions with 30-oxobetulinic acid (**2**). Reagents and conditions: i) SeO_2, 2-methoxyethanol, 110 °C, 4 h; ii) PPh_3, toluene/acetonitrile, reflux, overnight; iii) *t*-BuOK, THF, r. t., 30 min; iv) THF, 0 °C, then r. t., 2–18 h. *Yields for compounds **12.2** and **12.29** are given for a mixture of both *E*/*Z* isomers. Derivatives **12.5**–**12.10**, **12.31**, **12.44**, **12.45**, **12.50**, and **12.51** were not obtained.

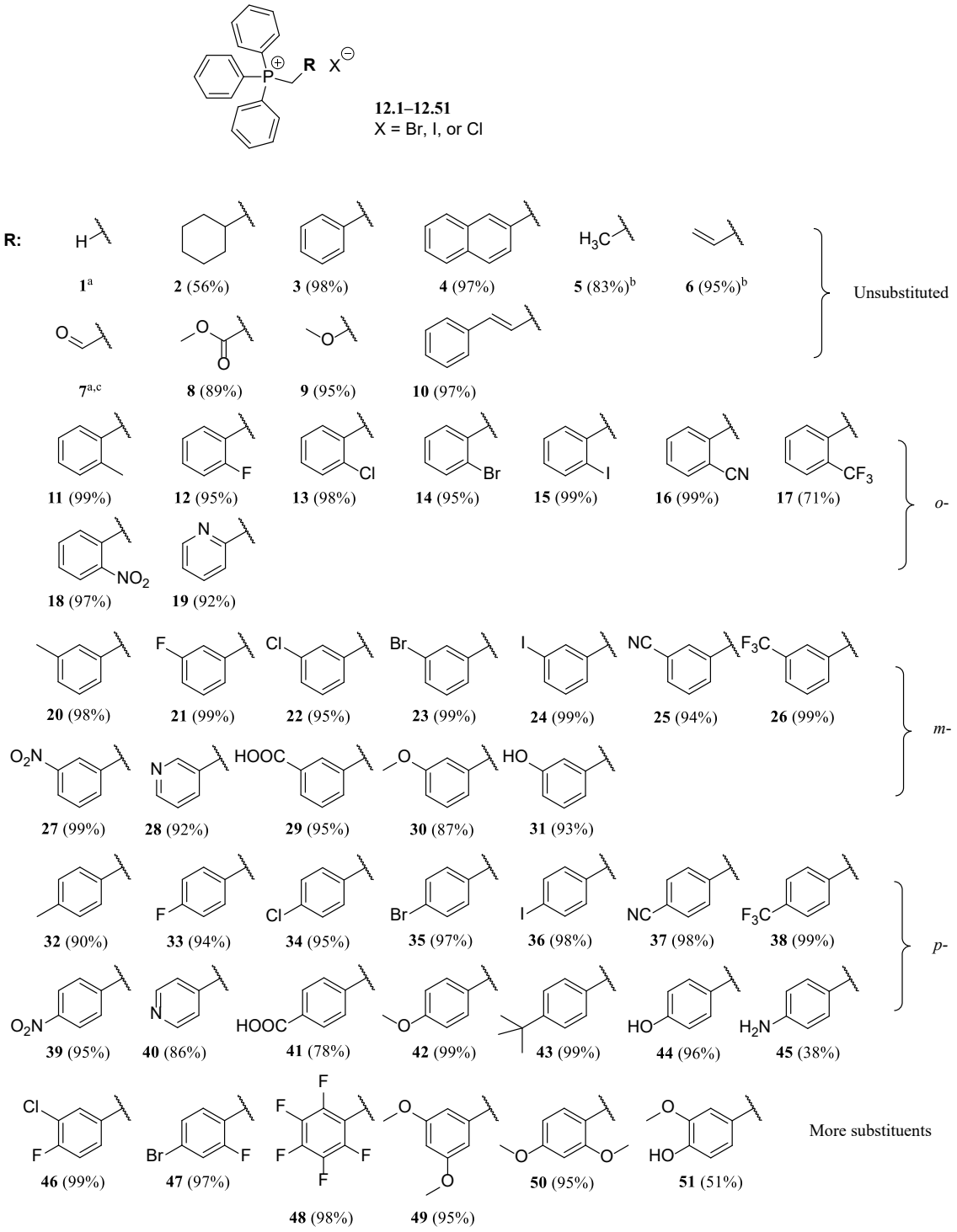


Fig. 48: List of triphenylphosphonium salts prepared in this project with corresponding yields. [a] Purchased salt; [b] Iodide salt; [c] Chloride salt.

the prepared ylide markedly helped to decrease the amount of the *Z* isomer, while the formation of the *E* isomer was predominant (at least 15 : 1 for *E*/*Z* at 0 °C in THF). Using these optimized conditions, most of the salts **12.1**–**12.51** were successful in the Wittig reaction giving the pure *E* isomer in moderate – high yield. However, in contrast with the *m*-nitro derivative **13.27** which reacted smoothly at

0 °C, this procedure was not successful for *o*-nitro and *p*-nitro derivatives **13.18** and **13.39**, where the reaction temperature at 0 °C was insufficient to complete the reaction, and the starting material remained unreacted in the reaction mixture. The reaction was finished in refluxing THF for *p*-nitro derivative **13.39**, while the conversion for the *o*-nitro derivative **13.18** reached only 40 % (visual TLC analysis), and no further change in the procedure helped to increase the conversion nor yield. Surprisingly, almost pure *E* isomers were formed at higher temperatures in both cases. Another exception was observed for derivatives **13.29** (*m*-COOH) and **13.2** (cyclohexyl substituent) – both isomers were always formed in a ratio of approximately 2 : 1, or 2.5 : 1 (*E*/*Z*), respectively; the mixtures were inseparable.

Among the prepared derivatives, most of them were generally stable at room temperature and in a solution, however, there were some exceptions. As mentioned earlier, the 2,4-dimethoxy derivative **13.50** always decomposed in contrast with the similar 3,5-dimethoxy derivative **13.49**, nevertheless, its structure was proved by ^{1}H NMR immediately after the purification using column chromatography. Equally, the *p*-amino derivative **13.45** was unstable in silica gel, but its exact mass was proved in a reaction mixture by HRMS. The derivative **13.10** with a longer conjugated chain was obtained as a mixture of several isomers that were unstable and decomposed over time. Attempts to prepare derivatives **13.5**–**13.9** from stabilized and nonstabilized ylides were unsuccessful; the reactions did not proceed at all, or only traces of products were found in the isomeric mixtures. Triphenylphosphonium salts bearing -OH groups (**12.31**, **12.44**, **12.51**) were unreactive in the Wittig reaction due to the resonance deactivation of the active ylide form after deprotonation of the -OH group. In addition, this fact was supported visually as a decolorization of the originally colored reaction mixture (active ylide form) a while after the addition of the base.

Structures of the final derivatives **13.1**–**13.49** were confirmed by their characteristic signals in ^{1}H NMR spectra:

- five singlets in the range of 0.75–1.00 ppm, all with integrals 3, were assigned to five methyl groups at the lupane skeleton,
- doublet of doublets around 3.20 ppm with integral 1 and the coupling constants about 11.4 Hz and 4.8 Hz was assigned to H-3α,
- a triplet of doublets around 3.30 ppm with integral 1 and the coupling constants about 11.2 Hz and 4.8 Hz was assigned to H-19β,
- two singlets around 5.12 ppm and 5.14 ppm with integrals 1 were assigned to the H-29 protons,
- two doublets around 6.65 ppm and 6.80 ppm (depending on the substituent on the aromatic ring) with integrals 1 and the coupling constant 16.2 Hz were assigned to the H-30 and H-31 protons, characteristic for the new double bond,
- rest of the peaks were characteristic for each substituent,

- *E* and *Z* isomers were identified in the ^{1}H NMR spectra by differences in coupling constants and chemical shifts of corresponding peaks as is visible in Fig. 49 – the amount of the *E* isomer was always higher with a typical larger coupling constant around 16 Hz while the *Z* isomer was formed in a minor amount, the two doublets were always more upfield and had coupling constants around 12 Hz.

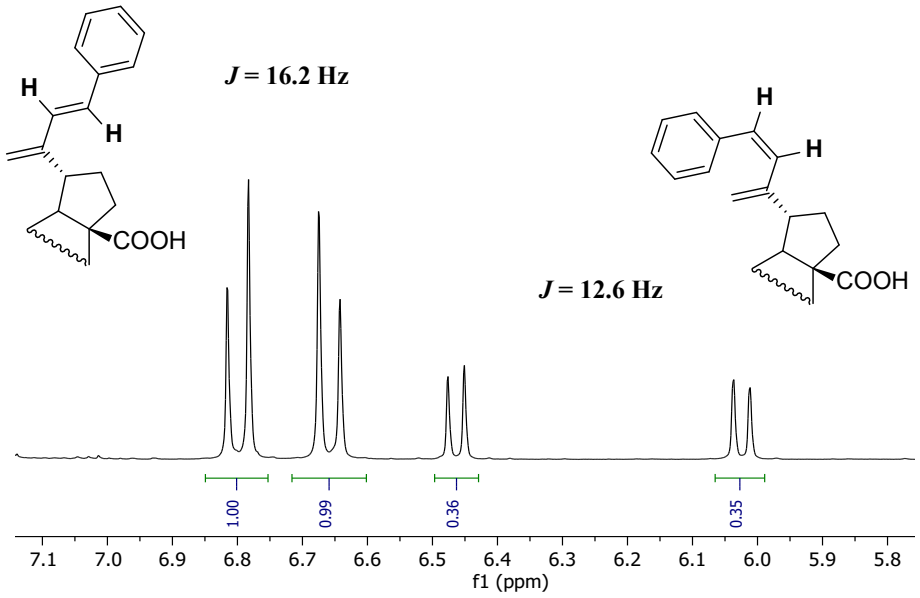


Fig. 49: An enlarged area of ^{1}H NMR spectra showing signals of the protons H-30 and H-31 of both *E* and *Z* isomers with corresponding coupling constants.

Biology

All prepared derivatives **13.1**–**13.49** with the parent aldehyde **2** were tested for their cytotoxic activities *in vitro* using standard MTS assay against a panel of eight cancer cell lines (CCRF-CEM, CEM-DNR, HCT116, HCT116 p53$^{-/-}$, K562, K562-TAX, A549, U2OS), and two human normal fibroblasts (BJ, MRC-5). Results of the IC_{50} values are displayed in Tab. 2. Derivatives are considered inactive when $IC_{50} > 50$ µmol · L^{-1}.

Compounds **13.22** and **13.39** showed the best results in primary screening with the highest cytotoxic activity against CCRF-CEM (IC_{50} = 3.2 µmol/L for **13.22**, and IC_{50} = 3.6 µmol/L for **13.39**, respectively) and favorable selectivity index (SI > 13.0 for **13.22**), therefore, they were subjected for further biological testing (cell cycle analysis, synthesis of DNA and RNA, protein expression, cell death, pharmacological parameters) to evaluate their mechanism of action. It was found that both compounds trigger selective apoptosis in cancer cells *via* intrinsic pathway. Pharmacological parameters of **13.22** were better than for **13.39**, therefore, the derivative **13.22** was the finally selected candidate for further preclinical development as anticancer drug. For more details, please see the original article in Appendix III.

Tab. 2: Cytotoxic activities of prepared derivatives **13.1**–**13.49** and the parent compound **2** in eight tumor and two normal fibroblast cell lines. The most active derivatives **13.22** and **13.39** are highlighted.

Comp.	IC_{50} (μmol · L^{-1})[a]										
	CCRF-CEM	CEM-DNR	HCT 116	HCT116 $p53^{-/-}$	K562	K562-TAX	A549	U2OS	BJ	MRC-5	SI[b]
2[c]	**0.8**	**3.8**	**1.9**	**1.9**	**3.4**	**1.3**	>50	**2.2**	6.7	**2.6**	5.7
13.1	11.1	25.1	24.1	24.3	15.7	26.2	23.9	24.1	44.6	24.3	3.1
13.2[d]	8.0	45.7	15.2	>50	>50	43.6	>50	>50	>50	>50	>6.3
13.3	6.5	30.2	35.5	39.5	40.0	25.3	41.8	24.2	>50	>50	>7.7
13.4	11.8	47.0	30.2	26.3	>50	45.8	32.9	27.2	>50	43.1	>3.9
13.11	19.1	22.8	29.7	25.9	28.1	25.9	26.8	28.0	33.0	31.5	1.7
13.12	15.4	23.2	24.5	22.6	21.8	25.1	29.7	27.7	42.1	31.7	2.4
13.13	18.7	42.1	26.6	24.8	23.9	39.3	29.4	28.1	42.3	32.6	2.0
13.14	16.8	49.1	27.2	24.5	21.6	47.9	28.6	27.8	46.7	35.3	2.4
13.15	20.6	>50	29.6	29.5	25.1	>50	29.7	28.5	>50	29.5	>1.9
13.16	23.2	26.8	36.6	47.0	>50	24.8	>50	40.4	>50	>50	>2.2
13.17	21.8	>50	33.0	36.1	48.7	>50	37.0	29.6	>50	>50	>2.3
13.18	23.7	30.6	34.8	39.3	>50	29.8	45.2	33.1	>50	>50	>2.1
13.19	19.7	27.7	>50	>50	>50	29.2	>50	>50	>50	>50	>2.5
13.20	9.5	21.4	24.7	22.3	24.9	23.6	36.5	18.2	>50	42.2	>4.9
13.21	11.7	23.1	23.0	21.0	21.6	21.9	28.7	27.7	41.5	32.1	3.1
13.22	**3.2**	25.5	16.4	15.2	27.7	25.6	29.2	17.3	>50	32.6	>13.0
13.23	8.8	35.9	25.3	26.8	23.6	32.8	33.1	27.6	>50	>50	>5.7
13.24	11.6	>50	27.3	29.5	25.2	48.4	38.7	28.4	>50	>50	>4.3
13.25	12.1	21.6	35.1	29.4	>50	22.7	>50	>50	>50	>50	>4.1
13.26	8.0	28.1	23.1	24.8	26.5	25.1	35.1	24.6	>50	42.9	>5.8
13.27	12.4	25.3	37.5	35.5	>50	24.3	48.8	29.3	>50	45.1	>3.8
13.28	11.3	17.4	30.9	32.8	>50	14.2	26.2	30.9	>50	>50	>4.4
13.29[d]	20.1	33.9	43.4	43.1	>50	36.7	>50	>50	>50	>50	>2.5
13.30	**4.6**	30.6	34.6	39.6	41.4	25.6	40.1	25.2	>50	44.1	>10.3
13.32	5.9	26.1	21.4	18.2	>50	26.3	>50	>50	>50	>50	>8.5
13.33	**4.9**	31.4	35.4	41.0	36.2	29.8	40.6	23.9	44.6	38.3	8.5
13.34	5.4	33.1	39.5	38.5	46.3	29.2	45.4	25.8	>50	>50	>9.2
13.35	6.5	28.9	33.2	31.8	>50	29.3	>50	27.0	>50	35.2	>6.6
13.36	16.0	>50	30.7	33.3	>50	>50	>50	32.2	>50	41.2	>2.9
13.37	23.8	27.0	39.1	43.3	>50	27.2	>50	44.1	>50	49.7	>2.1
13.38	21.5	40.9	40.9	31.9	40.5	45.6	41.4	28.2	49.5	42.4	2.1
13.39	**3.6**	22.9	18.6	15.7	11.5	23.4	14.3	19.5	24.3	19.2	6.1
13.40	5.9	20.5	35.0	30.6	43.4	20.6	46.0	29.7	>50	>50	>8.4
13.41	17.5	32.0	39.0	42.5	>50	39.2	>50	49.0	>50	>50	>2.9
13.42	20.9	>50	42.4	48.9	>50	48.6	>50	48.6	>50	>50	>2.4
13.43	45.6	>50	>50	>50	>50	>50	>50	45.1	>50	>50	>1.1
13.46	5.5	27.8	21.8	20.8	40.6	25.2	45.5	26.5	>50	32.4	>7.5
13.47	22.4	>50	33.5	33.6	26.0	>50	32.5	29.7	43.0	33.7	1.7
13.48	22.1	>50	32.2	36.3	29.8	>50	32.2	30.2	>50	43.2	>2.1
13.49	10.0	13.7	17.3	20.8	22.2	16.0	19.0	22.3	47.6	29.2	3.8

[a] The lowest concentration that kills 50 % of cells. The maximum standard deviation in cytotoxicity assays is typically 15 % of the average value. Compounds with IC_{50} > 50 μmol/L were considered inactive. [b] Selectivity index is calculated for IC_{50} of CCRF-CEM line vs. the average IC_{50} values of both fibroblasts (BJ and MRC-5). [c] Parent compound is used as a standard. [d] IC_{50} value was measured for a mixture of both *E*/*Z* isomers.

4.1.3 CuAAC reactions at the position C-30

The theoretical background of this project was described in Chapter 2.3 and in the review article:

Pokorný, J.; Borková, L.; Urban, M., Click Reactions in Chemistry of Triterpenes – Advances Towards Development of Potential Therapeutics, *Current Med. Chem.* **2018**, *25* (5), 636–658.

The project follows publications below (see Appendices IV, V):

Šidová, V.; Zoufalý, P.; **Pokorný, J.**; Džubák, P.; Hajdúch, M.; Popa, I.; Urban, M., Cytotoxic conjugates of betulinic acid and substituted triazoles prepared by Huisgen Cycloaddition from 30-azidoderivatives, *PLoS ONE* **2017**, 12, 2, e0171621.

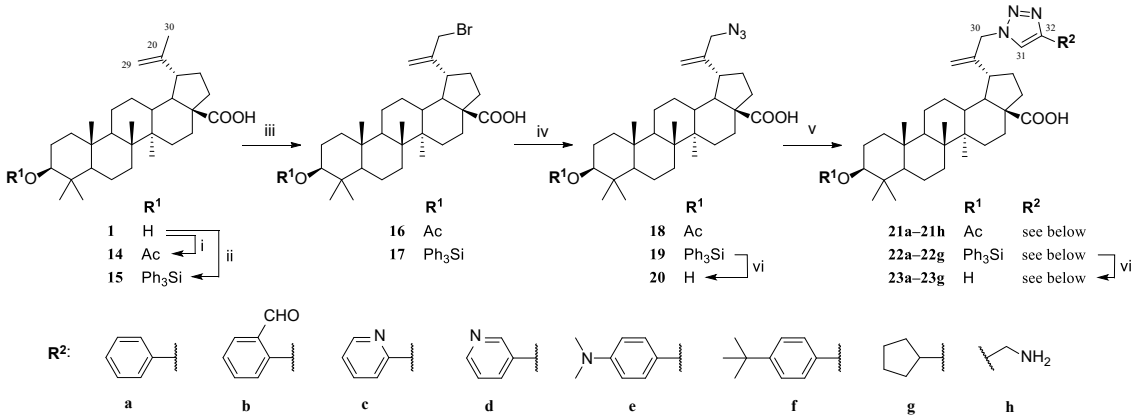
and

Pokorný, J.; Horka V.; Šidová V.; Urban M., Synthesis and characterization of new conjugates of betulin diacetate and bis(triphenysilyl) betulin with substituted triazoles, *Monatsh. Chem.* **2018**, *149* (4), 839–845.

In these two studies, three sets of betulinic acid derivatives with the free C-28 carboxyl group and variously protected/not protected C-3 hydroxyl group, followed by two sets of both OH-protected betulin derivatives were prepared using CuAAC leading to new conjugates, containing a variously substituted triazole ring at the position C-30. Most of the compounds in both series were synthetized by students under my supervision. All derivatives were then subjected to cytotoxicity testing against 8 tumor cell lines and 2 noncancer fibroblasts.

In the first study[321], betulinic acid (**1**, Scheme 23) was firstly protected by acetate (**14**) or triphenylsilyl group (**15**) at 3β-OH as the protection facilitated the solubility of the triterpenic substrate for subsequent bomination at the position C-30. This was done by a standard procedure using NBS as a bromination agent, AIBN as a source of radicals, and CCl_4 as a solvent. Bromoderivatives **16** and **17** were then treated with sodium azide in DMSO to yield corresponding 30-azidoderivatives **18** and **19**. The silylether **19** was deprotected by TBAF in THF to obtain the unprotected azide **20**. Final derivatives **21a**–**21h** and **22a**–**22g** with the protection at C-3 were prepared by CuAAC using alkynes **a**–**h** in moderate to high yields. An exception was the reaction with propargylamine (**h**) which was successful only in the reaction with the acetate **18** giving the product **21h** in a yield of 10 %; protection of the -NH_2 group of propargylamine by FMOC or BOC did not solve the problem. Unprotected derivatives **23a**–**23g** were then prepared from their silylated analogs using either TBAF in THF or HCl in DCM; both ways were possible giving moderate yields. Derivatives **23c** and **23d** were prepared also directly from the

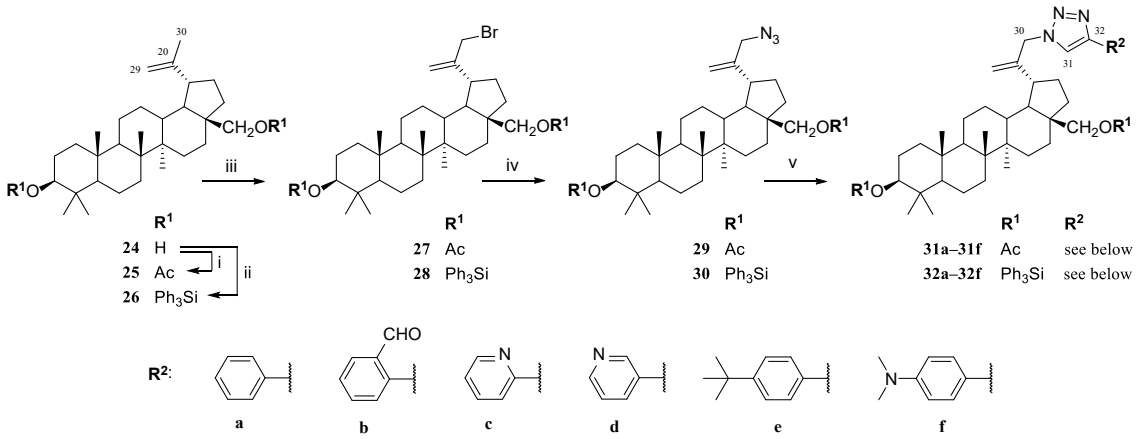
unprotected azide **20**. A total of 22 final molecules with a triazole ring were synthetized.



Scheme 23: Derivatives of betulinic acid (**1**) prepared by CuAAC reaction. Reagents and conditions: i) Ac_2O, pyridine, r. t., 16 h; ii) Ph_3SiCl, imidazole, DMF, r. t., 36 h; iii) NBS, AIBN, CCl_4, 75 °C for 1 h, then 50 °C for 3 h; iv) NaN_3, DMSO, r. t., 36 h; v) $CuSO_4 \cdot 5H_2O$, sodium L-ascorbate, alkynes **a**–**h**, DMF, r. t. or 50 °C; vi) TBAF, THF, r. t. 18–32 h, or HCl, DCM, r. t., 5–11 h.[321]

Cytotoxicity testing revealed that acetates **21a**–**21h** were highly active on multiple cancer cell lines with IC_{50} values in low micromolar ranges in contrast with their less active unprotected analogs **23a**–**23g** with the free 3β-OH, which we expected to have the potential highest activity. Moreover, the 3β-*O*-acetate precursors **16** and **18** showed significant cytotoxicity. The most active derivative **21b** with benzaldehyde connected to the triazole ring had $IC_{50} = 3.3\ \mu mol \cdot L^{-1}$ against the CCRF-CEM cell line. Results suggested that both the acetate and triazole probably positively influence the cellular uptake while the triazole may also become part of the pharmacophore.

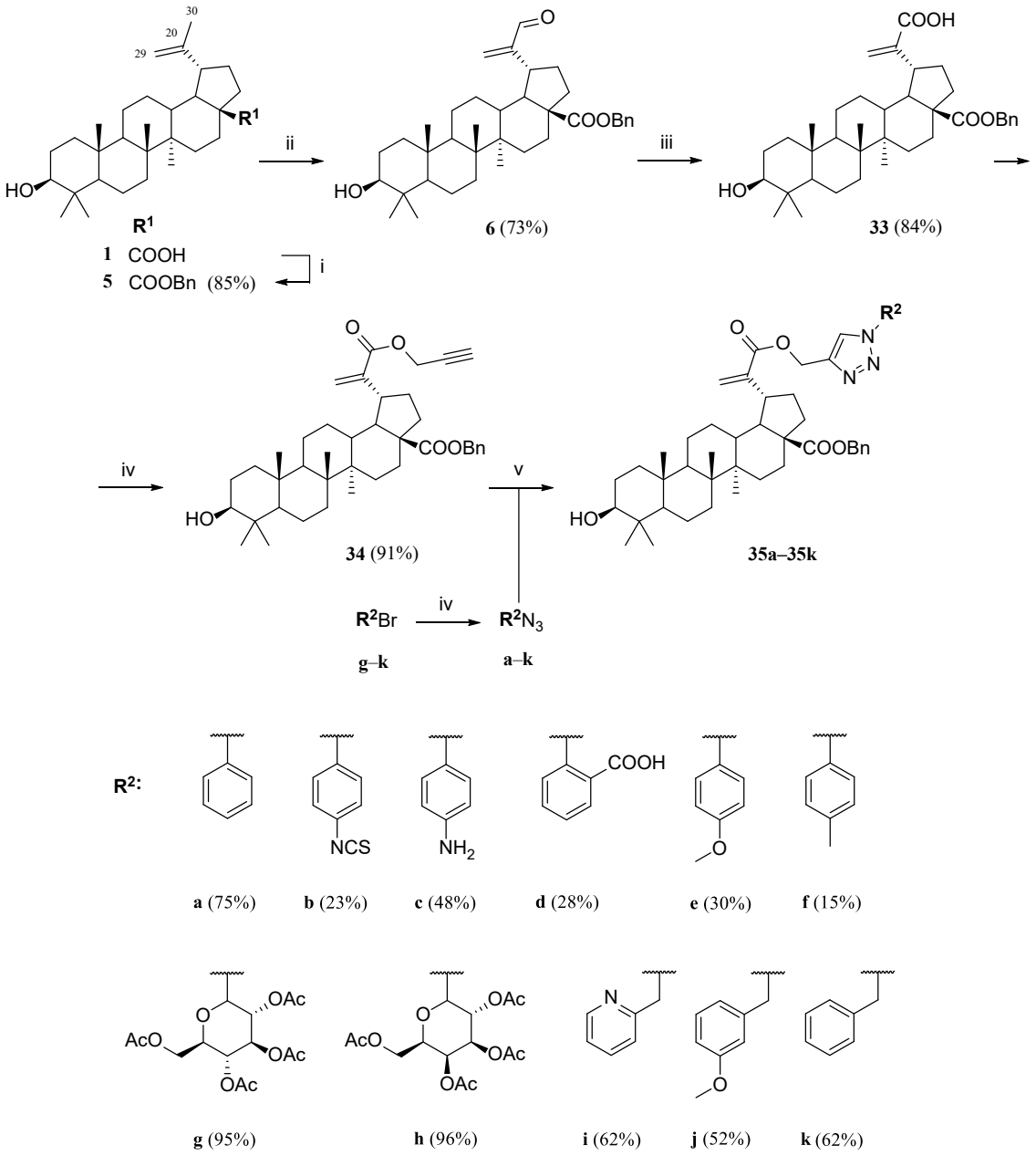
In the second article[260], similar two sets of betulin derivatives **31a**–**31f** and **32a**–**32f** (Scheme 24) were synthesized for the SAR study to extend the previously prepared library of betulinic acid derivatives. Using the same procedures as described above, betulin diacetate **25** and bis(triphenylsilyl)betulin **26** were prepared by the protection of betulin **24** to serve as starting materials for subsequent bromination and substitution to obtain protected azides **27** and **28**, respectively. Six alkynes **a**–**f** then reacted in CuAAC with corresponding azides to give twelve new final conjugates – diacetates **31a**–**31f** and bis(triphenylsilyl) derivatives **32a**–**32f** in moderate-high yields. Unfortunately, the solubility of the final compounds was not sufficient to perform cytotoxicity assays. In addition, attempts to deprotect the derivatives that could help to improve their solubility in water-based media used in biological tests were unsuccessful, and therefore only their synthesis was published, and the compounds were not further developed.



Scheme 24: Derivatives of betulin (**24**) prepared by CuAAC reaction. Reagents and conditions: i) Ac_2O, pyridine, r. t., 16 h; ii) Ph_3SiCl, imidazole, DMF, 50 °C, 36 h; iii) NBS, AIBN, CCl_4, reflux; iv) NaN_3, DMSO, r. t., 36 h; v) $CuSO_4 \cdot 5H_2O$, sodium L-ascorbate, alkynes **a**–**h**, *t*-BuOH/H_2O 2 : 1, 50 °C, 16 h.[260]

In order to further extend existing biological information from the previous SAR study in the articles above, a new set of triterpenic triazoles was prepared from betulinic acid (**1**) using CuAAC. In this project, the connection between the terpene and the triazole ring was represented by an ester function, which could be easily cleaved by nonspecific esterases in a cell, and therefore, such compound could possibly serve as a prodrug. Moreover, some compounds from previous studies with the same type of connection between the terpene and the triazole ring showed strong neuroprotective activity.[249] Therefore, this new set of derivatives will be evaluated in neurodegeneration models. The following text summarizes the results that have not yet been published.

Synthesis of the series of eleven compounds **35a**–**35k** (Scheme 25) started from betulinic acid (**1**), which was firstly protected at C-28 carboxyl function by benzyl ester to form benzyl betulinate (**5**, Scheme 25). Protection of this position was needed for the selective alkylation of the second carboxyl group at C-30, which was introduced into the molecule in two steps. First, oxidation of the allyl position of the isopropenyl moiety using SeO_2 in 2-methoxyethanol under reflux provided 30-oxobenzyl betulinate (**6**), which was further oxidized by Pinnick oxidation using $NaClO_2$, KH_2PO_4, and 2-methyl-2-butene in a mixture of *t*-BuOH/H_2O (1 : 1) to diacid **33** with 84% yield. Subsequently, alkylation of the compound **33** by propargyl bromide in THF afforded the propargyl ester **34** (91% yield) which served as a substrate for cycloaddition reactions. In this case, the alkyne was a part of the triterpenoid (**34**), and it reacted with variously substituted aromatic azides (**a**–**f**, commercially available), or azides prepared by substitution reaction (**g**–**k**) from corresponding bromides in DMF at r. t.



Scheme 25: CuAAC reactions on betulinic acid (**1**). Reagents and conditions: i) BnBr, K_2CO_3, THF, reflux, 3 h; ii) SeO_2, 2-methoxyethanol, 110 °C, 4 h; iii) $NaClO_2$, KH_2PO_4, 2-methyl-2-butene, *t*-BuOH/H_2O 1 : 1, r. t. 5 h; iv) propargyl bromide, K_2CO_3, THF, reflux, 4 h; v) corresponding azide, $CuSO_4 \cdot 5H_2O$, sodium ascorbate, *t*-BuOH/H_2O 2 : 1, 50 °C, 18 h; iv) NaN_3, DMF, r. t., overnight.

The first attempt of the cycloaddition reaction was done in DMF at r. t. using azidobenzene (**a**, 1.2 eq) and CuI as a source of copper (I) catalyst according to the literature.[322] The reaction was running overnight, and the next day, full conversion of the starting triterpene **34** was observed as four new spots on TLC. The main spot, considered as a potential product, was separated and purified using column chromatography. Subsequent NMR analysis revealed the desired product **35a** in a low yield of 33 %. To increase the yield, CuI as a catalyst was replaced by a commonly used combination – $CuSO_4 \cdot 5H_2O$ and sodium L-ascorbate (CuI

seemed to be of poor quality, possibly partially oxidized to CuI_2). The second reaction was done with 4-azidophenyl isothiocyanate (**b**, 1.2 equivalents) in DMF at r. t., but after 3 days, full conversion was still not achieved. The reaction afforded the triazole **35b** in a very low yield of 23 %. Therefore, a change in the used solvent was done and the next reactions were set up in a mixture of *t*-BuOH/H_2O in a ratio of 2 : 1 as described in the literature.[323] These reaction conditions were successfully applied in the reaction with azidobenzene (**a**, 1.5 eq), $CuSO_4 \cdot 5H_2O$ (0.4 eq), and sodium ascorbate (0.8 eq). Full conversion of the starting material **34** without any sideproducts was achieved in 18 h at 65 °C, and after purification, the product **35a** was obtained in a 75% yield, indicating significant improvement compared to the first case. Having better reaction conditions, the reaction with 4-azidophenyl isothiocyanate (**b**, 1.5 eq) was repeated at 65 °C overnight, unfortunately, the next day only a partial conversion of the starting propargyl ester **34** was observed (ca 20 % of the material remained unreacted, visual analysis from TLC). After purification on the column chromatography, the yield of the reaction reached 23 % as in the previous case. Such a low yield may indicate poor quality of the azide, which was purchased and stored for a longer time (5 months) in a freezer. Unfortunately, similar bad experiences with commercially available azides were observed in further experiments (**d**–**f**), where low yields were always achieved. Triazole **35c** was prepared from 4-azidoaniline hydrochloride (**c**) with a moderate yield of 48 %, while the conversion of the starting terpene **34** was not complete. A similar situation was observed for 2-azidobenzoic acid (**d**) where completion of the reaction was achieved by an excess of the azide and sodium L-ascorbate. The low yield of 28 % was caused by a sideproduct (significant unknown spot on TLC) and difficult purification, since the product was very polar, sitting almost at the start of TLC in the solvent mixture DCM/MeOH 10 : 1. The derivative **35d** was finally purified using the Auto-Purification LC-MS instrument (Waters). For the derivatives **35e** and **35f**, fresh azides were bought and used immediately after delivery. Unfortunately, a conversion of only about 40 % (visual TLC analysis) was reached for both cases, leading to small yields of 30 % for **35e**, and only 15 % for **35f**. During the reactions, 0.5 eq of each azide, and 0.2 eq of sodium L-ascorbate were added into the reaction mixture to a total of 2 equivalents of azide, unfortunately, it did not help to complete the reactions; instead, an unknown polar sideproduct was formed. These two reactions were repeated with the same unsatisfactory results. Since the amount of the products was sufficient for the biological tests, the yields were not further optimized. To do so in the future, it would be desirable to prepare fresh azides and to use them immediately in the CuAAC.

For another five derivatives, fresh azides **g**–**k** were prepared from corresponding bromides by a nucleophilic substitution using sodium azide in DMF at r. t. Acetobromo-α-D-glucose (**g**) and acetobromo-α-D-galactose (**h**) were treated with NaN_3

overnight, next day, the products were extracted and purified on the column chromatography, affording white crystalline azide **g** of acetylated glucose (83% yield), and colorless oily substance of acetylated galactose. CuAAC reactions provided derivatives **35g** in excellent yield 95 %, and **35h** with a similar yield of 96 %. It is worth mentioning that these two azides were freshly prepared and pure (after column chromatography), which resulted in great yields of cycloaddition products without any sideproducts or problems with conversion of the starting triterpene. The pyridine derivative **35i** was prepared using 2-(bromomethyl)pyridine hydrobromide, which was converted to corresponding azide (yellow oily substance) that was immediately used in the cycloaddition reaction without purification. Conversion of the starting material was not complete (ca 30 % of the mass remained unreacted, visual TLC analysis), however, the yield of the reaction reached 62 % after purification of the product **35i**. Derivatives **35j** and **35k** were prepared in a similar manner from corresponding bromides, which were transformed to azides (**j** and **k**) and used subsequently without purification. Moderate yields of 52 % for **35j**, and 62 % for **35k** were achieved due to ca 60% conversion of the starting terpene only.

It can be speculated that the purity of the azide significantly affects the yield of the cycloaddition. Only moderate–low yields were achieved with commercially available azides, while fresh purified azides (**g**, **h**) afforded products in almost quantitative yields with 100% conversion of the starting material as is typical for the click chemistry. This fact is supported by the observations of our reactions with azides, which were freshly prepared but not purified (**i**–**k**), and then gave only medium yields of about 60%, while the conversion was still not complete. Moreover, the addition of the azide and other reactants did not always help to complete the reaction, instead, unknown sideproducts were formed in minor amounts. Thus, the use of CuAAC in this case cannot be considered as the typical example of click chemistry due to lower yields, longer reaction times and higher reaction temperatures.

Structures of the final derivatives **35a**–**35k** were confirmed by their characteristic signals in ^{1}H NMR spectra:

- five singlets in the range of 0.65–0.95 ppm, all with integrals 3, were assigned to five methyl groups at the lupane skeleton,
- doublet of doublets around 3.15 ppm with integral 1 and the coupling constants about 11 Hz and 4.8 Hz was assigned to H-3α,
- a triplet of doublets around 3.30 ppm with integral 1 and the coupling constants about 11 Hz and 4.9 Hz was assigned to H-19β,
- two doublets around 5.08 ppm and 5.14 ppm with integrals 1 and the coupling constants about 12.3 Hz, and a multiplet in the range of 7.27–7.38 ppm with integral 5 were assigned to the benzyl group,

- two doublets around 5.30 ppm and 5.40 ppm with integrals 1 and coupling constants about 12.7 Hz were assigned to –CH_2– ester bridge to the triazole ring,
- two singlets around 5.5 ppm and 6.0 ppm with integrals 1 were assigned to the protons H-29a and H-29b,
- one singlet in the range of 7.7–8.0 ppm with integral 1 was assigned to the proton on the triazole ring,
- three multiplets around 7.73 ppm (integral 2), 7.51 ppm (integral 2), and 7.43 ppm (integral 1) were assigned to the aromatic substituent **a**, attached to the triazole ring,
- two multiplets around 7.76 ppm and 7.35 ppm, both with integral 2, were assigned to the aromatic substituent **b** (4-isothiocyanate), attached to the triazole ring,
- two multiplets around 7.46 ppm and 6.75 ppm, both with integral 2, were assigned to the aromatic substituent **c** (4-amino), attached to the triazole ring,
- three multiplets around 7.85 ppm (integral 1), 7.64 ppm (integral 2), and 7.55 ppm (integral 1) were assigned to the aromatic substituent **d** (2-carboxy), attached to the triazole ring,
- two multiplets around 7.63 ppm and 7.01 ppm, both with integral 2, and a singlet at 3.86 ppm with integral 3, all were assigned to the aromatic substituent **e** (4-methoxy), attached to the triazole ring,
- multiplet in the range of 7.27–7.43 ppm with integral 4, and a singlet at 2.22 ppm with integral 3 were assigned to the aromatic substituent **f** (4-methyl), attached to the triazole ring,
- four singlets at 1.85 ppm, 2.02 ppm, 2.06 ppm, and 2.07 ppm, each with integral 3 (4 × acetate group), and a doublet at 5.87 ppm with integral 1, multiplet at 5.42 ppm with integral 2, multiplet at 5.23 ppm with integral 1, doublet of doublets at 4.30 ppm with integral 1 and coupling constants 12.7 Hz and 4.9 Hz, multiplet at 4.13 ppm with integral 1, and multiplet at 3.99 ppm with integral 1, all were assigned to the substituent **g** (acetylated glucose), attached to the triazole ring,
- four singlets at 1.87 ppm, 2.01 ppm, 2.04 ppm, and 2.22 ppm, each with integral 3 (4 × acetate group), doublet at 5.83 ppm with integral 1, multiplet in the range of 5.53–8.58 ppm with integral 2, doublet of a doublet at 5.24 with integral 1 and coupling constants 10.3 Hz and 3.3 Hz, and multiplet in the range of 4.10–4.23 ppm with integral 3, all were assigned to the substituent **h** (acetylated galactose), attached to the triazole ring,
- multiplet around 8.57 ppm with integral 1, multiplet around 7.67 ppm with integral 1, multiplet around 7.24 ppm with integral 1, and multiplet around 7.19 ppm with integral 1 (4 × H of pyridine), one singlet at 5.63 ppm with integral 2 (the –CH_2– bridge between pyridine and triazole ring), all were assigned to the substituent **i** (pyridine), attached to the triazole ring,

- multiplet around 7.27 ppm with an integral 1, multiplet around 6.86 ppm with an integral 1, multiplet around 6.83 with an integral 1, and multiplet around 6.78 ppm with an integral 1 (4 × H of aromatic ring), singlet at 5.47 ppm with an integral 2 (the –CH_2– bridge between pyridine and triazole ring), and a singlet at 3.76 ppm with an integral 3 (methoxy group), all were assigned to the aromatic substituent **j** (3-methoxy), attached to the triazole ring,
- multiplet in the range of 7.3–7.4 ppm with an integral 3, multiplet around 7.27 ppm with an integral 2, and a singlet at 5.51 ppm with an integral 2, all were assigned to the benzyl group **k**, attached to the triazole ring.

Biology

Prepared triazole derivatives **35a–35k** were tested for their cytotoxic activity *in vitro* using standard MTS assay against a panel of eight cancer cell lines (CCRF-CEM, CEM-DNR, HCT116, HCT116 $p53^{-/-}$, K562, K562-TAX, A549, U2OS), and two human normal fibroblasts (BJ, MRC-5). Results of the IC_{50} values are displayed in Tab. 3. Derivatives are considered inactive when $IC_{50} > 50$ µmol · L^{-1}.

Tab. 3: Cytotoxic activities of prepared triazole derivatives **35a**–**35k** in eight tumor and two normal fibroblast cell lines. The most active derivative **35i** is highlighted.

Comp.	IC_{50} (µmol · L^{-1})[a]										
	CCRF-CEM	CEM-DNR	HCT 116	HCT116 $p53^{-/-}$	K562	K562-TAX	A549	U2OS	BJ	MRC-5	SI[b]
1[c]	8.1	14.0	15.8	16.0	**4.3**	14.1	9.4	20.8	24.2	28.2	3.2
35a	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	-
35b	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	-
35c	30.4	>50	>50	49.3	>50	14.9	>50	15.2	>50	>50	>1.6
35d	14.6	29.8	29.5	28.8	27.1	21.7	29.1	23.2	29.3	25.7	1.9
35e	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	-
35f	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	-
35g	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	-
35h	44.1	>50	>50	>50	>50	>50	>50	>50	>50	>50	>1.1
35i	5.9	23.9	>50	>50	>50	8.7	>50	>50	>50	>50	>8.5
35j	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	-
35k	19.6	>50	>50	>50	>50	42.3	>50	>50	>50	>50	>2.6

[a] The lowest concentration that kills 50 % of cells. The maximum standard deviation in cytotoxicity assays is typically 15% of the average value. [b] Selectivity index is calculated for the IC_{50} of the CCRF-CEM line vs. average IC_{50} values of both fibroblasts (BJ and MRC-5). [c] Parent compound is used as a standard.

Results showed that only five derivatives **35c**, **35d**, **35h**, **35i**, and **35k** were active, however, this was expected due to the protection of the C-28 carboxyl group. Among them, compound **35d** showed general cytotoxicity in all tested cell lines in the moderate IC_{50} range of 14.6–29.8 µmol · L^{-1}. The rest of the active compounds

showed selective cytotoxicity against CCRF-CEM and K562-TAX cell lines, while they were inactive in human normal fibroblasts. The best candidate, derivative **35i**, was the most active in a low micromolar range in CCRF-CEM (IC_{50} = 5.9 $\mu mol \cdot L^{-1}$) and K562-TAX (IC_{50} = 8.7 $\mu mol \cdot L^{-1}$) cell lines with high selectivity (the selectivity index SI > 8.5), meaning significant improvement in comparison with the parent compound, betulinic acid **1**, which suffer from general toxicity (SI = 3.2). Cell cycle analysis and nucleic acid synthesis in the CCRF-CEM cell lines were evaluated for the compound **35i**, results are summarized in Tab. 4.

Tab. 4: Cell cycle analysis and influence on DNA and RNA synthesis in CCRF-CEM cancer cells treated with 1 × IC_{50} and 5 × IC_{50} of compound **35i**.

		Used conc. ($\mu mol \cdot L^{-1}$)	**Sub G1** (%)	**G0/G1** (%)	**S** (%)	**G2/M** (%)	**pH3^{Ser10}** (%)	**DNA synthesis** (%)	**RNA synthesis** (%)
Control		0	2.22	40.73	41.22	18.06	1.92	36.39	32.41
35i	1 × IC_{50}	5.86	6.52	38.84	41.28	19.89	1.72	32.07	19.48
	5 × IC_{50}	29.30	9.75	44.77	38.44	16.80	1.09	27.87	23.91

The influence of the compound **35i** on cell cycle and DNA/RNA synthesis is not significant, however, inhibition of DNA and RNA synthesis is obvious at 5 × IC_{50} concentration. Compound **35i** led to the accumulation of the cells in the G0/G1 cell cycle phase at 5 × IC_{50}.

4.2 Modifications of the double bonds at the A-ring

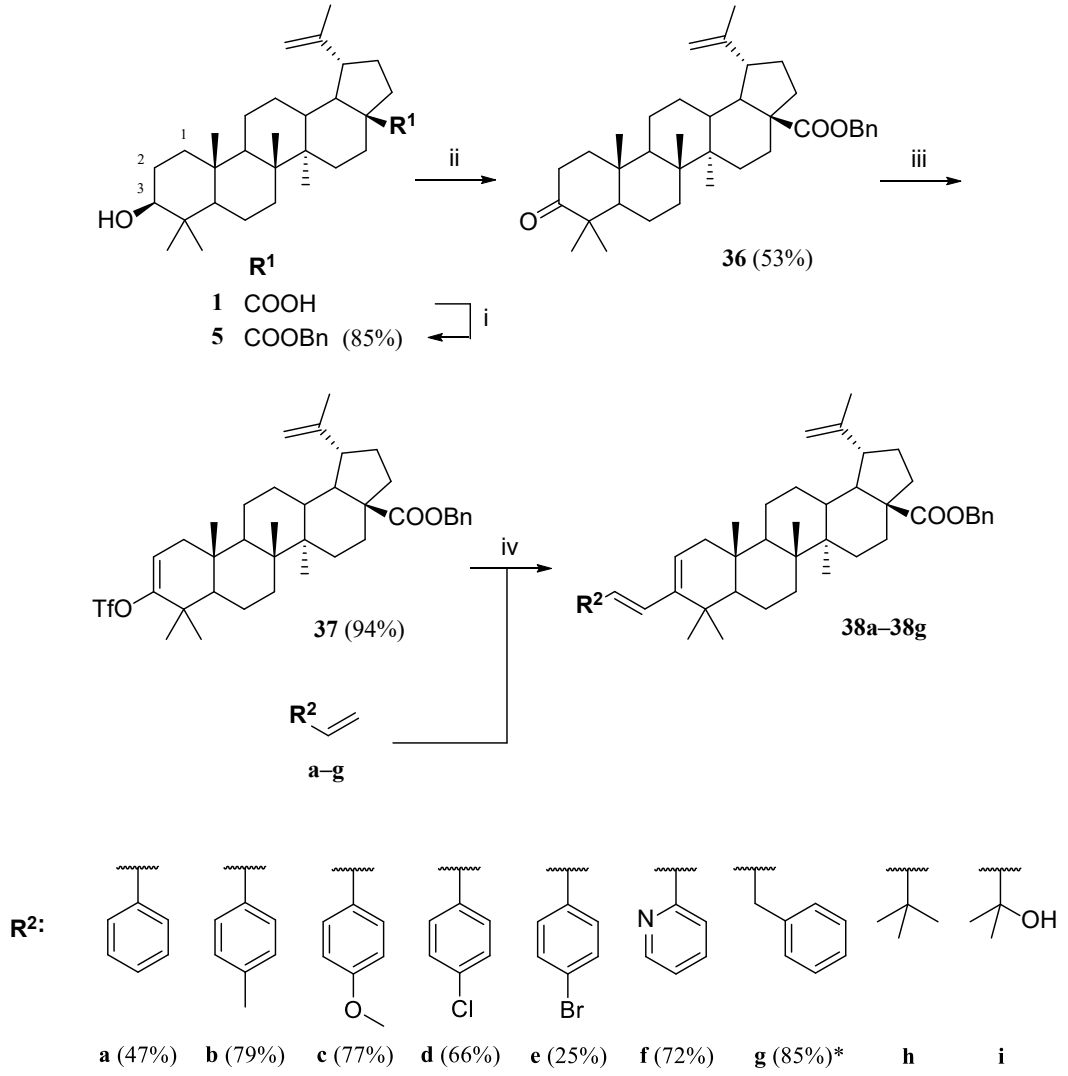
Unsuccessful attempts to derivatize the isopropenyl unit by the Heck reaction were performed in the study of Zhu et al.[110] with very low yields, problems with conversion, stereoselectivity, and a special $Fe(OTf)_3$ catalyst (see Fig. 13, p. 21). We decided to verify the standard Heck procedure in a less hindered position of the lupane skeleton. From the literature,[191–194] it is known that the Suzuki-Miyaura cross-coupling reaction works at the position C-3 at the A-ring. Therefore, this approach was chosen to determine if the Heck reaction is suitable for an introduction of a new double bond into the molecule next to the A-ring. This work was focused on the scope and limitations of this reaction since it is promising for the potential synthesis of numerous new compounds in the future.

4.2.1 Exploration of the Heck reaction at the position C-3

For the exploration of the Heck reaction at the position C-3 of betulinic acid (**1**), the carboxyl group at C-28 was firstly protected by benzyl ester in the same way as described in the projects above, resulting in benzyl betulinate (**5**, Scheme 26). Then, oxidation of the 3β-OH group was done using $Na_2Cr_2O_7 \cdot 2H_2O$ in acidic conditions at r. t. in 18 h, resulting in benzyl betulonate (**36**, 53% yield). The synthesis of the triflate **37** was performed according to the literature[193] with an excellent yield of 94 %.

Having the starting triterpene substrate for the Heck reaction, searching for the optimal reaction conditions started with styrene (**a**, 200 mol %), palladium acetate as a Pd catalyst (15 mol %), together with an addition of triphenylphosphine (30 mol %), K_2CO_3 as a base (200 mol %), DMF as a solvent, and reaction temperature at 70 °C. After several test reactions on a small scale, the reaction seemed to proceed with one major and some minor products according to the TLC analysis, indicating a potential Heck product. Therefore, 50 mg of the starting triflate **37** was used, the reaction was monitored by TLC, and after 4 h at 70 °C, full consumption of the starting terpene **37** was observed. Then, extraction and purification of the major product by column chromatography afforded 20 mg of a white compound in a yield of 43 %. Subsequent ^{1}H NMR analysis proved the structure of the product **38a** containing a new double bond, which was identified as two doublets present at 6.80 ppm and 6.69 ppm with coupling constants 15.8 Hz, indicating unique *E* stereoselectivity of the new double bond as is typical for the Heck reaction.

In the next reaction of **37** with styrene (**a**), a different catalyst was tried – $PdCl_2(PPh_3)_2$ with triphenylphosphine ligands and higher reaction temperature at 85 °C led to 50% conversion of the starting triterpene only (visual TLC analysis), meaning no improvement.



Scheme 26: Heck reactions at the position C-3. Reagents and conditions: i) BnBr, K_2CO_3, THF, reflux, 3 h; ii) $Na_2Cr_2O_7 \cdot 2H_2O$, AcOH, Ac_2O, $NaOAc \cdot 3H_2O$, r. t., 18 h; iii) Tf_2NPh, KHMDS, THF, −78 °C, 4 h; iv) styrene **a**–**f** or allylbenzene **g**, PPh_3, $Pd(OAc)_2$, piperidine, DMF, 85 °C, 1–4 h. *The product **38g** was obtained as a mixture of *E* and *Z* isomers.

The reaction using palladium acetate was further optimized using various bases. Triethylamine was employed as K_2CO_3 was not sufficiently soluble in DMF. However, it did not help to increase the yield nor reduce the amount of formed side-products, moreover, the conversion was still not complete. For the next experiment with allylbenzene (**g**), which was chosen to check the scope of the reaction with a different double bond substrate, piperidine was used in the same concentration of 200 mol %. Surprisingly, the reaction was finished in 1 h at 85 °C, providing exclusively one spot on TLC with 100% conversion. Subsequent extraction followed

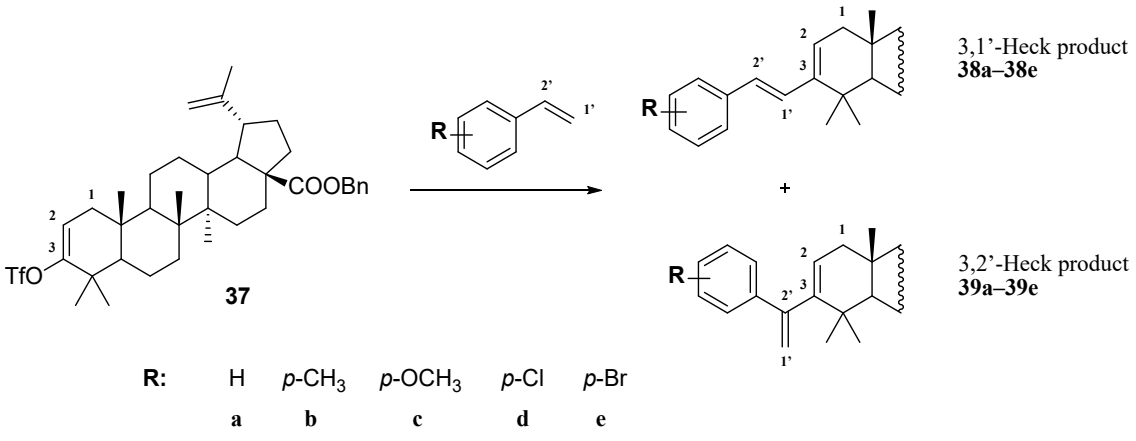
by purification of the product afforded the derivative **38g** in a yield of 85 %, meaning great improvement. However, the analysis of ^{1}H NMR spectra revealed a mixture of *E*/*Z* isomers in a ratio of 1.4 : 1, but the assignment of the proton signals to the respective isomers was not performed since it was impossible to separate the mixture of isomers for the performance of 2D-NMR spectra that would assign the E/Z isomers unambiguously. Disruption of the conjugation between the double bond and the aromatic ring probably resulted in low stereoselectivity leading to a formation of a mixture of isomers. This idea is supported by the fact that in all other reactions with styrene-based substituents only *E* isomer was always found. Nevertheless, it was revealed by this experiment that piperidine is the best choice for these reactions in comparison with Et_3N and K_2CO_3, giving full conversion of the starting triterpene in 1–4 h with a minimum amount of sideproducts.

The reaction with styrene (**a**) was therefore set up again at 85 °C using piperidine as a base, 15 mol % of $Pd(OAc)_2$, and 60 mol % of PPh_3, affording the product **38a** in 1 h with a yield of 47 % after purification. Compared to the first experiment, the yield was increased by only 3 %, but in further reactions with substituted styrenes (**b**–**d**) and 2-vinylpyridine (**f**), high yields of about 70 % were easily achieved together with 100% conversion of the starting material **37** under these conditions. In addition, it was found that the correct course of the reaction can be visually checked by the change in color of the reaction mixture. When it is done correctly, the initially yellow reaction mixture turns orange when heated, indicating the ongoing Heck process, then, when finished, it turns black (probably due to the aggregation of Pd^0 meaning the end of the catalytic cycle). In the case of 4-bromostyrene (**e**), only a partial conversion of about 50 % was observed, leading to a low yield of 25 % after column chromatography. The reaction has not been repeated, however, such a low yield could be caused due to a faulty reaction or by the fact that according to general Scheme 17, 4-bromostyrene (**e**) could react with itself to form undesired side-product. To verify this and increase the yield of the product **38e**, it would be desirable to add the 4-bromostyrene (**e**) to the reaction gradually.

Furthermore, aliphatic olefines were evaluated in small-scale test reactions with 3,3-dimethylbut-1-ene (**h**) and 2-methyl-3-buten-2-ol (**i**) to determine the scope of the reaction conditions. In the first case, full conversion of the triflate **37** was observed in four spots on TLC. The main product was separated by column chromatography, yielding 5 mg of a white compound (19 %). ^{1}H NMR analysis revealed a complex mixture of several products that were complicated to interpret; however, the desired Heck product was distinguishable. In the latter reaction with olefin **i**, full consumption of the triflate **37** was observed again, resulting in one polar spot and some decomposed mass on the TLC start. In this case, so little product was formed (visual TLC analysis) that it was not further isolated.

During NMR analyses, it was found out that in some cases different Heck product (**39a**–**39e**, Scheme 27) was formed in a smaller amount. At first, it seemed like

an impurity after imperfect chromatography, but then it was found that the same peaks appeared in other spectra. The possible result of the reaction is shown in Scheme 27, where the major 3,1'-regioisomer was formed, but also the 3,2'-product was observed. An enlarged area of the ^{1}H NMR analysis of the product **38b** is shown in Fig. 50, where two doublets at 6.76 ppm and 6.68 ppm with a coupling constant of 15.8 Hz were assigned to the protons H-1' and H-2' of the double bond of the 3,1'-regioisomer **38b**, together with a multiplet of the proton H-2 in the range of 5.87–5.81 ppm. Little upfield, the smaller multiplet in the range of 5.49–5.46 ppm was assigned to the H-2 proton of the 3,2'-regioisomer **39b**, together with two doublets at 5.33 ppm and 4.97 ppm with a coupling constant of 2.3 Hz, indicating geminal protons at H-1'. Two doublets at 5.18 ppm and 5.12 ppm were assigned to the $-CH_2-$ bridge of the benzyl ester protecting group. From Fig. 50, it is obvious that the integral intensity of the main 3,1'-product compared with the 3,2'-product is in the ratio of about 7 : 1, meaning that 13 % of the mass is a different compound, which was not directly visible as another spot on TLC, as it has almost identical R_f. Similar results were observed for **38c**, where almost 15 % of the mass was related to the 3,2'-regioisomer **39c**, as well as for **38d**. Smaller amounts of the 3,2'-Heck products were found for **38e** (compound **39e**, 11 %), and for **38a** (compound **39a**, 5 %). Compound **38f** was isolated as a pure 3,1'-Heck product. From this point of view, it is clear that there is still a lot of space to optimize the stereoselectivity of the reaction, which was found to be unspecific under these conditions. This will be the subject of further study, where different ligands will be used.



Scheme 27: Two regioisomers 3,1' and 3,2' as products of the Heck reaction.
Reagents and conditions: substituted styrene, PPh_3, $Pd(OAc)_2$, piperidine, DMF, 85 °C, 1–4 h.

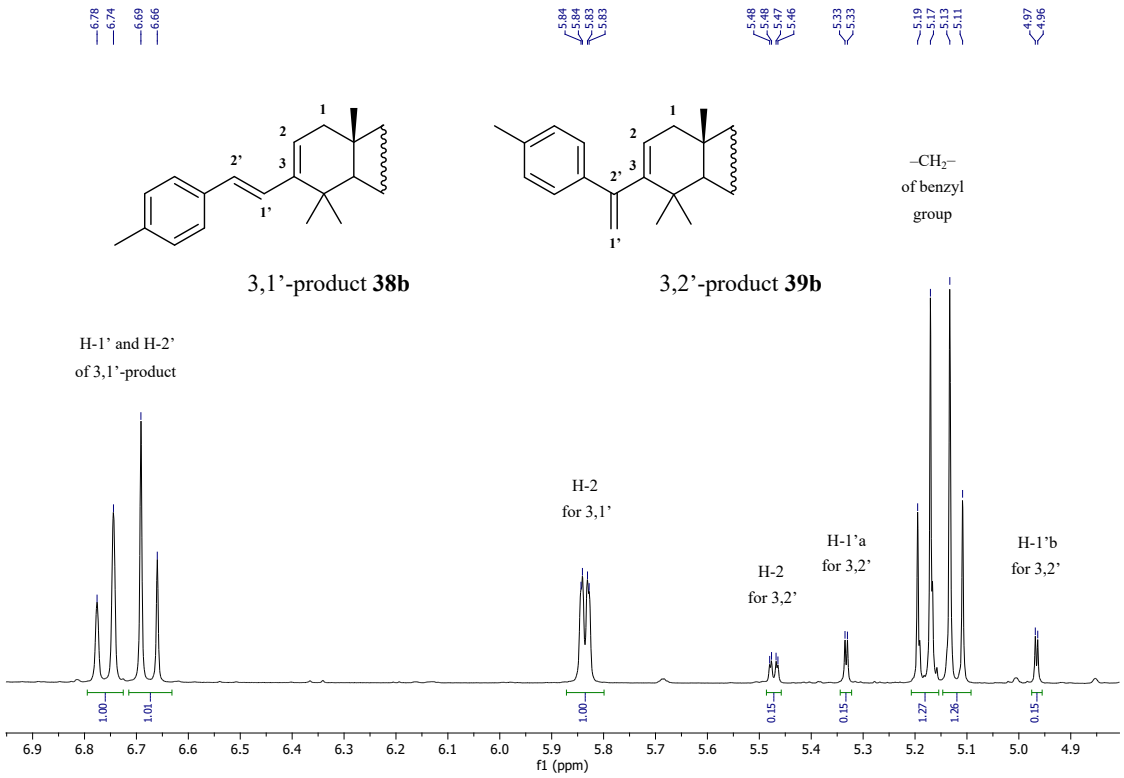


Fig. 50: An enlarged region of ^{1}H NMR spectra of the derivative **38b**.

Structures of the derivatives **38a**–**38g** were confirmed by their characteristic signals in ^{1}H NMR spectra:

- five singlets in the range of 0.80–1.15 ppm, all with integrals 3, were assigned to five methyl groups at the lupane skeleton,
- one singlet around 1.70 ppm with an integral 3 was assigned to the C-30 methyl group,
- a triplet of doublet around 3.05 ppm with an integral 1 and the coupling constants about 10.9 Hz and 4.7 Hz was assigned to H-19β,
- two singlets around 4.75 ppm and 4.60 ppm with integrals 1 were assigned to the protons H-29a and H-29b,
- two doublets around 5.17 ppm and 5.10 ppm with integrals 1 and the coupling constant about 12.3 Hz, and a multiplet in the range of 7.27–7.42 ppm with an integral 5 were assigned to the benzyl group,
- a multiplet in the range of 5.80–5.90 ppm with an integral 1 was assigned to the proton H-2,
- two doublets around 6.70 ppm and 6.80 ppm with integrals 1 and coupling constant 15.8 Hz were assigned to the protons of the introduced double bond,
- a multiplet in the range of 7.15–7.40 ppm with an integral 10 was assigned to two aromatic rings – the benzyl group and the substituent **a**,

- a singlet at 2.35 ppm with an integral 3, together with two multiplets around 7.12 ppm and 7.31 ppm with integrals 2 were assigned to the substituent **b** (4-methyl),
- a singlet at 3.81 ppm with an integral 3, together with two multiplets around 6.86 ppm and 7.34 ppm with integrals 2 were assigned to the substituent **c** (4-methoxy),
- two multiplets around 7.26 ppm and 7.32 ppm, each with an integral 2 were assigned to the substituent **d** (4-chloro),
- two multiplets around 7.25 ppm and 7.41 ppm, each with an integral 2 were assigned to the substituent **e** (4-bromo),
- four multiplets around 5.95 ppm, 7.08 ppm, 7.60 ppm, and 8.54 ppm, each with an integral 1 were assigned to the pyridine substituent **f**,
- a multiplet in the range of 7.15–7.39 ppm with an integral 10 was assigned to two aromatic rings – the benzyl group and the substituent **g**, together with a doublet at 2.89 ppm with an integral 1 and coupling constant 6.6 Hz.

5 Conclusions and future directions

The main aim of this Ph.D. thesis was to synthesize new derivatives of betulinic acid (**1**) with anticancer activity. Within the thesis, a theoretical review focused on modifications of the lupane-type triterpenoids containing double bonds with an emphasis on the biological activity of these compounds was performed. Synthesis, purification, and characterization of new derivatives were done according to the set goals:

1) The low selectivity of the 30-oxobetulinic acid (**2**) was successfully solved by the C-30 isosteric replacement approach:

   A. A small set of ten new highly cytotoxic triterpenic azines with the free carboxyl group **4a**–**4e**, and protected derivatives **7a**–**7e** (Scheme 21) was prepared in the first project, which was focused on masking of the Michael acceptor of the parent aldehyde **2** by condensation reactions with variously substituted hydrazones. The nonprotected compounds **4a**–**4e** showed great selectivity towards the CCRF-CEM cancer cell line while toxicity decreased significantly towards other cell lines including nonmalignant fibroblasts, meaning that the goal was achieved. The most active derivative **4d** had comparable cytotoxic activity to the parent 30-oxobetulinic acid (**2**). However, poor stability was the biggest drawback of this class of derivatives, together with low yields and sideproducts. This work was published in 2018 in the Future Medicinal Chemistry Journal (Appendix IV).

   B. A large library of forty new triterpenoid dienes **13.1**–**13.51** (Scheme 22) was prepared from the same substrate **2** using Wittig reactions. For this purpose, a wide palette of variously substituted triphenylphosphonium salts **12.1**–**12.51** (Fig. 48) was prepared. The main interest was focused on the improvement of inappropriate properties of previously prepared azines. An isosteric replacement of C=N by C=C bond yielded stable compounds which showed comparable anticancer activities and high selectivity compared to azines. Therefore, the lack of selectivity of the parent compound, 30-oxobetulinic acid (**2**), was diminished and stability was achieved. The most active derivatives were further analyzed for DNA and RNA synthesis,

cell cycle, protein expression, and pharmacological parameters to find out the mechanism of action. The compounds cause selective cell apoptosis particularly by the mitochondrial pathway. The derivative **13.22** was considered a potential candidate for further preclinical development. The work was published in 2021 in the European Journal of Medicinal Chemistry (Appendix V). In this article, ten derivatives were prepared by MSc. Denisa Olejníková under my supervision.

2) Copper(I)-catalyzed azide-alkyne cycloadditions were employed in the project focusing on the preparation of triterpenoid conjugates with a triazole ring. In previous studies, it was found that 3β-*O*-acetyl derivatives of betulinic acid (**1**) with the free C-28 carboxyl group (**21a**–**21h**, Scheme 23) were more active than their nonprotected analogs (**23a**–**23g**). Unfortunately, derivatives of betulin (**24**) with protection on both -OH groups (**31a**–**31f**, and **32a**–**32f**, Scheme 24) were not sufficiently soluble to perform cytotoxicity assays. Within my work, eleven new compounds **35a**–**35k** (Scheme 25) were prepared, fully characterized, and tested *in vitro* for their cytotoxic activity. The best derivative **35i** showed higher cytotoxicity against the CCRF-CEM cell line than the parent compound, betulinic acid (**1**); however, most of the compounds were inactive. In contrast, these compounds may be effective as neurodegenerative agents, which will be verified in further studies.

3) The Heck reaction was introduced to the chemistry of triterpenes in the last project focused on the exploration of possibilities to use this cross-coupling reaction for a modification at the A-ring of betulinic acid (**1**). Seven new derivatives **38a**–**38g** (Scheme 26) containing a new double bond have been prepared to date, however, further investigation to optimize the regioisomers formation is needed. This could be solved particularly by a change in the used ligand since appropriately selected ligands can sterically block the formation of unwanted isomers. Reducing the amount of catalyst used in the reaction will be also further optimized. This project will be followed up with a different protecting group to obtain final derivatives with the free C-28 carboxyl group which is desirable for higher cytotoxic activity.

In conclusion, four different synthetic targets dealing with modifications of betulinic acid (**1**) are part of this Ph.D. thesis. In summary, 68 new final triterpenoid compounds were synthesized, fully characterized by physical and spectral data, and most of them were tested for cytotoxic activity against eight cancer cell lines and two normal fibroblasts, providing valuable SAR information. Some of the most promising derivatives were further evaluated in the following biological assays. Cytotoxicity tests and biological assays were performed by other researchers at IMTM (Soňa Gurská, Ivo Frydrych, Barbora Lišková).

Future directions:

- The isosteric replacement at the position C-30 was successful in the improvement of selectivity of new compounds. This approach may be generally used in future projects.
- Newly synthesized dienes opened the triterpenoid skeleton for many new transformations – dienes are good substrates for Diels-Alder reactions which are already successfully investigated in our research group.
- The Heck reaction may be used for the preparation of a number of new compounds. In order to use these compounds in biology, it is necessary to find better protecting groups for the position C-28 that can be easily deprotected.

6 Experimental part

6.1 Materials and instruments

NMR measurements

All ^{1}H and ^{13}C NMR experiments were recorded at either 500 MHz (Jeol JNM-ECX-500) or 400 MHz (Jeol JNM-ECA400II) for ^{1}H NMR, and 126 MHz or 100 MHz for ^{13}C NMR, respectively, at 20 °C in $CDCl_3$, CD_3OD, or DMSO-d_6. Chemical shifts (δ ppm) are reported relative to the residual solvent peak (for $CDCl_3$ δH = 7.26 ppm, δC = 77.16 ppm; for CD_3OD δH = 3.31 ppm, δC = 49.00 ppm; for DMSO-d_6 δH = 2.50 ppm, δC = 39.52 ppm). Coupling constants J are reported in Hz. NMR spectra were processed in MestReNova 6.0.2-5475 and JEOL Delta 5.0.5.1 software.

HRMS analysis

HRMS analysis was performed using LC chromatograph Dionex UltiMate 3000 (Thermo Fischer Scientific, MA, USA) and mass spectrometer Exactive Plus Orbitrap high-resolution (Thermo Fischer Scientific, MA, USA). Source of ionization: electrospray or APCI. Spectra: positive and negative mode in a range of 400–700 m/z. Chromatographic separation: column Phenomenex Gemini (C18, 50 × 2 mm, 3 µm particle), isocratic elution, mobile phases: 80% ACN and 20% buffer (0.01 mol · L^{-1} ammonium acetate) or 95% MeOH + 5% water + 0.1% HCOOH. Sample preparation: dissolution in MeOH. Spectra were processed in the Thermo Scientific Xcalibur 4.1.31.9 software.

Melting point measurements

Melting points were determined using the Stuart Melting Point Apparatus SMP30 and are uncorrected.

Specific rotations

Specific rotations were measured in THF solutions at 22 °C using an Atago POL-1/2 instrument, sample concentrations (c) are given in grams per 100 mL.

IR measurements

IR spectra were recorded on a Nicolet Avatar 370 FTIR and processed in the OMNIC 9.8.372 software. DRIFT stands for Diffuse Reflectance Infrared Fourier Transform.

TLC analysis

TLC was carried out on Kieselgel 60 F_{254} plates (Merck) detected first by UV light (254 nm) and then by spraying with 10% aqueous H_2SO_4 and heating to 150–200 °C.

Column chromatography

Purification of compounds was performed using column chromatography on Silica gel 60 (Merck 7734). The mobile phases are reported as the volume ratio of solvents for each experiment separately.

Chemicals

Betulinic acid was obtained from the company Betulinines (www.betulinines.com). All other chemicals and solvents were obtained from the companies Sigma-Aldrich, Fluorochem, Lach-ner, VWR, or Across Organics in analytical quality, and were used as purchased.

Biological testing

Cytotoxicity testing, the study of the cell cycle, DNA and RNA synthesis were performed by the biological department at the Institute of Molecular and Translational Medicine, Faculty of Medicine, Palacký University Olomouc. The individual experimental procedures are described in the attached publications in Appendices I–IV.

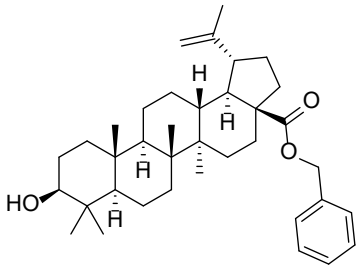
6.2 Azines

The experimental section is a part of the original article, for more information, please see Appendix II.

6.3 Wittig reactions

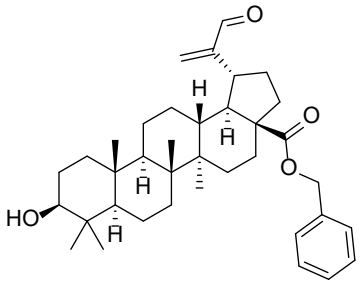
The experimental section is a part of the original article, for more information, please see Appendix III.

6.4 CuAAC reactions at the position C-30



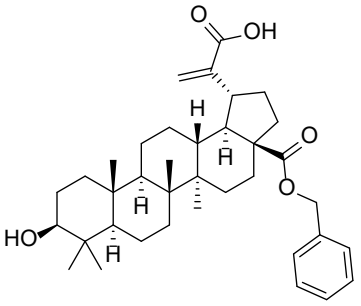
Chemical Formula: $C_{37}H_{54}O_3$
Exact Mass: 546,4073

Benzyl betulinate (**5**): 10 g (21 mmol) of betulinic acid was dissolved in 100 mL of THF, and 8.6 g (63 mmol) of K_2CO_3 was added. The mixture was placed to stir under reflux, after 10 min, 7.4 mL (63 mmol) of benzyl bromide was added. The reaction was monitored by TLC; after completion in 3 h, the reaction mixture was quenched by adding 100 mL of distilled water, followed by extraction to EtOAc. Organic layers were combined, washed with brine, dried over anhydrous $MgSO_4$, and evaporated. The crude product was purified on silica gel using flash column chromatography (Hex/EtOAc 3 : 1) affording 10.8 g (95 %) of white crystalline solid. **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.39–7.29 (m, 5H, arom. ring), 5.14 (d, $J = 12.3$ Hz, 1H, -CH_2- Bn group), 5.09 (d, $J = 12.3$ Hz, 1H, -CH_2- Bn group), 4.72 (s, 1H, H-29a), 4.59 (s, 1H, H-29b), 3.17 (dd, $J_1 = 11.4$ Hz, $J_2 = 4.8$ Hz, 1H, H-3α), 3.02 (td, $J_1 = 10.9$ Hz, $J_2 = 4.7$ Hz, 1H, H-19β), 2.31–2.24 (m, 1H), 2.18 (td, $J_1 = 12.7$ Hz, $J_2 = 3.6$ Hz, 1H), 1.67 (s, 3H, 30-CH_3), 0.95 (s, 3H), 0.94 (s, 3H), 0.80 (s, 3H), 0.76 (s, 3H), 0.75 (s, 3H) ppm.



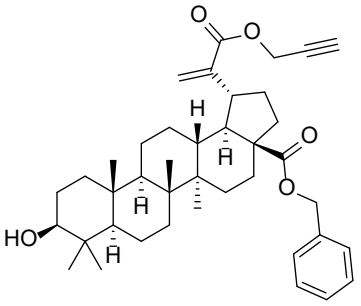
Chemical Formula: $C_{37}H_{52}O_4$
Exact Mass: 560,3866

Oxidation of the C-30 position to aldehyde (**6**): 9 g (15 mmol) of benzyl betulinate (**5**) was dissolved in 200 mL of 2-methoxyethanol, and 5 g (45 mmol) of SeO_2 was added. The reaction was placed to stir and heated at 110 °C. After completion in 4 h, the hot black reaction mixture was immediately filtered over a paper filter into 1 L of icy water where the product precipitated as white solid. The precipitate was then filtered using a Büchner funnel, and the filter cake was dried at r. t. for 3 days. When dried, the product was purified by flash column chromatography (Hex/EtOAc 6 : 1), affording 6.7 g (73 %) of white crystalline solid. **^{1}H NMR** (500 MHz, r$CDCl_3$) δ 9.51 (s, 1H, H-30), 7.39–7.28 (m, 5H, arom. ring), 6.25 (s, 1H, H-29a), 5.88 (s, 1H, H-29b), 5.15 (d, $J = 12.3$ Hz, 1H, -CH_2- Bn group), 5.11 (d, $J = 12.3$ Hz, 1H, -CH_2- Bn group), 3.35 (td, $J_1 = 11.1$ Hz, $J_2 = 4.8$ Hz, 1H, H-19β), 3.16 (dd, $J_1 = 11.3$ Hz, $J_2 = 4.8$ Hz, 1H, H-3α), 2.30 (dt, $J_1 = 12.6$ Hz, $J_2 = 3.0$ Hz, 1H), 2.15 (td, $J_1 = 12.6$ Hz, $J_2 = 3.5$ Hz, 1H), 1.90 (dd, $J_1 = 12.4$ Hz, $J_2 = 7.9$ Hz, 1H), 0.95 (s, 3H), 0.90 (s, 3H), 0.78 (s, 3H), 0.74 (s, 3H), 0.73 (s, 3H) ppm.



Chemical Formula: $C_{37}H_{52}O_5$
Exact Mass: 576,3815

Oxidation of the C-30 position to the carboxyl group (**33**): 6.7 g (12 mmol) of the protected aldehyde (**6**) was dissolved in 150 mL of *t*-BuOH, and 30 mL (220 mmol) of 2-methyl-2-butene was added, followed by 4.8 g (44 mmol) of $NaClO_2$, 6.1 g (44 mmol) of KH_2PO_4 and 75 mL of water. The reaction mixture was stirred vigorously at r. t. for 5 h. After completion, the product was extracted using saturated NH_4Cl solution and EtOAc, organic layers were combined, washed with brine and water, dried over anhydrous $MgSO_4$, and evaporated. The crude product was purified by flash column chromatography (DCM/EtOAc/AcOH 6 : 1 : 0.04), affording 5.8 g (84 %) of white solid. **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.42–7.28 (m, 5H, arom. ring), 6.21 (s, 1H, H-29a), 5.66 (s, 1H, H-29b), 5.16 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 5.10 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 3.37 (td, J_1 = 11.2 Hz, J_2 = 4.9 Hz, 1H, H-19β), 3.18 (dd, J_1 = 11.1 Hz, J_2 = 4.2 Hz, 1H, H-3α), 2.36–2.25 (m, 1H), 2.17 (td, J_1 = 12.8 Hz, J_2 = 3.5 Hz, 1H), 0.95 (s, 3H), 0.92 (s, 3H), 0.79 (s, 3H), 0.75 (s, 3H), 0.74 (s, 3H) ppm.



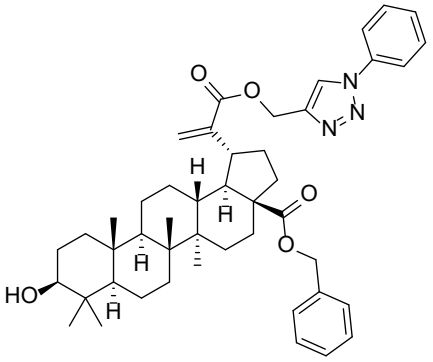
Chemical Formula: $C_{40}H_{54}O_5$
Exact Mass: 614,3971

Esterification of the 30-COOH (**34**): 0.47 g (0.8 mmol) of the 30-acid (**33**) was dissolved in 40 mL of THF, 0.34 g (2.5 mmol) of K_2CO_3 was added, and the reaction mixture was placed to stir under reflux. After 10 min, 265 µL (2.5 mmol) of propargyl bromide was added. The reaction was monitored by TLC, after completion in 4 h, the mixture was extracted by EtOAc, organic layers were combined, washed with brine, dried over anhydrous $MgSO_4$, and evaporated. The product was purified by flash column chromatography (Hex/EtOAc 5 : 2), affording 0.46 g (91 %) of white solid. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.40–7.29 (m, 5H, arom. ring), 6.09 (s, 1H, H-29a), 5.61 (s, 1H, H-29b), 5.15 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 5.10 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 4.81–4.75 (m, 1H, -CH_2- propargyl group), 4.75–4.68 (m, 1H, -CH_2- propargyl group), 3.34 (td, J_1 = 11.1 Hz, J_2 = 4.8 Hz, 1H, H-19β), 3.16 (dd, J_1 = 11.2 Hz, J_2 = 4.9 Hz, 1H, H-3α), 2.47 (t, J = 2.5 Hz, 1H, terminal alkyne H), 2.30 (dt, J_1 = 12.3 Hz, J_2 = 2.9 Hz, 1H), 2.16 (td, J_1 = 12.6 Hz, J_2 = 3.5 Hz, 1H), 0.95 (s, 3H), 0.90 (s, 3H), 0.78 (s, 3H), 0.74 (s, 3H), 0.74 (s, 3H) ppm.

General procedure for the azide preparation: 0.5 mmol of the corresponding bromide was dissolved in DMF, and 1.0 mmol of NaN_3 was added. The reaction mixture was stirred overnight, when complete (TLC analysis), the product was extracted using DCM, washed with brine and water, dried over anhydrous $MgSO_4$

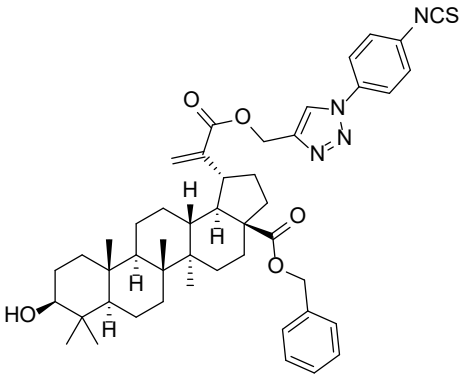
and evaporated. The prepared azide was immediately used in the next step without purification, or it was purified by column chromatography (for sugar azides **g**, **h**) using a mixture of toluene/diethyl ether 1 : 1 as a mobile phase.

General procedure for the CuAAC reactions: 0.1 g (0.16 mmol) of the propargyl ester (**34**) was dissolved in 8 mL of *t*-BuOH, followed by addition of 1.5 equivalents of the corresponding azide **a**–**k**, 0.4 equivalent of $CuSO_4 \cdot 5H_2O$, 0.8 equivalent of sodium ascorbate, and 4 mL of distilled water. The suspension was placed to stir at 50 °C and monitored by TLC. After completion, the product was extracted using DCM, organic layers were combined, washed with brine, dried over anhydrous $MgSO_4$, and evaporated. The product was purified by flash column chromatography (Hex/EtOAc, Tol/Et_2O, or $CHCl_3$/EtOAc), affording final conjugates **35a**–**35k** in moderate to high yields.



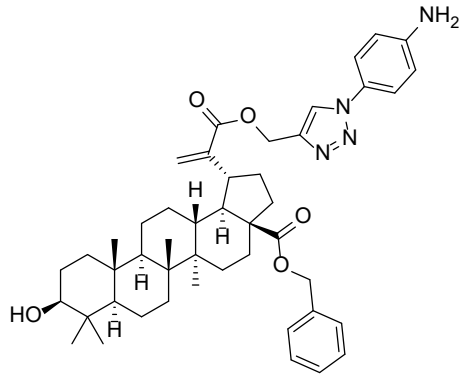
Chemical Formula: $C_{46}H_{59}N_3O_5$
Exact Mass: 733,4455

The compound **35a** was obtained as yellowish solid, 90 mg (75 %) by the general procedure, while the reaction time was 18 h: m. p. 78–81 °C; **^{1}H NMR** (500 MHz, $CHCl_3$) δ 8.08 (s, 1H, triazole ring), 7.77–7.71 (m, 2H, arom. ring), 7.54–7.48 (m, 2H, arom. ring), 7.46–7.41 (m, 1H, arom. ring), 7.37–7.27 (m, 5H, arom. Bn group), 6.09 (s, 1H, H-29a), 5.58 (s, 1H, H-29b), 5.40 (d, J = 12.7 Hz, 1H, -CH_2- ester group), 5.36 (d, J = 12.7 Hz, 1H, -CH_2- ester group), 5.14 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 5.08 (d, J = 12.2 Hz, 1H, -CH_2- Bn group), 3.36 (td, J_1 = 11.1, J_2 = 4.9 Hz, 1H, H-19β), 3.13 (dd, J_1 = 11.2, J_2 = 4.8 Hz, 1H, H-3α), 2.30–2.23 (m, 1H), 2.11 (td, J_1 = 12.6, J_2 = 3.5 Hz, 1H), 1.89 (dd, J_1 = 12.4, J_2 = 8.0 Hz, 1H), 1.82 (t, J = 11.4 Hz, 1H), 0.93 (s, 3H), 0.77 (s, 3H), 0.72 (s, 3H), 0.71 (s, 3H), 0.69 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 175.75, 167.28, 146.49, 143.90, 136.99, 136.50, 129.86 (2C), 128.95, 128.57 (2C), 128.33 (2C), 128.15, 124.75, 122.10, 120.55 (2C), 78.95, 65.84, 57.67, 56.59, 55.37, 51.56, 50.37, 42.32, 42.19, 40.61, 38.90, 38.67, 37.96, 37.14, 36.63, 34.34, 32.40, 32.04, 29.52, 28.09, 27.45, 27.09, 20.85, 18.32, 16.10, 15.83, 15.44, 14.55 ppm; **HRMS** (ESI$^+$) *m/z* calcd for $C_{46}H_{60}O_5N_3$ $[M+H]^+$ 734.4527, found 734.4528; **IR** (DRIFT) ν_{max} 3428, 2940, 2869, 1716, 1621, 1600, 1502, 1454 cm^{-1}.



Chemical Formula: $C_{47}H_{58}N_4O_5S$
Exact Mass: 790,4128

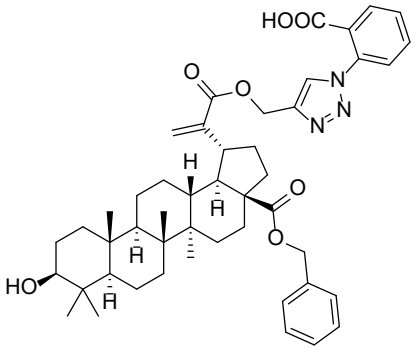
The compound **35b** was obtained as yellow solid, 29 mg (23 %) by the general procedure, while the reaction time was 18 h: m. p. 119–122 °C; **^{1}H NMR** (500 MHz, $CDCl_3$) δ 8.08 (s, 1H), 7.78–7.74 (m, 2H, arom. ring), 7.39–7.29 (m, 8H, arom. ring), 6.10 (s, 1H, H-29a), 5.59 (s, 1H, H-29b), 5.40 (d, J = 13.0 Hz, 1H, -CH_2- ester group), 5.36 (d, J = 12.8 Hz, 1H, -CH_2- ester group), 5.14 (d, J = 12.4 Hz, 1H, -CH_2- Bn group), 5.08 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 3.36 (td, J = 11.1, 4.9 Hz, 1H, H-19β), 3.14 (dd, J_1 = 11.2, J_2 = 5.0 Hz, 1H, H-3α), 2.31–2.24 (m, 1H), 2.12 (td, J_1 = 12.7, J_2 = 3.5 Hz, 1H), 1.89 (dd, J_1 = 12.3, J_2 = 7.9 Hz, 1H), 1.81 (t, J = 11.2 Hz, 1H), 0.94 (s, 3H), 0.78 (s, 4H), 0.73 (s, 2H), 0.72 (s, 3H), 0.70 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 175.77, 167.34, 146.48, 144.34, 138.12, 136.53, 135.33, 132.16, 128.64 (2C), 128.36 (2C), 128.22, 127.17 (2C), 124.77, 122.00, 121.58 (2C), 79.05, 65.91, 57.63, 56.64, 55.49, 51.61, 50.46, 42.37, 41.99, 40.67, 38.96, 38.78, 38.00, 37.21, 36.66, 34.40, 32.50, 32.08, 29.58, 28.15, 27.49, 27.21, 20.92, 18.37, 16.16, 15.88, 15.48, 14.60 ppm; **HRMS** (ESI$^+$) *m/z* calcd for $C_{47}H_{59}O_5N_4S$ $[M+H]^+$ 791.4201, found 791.4198; **IR** (DRIFT) ν_{max} 3349, 2941, 2868, 2033, 1717, 1619, 1515, 1453 cm^{-1}.



Chemical Formula: $C_{46}H_{60}N_4O_5$
Exact Mass: 748,4564

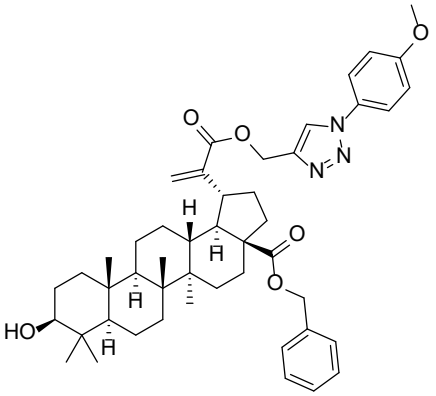
The compound **35c** was obtained as brown solid, 58 mg (48 %) by the general procedure, while the reaction time was 18 h: m. p. 103–106 °C; **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.93 (s, 1H, triazole ring), 7.49–7.44 (m, 2H, arom. ring), 7.37–7.29 (m, 5H, arom. ring of Bn group), 6.77–6.72 (m, 2H, arom. ring), 6.08 (s, 1H, H-29a), 5.57 (s, 1H, H-29b), 5.38 (d, J = 12.7 Hz, 1H, -CH_2- ester group), 5.35 (d, J = 12.7 Hz, 1H, -CH_2- ester group), 5.14 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 5.08 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 3.35 (td, J_1 = 11.2, J_2 = 5.0 Hz, 1H, H-19β), 3.14 (dd, J_1 = 11.1, J_2 = 4.7 Hz, 1H, H-3α), 2.27 (dt, J_1 = 12.4, J_2 = 2.9 Hz, 1H), 2.11 (td, J_1 = 12.7, J_2 = 3.6 Hz, 1H), 1.88 (dd, J_1 = 12.5, J_2 = 7.9 Hz, 1H), 1.83 (t, J = 11.6 Hz, 1H), 0.94 (s, 3H), 0.77 (s, 3H), 0.73 (s, 3H), 0.72 (s, 3H), 0.70 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 175.84, 167.34, 147.37, 146.52, 143.46, 136.55, 128.62 (2C), 128.56, 128.38 (2C), 128.19, 124.74, 122.28 (2C), 122.09, 115.35 (2C), 79.06, 65.89, 57.81, 56.62, 55.43, 51.45, 50.43, 42.37, 42.25, 40.66, 38.94, 38.74, 38.01, 37.20, 36.66, 34.39, 32.37, 32.09, 29.57, 28.12, 27.50, 27.10, 20.90, 18.37, 16.13, 15.88,

15.46, 14.59 ppm; **HRMS** (ESI$^+$) *m/z* calcd for $C_{46}H_{61}O_5N_4$ $[M+H]^+$ 749.4636, found 749.4634; **IR** (DRIFT) ν_{max} 3459, 3365, 3231, 2940, 2868, 1714, 1624, 1522, 1453 cm^{-1}.



Chemical Formula: $C_{47}H_{59}N_3O_7$
Exact Mass: 777,4353

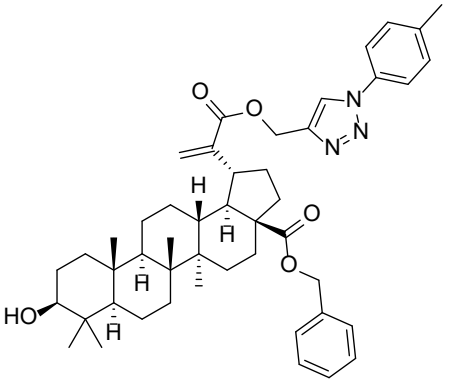
The compound **35d** was obtained as white solid, 55 mg (28 %) by the general procedure, while the reaction time was 18 h: m. p. 92–94 °C; **^{1}H NMR** (500 MHz, DMSO-d_6) δ 8.48 (s, 1H, triazole ring), 7.89–7.82 (m, 1H, arom. ring), 7.69–7.60 (m, 2H, arom. ring), 7.58–7.53 (m, 1H, arom. ring), 7.38–7.29 (m, 5H, arom. ring of Bn group), 6.03 (s, 1H, H-29a), 5.71 (s, 1H, H-29b), 5.32 (d, J = 12.9 Hz, 1H, -CH_2- ester group), 5.29 (d, J = 12.9 Hz, 1H, -CH_2- ester group), 5.12 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 5.08 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 3.28 (td, J_1 = 11.1, J_2 = 4.8 Hz, 1H, H-19β), 2.97–2.91 (m, 1H, H-3α), 2.19–2.12 (m, 1H), 2.05 (td, J_1 = 12.5, J_2 = 3.2 Hz, 1H), 0.85 (s, 3H), 0.79 (s, 4H), 0.69 (s, 3H), 0.64 (s, 3H), 0.63 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 176.10, 168.09, 167.18, 146.40, 142.66, 136.41, 135.94, 132.43, 132.30, 131.82, 130.02, 128.62 (2C), 128.40 (2C), 128.23, 126.81, 126.26, 124.38, 79.14, 66.05, 57.83, 56.73, 55.45, 51.22, 50.47, 42.41, 41.83, 40.68, 38.91, 38.80, 38.11, 37.22, 36.62, 34.41, 32.49, 32.04, 29.62, 28.10, 27.35, 27.28, 20.96, 18.37, 16.19, 15.89, 15.49, 14.70 ppm; **HRMS** (ESI$^+$) *m/z* calcd for $C_{47}H_{60}O_7N_3$ $[M+H]^+$ 778.4426, found 778.4424; **IR** (DRIFT) ν_{max} 3366, 2941, 2869, 1717, 1604, 1568, 1498, 1454 cm^{-1}.



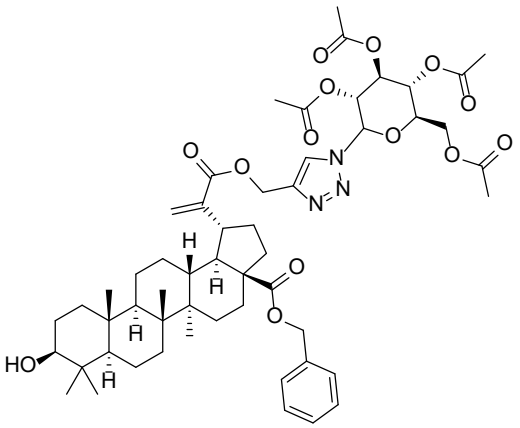
Chemical Formula: $C_{47}H_{61}N_3O_6$
Exact Mass: 763,4560

The compound **35e** was obtained as white solid, 37 mg (30 %) by the general procedure, while the reaction time was 18 h: m. p. 85–88 °C; **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.98 (s, 1H, triazole ring), 7.66–7.62 (m, 2H, arom. ring), 7.37–7.29 (m, 5H, arom. ring of Bn group), 7.03–6.99 (m, 2H, arom. ring), 6.09 (s, 1H, H-29a), 5.58 (s, 1H, H-29b), 5.40 (d, J = 12.7 Hz, 1H, -CH_2- ester group), 5.36 (d, J = 12.7 Hz, 1H, -CH_2- ester group), 5.14 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 5.09 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 3.86 (s, 3H, CH_3- methoxy group), 3.36 (td, J_1 = 11.2, J_2 = 5.0 Hz, 1H, H-19β), 3.13 (dd, J_1 = 10.9, J_2 = 4.7 Hz, 1H, H-3α), 2.27 (dt, J_1 = 12.5, J_2 = 3.1 Hz, 1H), 2.12 (td, J_1 = 12.7, J_2 = 3.6 Hz, 1H), 2.06–1.96 (m, 1H), 1.89 (dd, J_1 = 12.5, J_2 = 7.7 Hz, 1H), 1.84 (t, J = 11.5 Hz, 1H), 0.94 (s, 3H), 0.78 (s, 3H), 0.73 (s, 3H), 0.73 (s, 3H), 0.70 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 175.83, 167.35, 160.06, 146.52, 143.74, 136.56,

130.50, 128.63 (2C), 128.39 (2C), 128.21, 124.75, 122.24 (3C), 114.93 (2C), 79.06, 65.90, 57.79, 56.65, 55.78, 55.45, 51.48, 50.45, 42.39, 42.20, 40.68, 38.95, 38.76, 38.03, 37.22, 36.68, 34.40, 32.42, 32.11, 29.59, 28.11, 27.50, 27.14, 20.92, 18.38, 16.16, 15.89, 15.47, 14.61 ppm; **HRMS** (ESI$^+$) *m/z* calcd for $C_{47}H_{62}O_6N_3$ $[M+H]^+$ 764.4633, found 764.4633; **IR** (DRIFT) ν_{max} 3434, 2940, 2868, 1717, 1628, 1613, 1519, 1453 cm^{-1}.

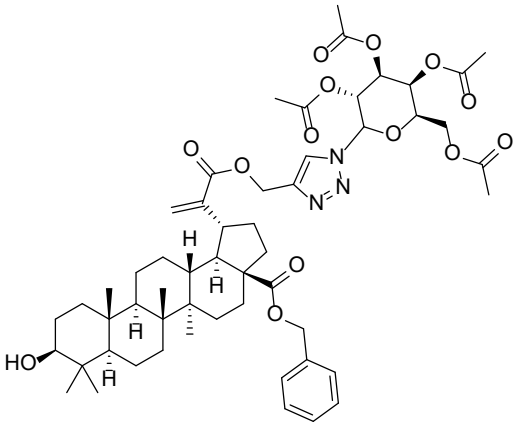

Chemical Formula: $C_{47}H_{61}N_3O_5$
Exact Mass: 747,4611

The compound **35f** was obtained as yellowish solid, 55 mg (15 %) by the general procedure, while the reaction time was 18 h: m. p. 81–84 °C; **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.81 (s, 1H, triazole ring), 7.43–7.27 (m, 10H, arom. rings), 6.10 (s, 1H, H-29a), 5.59 (s, 1H, H-29b), 5.42 (d, J = 12.8 Hz, 1H, -CH_2- ester group), 5.38 (d, J = 12.7 Hz, 1H, -CH_2- ester group), 5.14 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 5.08 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 3.36 (td, J_1 = 11.2, J_2 = 4.9 Hz, 1H, H-19β), 3.15 (dd, J_1 = 11.4, J_2 = 4.4 Hz, 1H, H-3α), 2.28 (dt, J_1 = 12.5, J_2 = 3.1 Hz, 1H), 2.22 (s, 3H, CH_3- group on arom. ring), 2.14 (td, J_1 = 12.7, J_2 = 3.7 Hz, 1H), 0.94 (s, 3H), 0.83 (s, 3H), 0.76 (s, 3H), 0.73 (s, 3H), 0.72 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 175.80, 167.23, 146.36, 143.10, 136.54, 136.46, 133.68, 131.67, 130.05, 128.61 (2C), 128.37 (2C), 128.19, 126.98, 126.03, 125.35, 124.84, 79.05, 65.88, 57.87, 56.67, 55.45, 51.14, 50.47, 42.42, 42.30, 40.69, 38.96, 38.80, 38.08, 37.24, 36.64, 34.40, 32.32, 32.08, 29.59, 28.12, 27.49, 27.17, 20.95, 18.38, 18.05, 16.17, 15.89, 15.47, 14.67 ppm; **HRMS** (ESI$^+$) *m/z* calcd for $C_{47}H_{62}O_5N_3$ $[M+H]^+$ 748.4684, found 748.4686; **IR** (DRIFT) ν_{max} 3434, 2940, 2868, 1717, 1620, 1500, 1453 cm^{-1}.

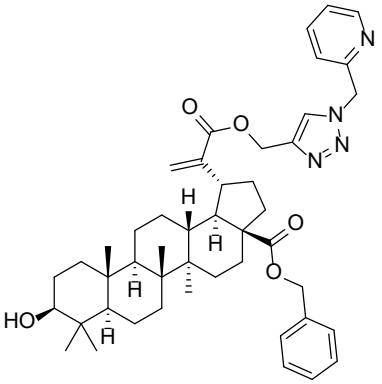

Chemical Formula: $C_{54}H_{73}N_3O_{14}$
Exact Mass: 987,5093

The compound **35g** was obtained as white solid, 155 mg (95 %) by the general procedure, while the reaction time was 18 h: m. p. 167–170 °C; **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.85 (s, 1H), 7.38–7.28 (m, 5H, arom. ring), 6.05 (s, 1H, H-29a), 5.90–5.83 (m, 1H), 5.57 (s, 1H, H-29b), 5.46–5.38 (m, 2H), 5.33–5.21 (m, 3H), 5.13 (d, J = 12.2 Hz, 1H, -CH_2- Bn group), 5.08 (d, J = 12.2 Hz, 1H, -CH_2- Bn group), 4.30 (dd, J_1 = 12.7, J_2 = 4.9 Hz, 1H), 4.16–4.11 (m, 1H), 4.03–3.96 (m, 1H), 3.32 (td, J_1 = 11.2, J_2 = 4.8 Hz, 1H, H-19β), 3.16 (dd, J_1 = 11.3, J_2 = 4.8 Hz, 1H, H-3α), 2.30–2.24 (m, 1H),

2.14 (td, $J_1 = 12.5$, $J_2 = 3.3$ Hz, 1H), 2.07 (s, 3H, Ac group), 2.06 (s, 3H, Ac group), 2.02 (s, 3H, Ac group), 1.85 (s, 3H, Ac group), 0.94 (s, 3H), 0.86 (s, 3H), 0.77 (s, 3H), 0.73 (s, 3H), 0.71 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 175.81, 170.60, 170.04, 169.46, 168.93, 166.98, 146.10, 143.83, 136.52, 128.62 (2C), 128.39 (2C), 128.20, 124.61, 122.28, 85.87, 79.04, 75.31, 72.70, 70.33, 67.72, 65.88, 61.59, 57.59, 56.67, 55.40, 50.69, 50.43, 42.42, 42.29, 40.67, 38.95, 38.79, 38.07, 37.24, 36.61, 34.37, 32.18, 32.05, 29.61, 28.08, 27.48, 27.16, 20.94, 20.81, 20.66, 20.64, 20.25, 18.37, 16.20, 15.88, 15.48, 14.67 ppm; **HRMS** (ESI$^+$) *m/z* calcd for $C_{54}H_{74}O_{14}N_3$ $[M+H]^+$ 988.5165, found 988.5167; **IR** (DRIFT) ν_{max} 3478, 2942, 2873, 2118, 1753, 1716, 1624, 1553, 1438 cm^{-1}.

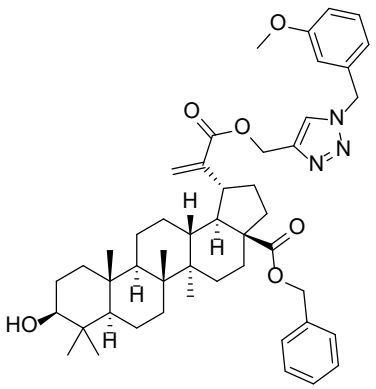

Chemical Formula: $C_{54}H_{73}N_3O_{14}$
Exact Mass: 987,5093

The compound **35h** was obtained as white solid, 154 mg (96 %) by the general procedure, while the reaction time was 18 h: m. p. 86–88 °C; **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.93 (s, 1H, triazole ring), 7.39–7.28 (m, 5H, arom. ring), 6.07 (s, 1H, H-29), 5.83 (d, J = 9.3 Hz, 1H), 5.59–5.52 (m, 3H), 5.30 (d, J = 12.8 Hz, 1H, -CH_2- ester group), 5.27 (d, J = 12.9 Hz, 1H, -CH_2- ester group), 5.24 (dd, J_1 = 10.3, J_2 = 3.3 Hz, (d, J = 12.4 Hz, 1H, -CH_2- Bn group), 5.09 (d, J = 12.2 Hz, 1H, -CH_2- Bn group), 4.25–4.08 (m, 3H), 3.33 (td, J_1 = 11.1, J_2 = 4.7 Hz, 1H, H-19β), 3.16 (dd, J_1 = 11.3, J_2 = 4.8 Hz, 1H, H-3α), 2.31–2.26 (m, 1H), 2.22 (s, 3H, Ac group), 2.15 (td, J_1 = 12.3, J_2 = 3.5 Hz, 1H), 2.04 (s, 3H, Ac group), 2.01 (s, 3H, Ac group), 1.87 (s, 3H, Ac group), 0.94 (s, 3H), 0.87 (s, 3H), 0.78 (s, 3H), 0.74 (s, 3H), 0.72 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 175.82, 170.46, 170.14, 169.96, 169.12, 167.08, 146.14, 143.69, 136.54, 128.64 (2C), 128.42 (2C), 128.22, 124.64, 122.53, 86.45, 79.09, 74.23, 70.88, 67.92, 66.91, 65.90, 61.24, 57.58, 56.69, 55.42, 50.72, 50.45, 42.45, 42.32, 40.70, 38.97, 38.81, 38.08, 37.27, 36.62, 34.39, 32.20, 32.08, 29.63, 28.10, 27.50, 27.18, 20.97, 20.84, 20.79, 20.65, 20.37, 18.39, 16.22, 15.91, 15.49, 14.69 ppm; **HRMS** (ESI$^+$) *m/z* calcd for $C_{54}H_{74}O_{14}N_3$ $[M+H]^+$ 988.5165, found 988.5167; **IR** (DRIFT) ν_{max} 3522, 2943, 2870, 1751, 1718, 1622, 1557, 1498, 1454 cm^{-1}.



Chemical Formula: $C_{46}H_{60}N_4O_5$
Exact Mass: 748,4564

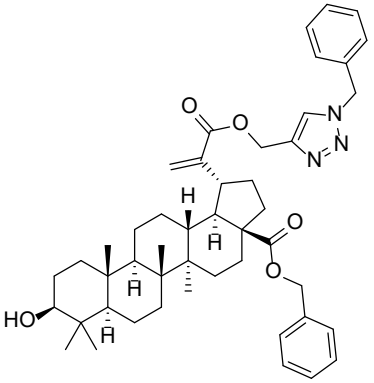
The compound **35i** was obtained as white solid, 76 mg (62 %) by the general procedure, while the reaction time was 18 h: m. p. 73–76 °C; **^{1}H NMR** (500 MHz, $CDCl_3$) δ 8.59–8.56 (m, 1H, pyridine), 7.78 (s, 1H, triazole ring), 7.70–7.65 (m, 1H, pyridine), 7.35–7.29 (m, 5H, arom. ring of Bn group), 7.26–7.23 (m, 1H, pyridine), 7.20–7.17 (m, 1H, pyridine), 6.03 (s, 1H, H-29a), 5.63 (s, 2H, -CH2- group), 5.53 (s, 1H, H-29b), 5.31 (d, J = 12.7 Hz, 1H, -CH_2- group), 5.24 (d, J = 12.7 Hz, 1H, -CH_2- group), 5.12 (d, J = 12.3 Hz, 1H, -CH_2- group), 5.07 (d, J = 12.3 Hz, 1H, -CH_2- group), 3.30 (td, J_1 = 11.1, J_2 = 4.8 Hz, 1H, H-19β), 3.16 (dd, J_1 = 11.3, J_2 = 4.7 Hz, 1H, H-3α), 2.27–2.22 (m, 1H), 2.12 (td, J_1 = 12.6, J_2 = 3.5 Hz, 1H), 0.94 (s, 3H), 0.80 (s, 3H), 0.76 (s, 3H), 0.73 (s, 3H), 0.71 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 175.79, 167.14, 154.30, 149.83, 146.23, 143.48, 137.52, 136.52, 128.58 (2C), 128.34 (2C), 128.16, 124.59, 124.43, 123.59, 122.57, 79.00, 65.84, 57.79, 56.64, 55.65, 55.42, 50.78, 50.42, 42.52, 42.40, 40.66, 38.94, 38.81, 38.06, 37.23, 36.57, 34.37, 32.15, 32.02, 29.56, 28.10, 27.48, 27.08, 20.91, 18.37, 16.17, 15.88, 15.47, 14.56 ppm; **HRMS** (ESI$^+$) *m/z* calcd for $C_{46}H_{61}O_5N_4$ $[M+H]^+$ 749.4636, found 749.4633; **IR** (DRIFT) ν_{max} 3399, 2941, 2868, 2245, 1716, 1621, 1595, 1573, 1497, 1454 cm^{-1}.



Chemical Formula: $C_{48}H_{63}N_3O_6$
Exact Mass: 777,4717

The compound **35j** was obtained as white solid, 65 mg (52 %) by the general procedure, while the reaction time was 18 h: m. p. 65–67 °C; **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.54 (s, 1H, triazole ring), 7.36–7.29 (m, 5H, arom. ring of Bn group), 7.29–7.25 (m, 1H, arom. ring), 6.89–6.85 (m, 1H, arom. ring), 6.85–6.82 (m, 1H, arom. ring), 6.80–6.77 (m, 1H, arom. ring), 6.02 (s, 1H, H-29a), 5.53 (s, 1H, H-29b), 5.47 (s, 2H, -CH_2- group), 5.29 (d, J = 12.7 Hz, 1H, -CH_2- group), 5.22 (d, J = 12.7 Hz, 1H, -CH_2- group), 5.13 (d, J = 12.3 Hz, 1H, -CH_2- group), 5.08 (d, J = 12.3 Hz, 1H, -CH_2- group), 3.76 (s, 3H, CH_3- methoxy group), 3.30 (td, J_1 = 11.2, J_2 = 4.8 Hz, 1H, H-19β), 3.16 (dd, J_1 = 11.3, J_2 = 4.7 Hz, 1H, H-3α), 2.28–2.22 (m, 1H), 2.12 (td, J_1 = 12.6, J_2 = 3.5 Hz, 1H), 0.95 (s, 3H), 0.80 (s, 3H), 0.77 (s, 3H), 0.74 (s, 3H), 0.71 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 175.79, 167.19, 160.23, 146.23, 143.45, 136.52, 135.93, 130.31, 128.59 (2C), 128.34 (2C), 128.16, 124.59, 123.77, 120.33, 114.36, 113.84, 79.02, 65.85, 57.78, 56.64, 55.42, 55.38, 54.24, 50.80, 50.42, 42.44, 42.39, 40.67, 38.94, 38.81, 38.06, 37.23, 36.56, 34.38, 32.16, 32.02, 29.56, 28.09, 27.46, 27.09, 20.91, 18.37, 16.17, 15.88, 15.47, 14.55 ppm; **HRMS** (ESI$^+$) *m/z* calcd

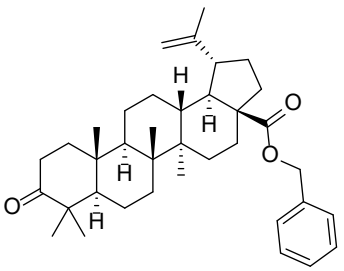
for $C_{48}H_{64}O_6N_3$ $[M+H]^+$ 778.4790, found 778.4786; **IR** (DRIFT) ν_{max} 3427, 2939, 2868, 1716, 1613, 1602, 1587, 1491, 1454 cm^{-1}.


Chemical Formula: $C_{47}H_{61}N_3O_5$
Exact Mass: 747,4611

The compound **35k** was obtained as white solid, 75 mg (62 %) by the general procedure, while the reaction time was 18 h: m. p. 69–71 °C; **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.53 (s, 1H, triazole ring), 7.37–7.32 (m, 8H, arom. ring), 7.27–7.25 (m, 2H, arom. ring), 6.03 (s, 1H, H-29a), 5.54 (s, 1H, H-29b), 5.51 (s, 2H, -CH_2- group), 5.29 (d, J = 12.7 Hz, 1H, -CH_2- group), 5.22 (d, J = 12.7 Hz, 1H, -CH_2- group), 5.13 (d, J = 12.3 Hz, 1H, -CH_2- group), 5.08 (d, J = 12.3 Hz, 1H, -CH_2- group), 3.30 (td, J_1 = 11.1, J_2 = 4.8 Hz, 1H, H-19β), 3.17 (dd, J_1 = 11.4, J_2 = 4.7 Hz, 1H, H-3α), 2.29–2.23 (m, 1H), 2.12 (td, J_1 = 12.6, J_2 = 3.5 Hz, 1H), 0.95 (s, 3H), 0.80 (s, 3H), 0.77 (s, 3H), 0.74 (s, 3H), 0.72 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 175.81, 167.22, 146.25, 143.50, 136.54, 134.53, 129.27 (2C), 128.95, 128.61 (2C), 128.37 (2C), 128.20 (3C), 124.64, 123.77, 79.07, 65.87, 57.80, 56.67, 55.46, 54.33, 50.83, 50.45, 42.42, 42.29, 40.69, 38.96, 38.84, 38.08, 37.26, 36.59, 34.40, 32.19, 32.05, 29.59, 28.12, 27.49, 27.12, 20.94, 18.39, 16.20, 15.90, 15.48, 14.58 ppm; **HRMS** (ESI$^+$) *m/z* calcd for $C_{47}H_{62}O_5N_3$ $[M+H]^+$ 748.4684, found 748.4686; **IR** (DRIFT) ν_{max} 3427, 2940, 2869, 1717, 1622, 1587, 1497, 1455 cm^{-1}.

6.5 Optimization of the Heck reaction at the position C-3

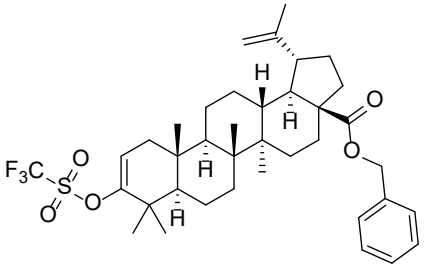
Benzyl betulinate (**5**): The compound was prepared according to the experimental procedure described above in Chapter 6.4.


Chemical Formula: $C_{37}H_{52}O_3$
Exact Mass: 544,3916

Benzyl betulonate (**36**): 3 g (5.5 mmol) of benzyl betulinate (**5**) was dissolved in 60 mL of 1,4-dioxane. Then, 24 mL of acetic acid, 7.5 mL of acetic anhydride, 1.5 g (11 mmol) of sodium acetate trihydrate, and 2.3 g (7.7 mmol) of $Na_2Cr_2O_7 \cdot 2H_2O$ were added. The reaction was stirred at r. t. 18 h and monitored by TLC. When finished, the dark reaction mixture was slowly neutralized with a saturated solution of $KHCO_3$, extracted by EtOAc, several times washed with water, organic layers were combined, dried over anhydrous $MgSO_4$, and evaporated. The crude product was purified by flash column chromatography (Hex/EtOAc 10 : 1) affording 1.57 g

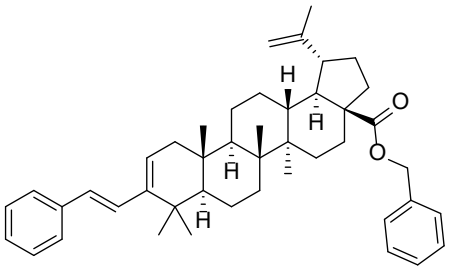
(53 %) of white crystalline compound. **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.39–7.29 (m, 5H, arom. ring), 5.15 (d, $J = 12.4$ Hz, 1H? -CH_2- Bn group), 5.09 (d, $J = 12.3$ Hz, 1H, -CH_2- Bn group), 4.72 (s, 1H, H-29a), 4.60 (s, 1H, H-29b), 3.02 (td, $J_1 = 11.0$ Hz, $J_2 = 4.8$ Hz, 1H, H-19β), 2.29 (dt, $J_1 = 12.4$ Hz, $J_2 = 3.1$ Hz, 1H), 2.22 (td, $J_1 = 12.7$ Hz, $J_2 = 3.6$ Hz, 1H), 1.68 (s, 3H, 30-CH_3), 1.60 (t, $J = 11.4$ Hz, 1H), 1.12 (dt, $J_1 = 13.5$ Hz, $J_2 = 3.0$ Hz, 1H), 1.06 (s, 3H), 1.01 (s, 3H), 0.95 (s, 3H), 0.90 (s, 3H), 0.79 (s, 3H) ppm.

Triflate (**37**): 1.5 g (2.75 mmol) of benzyl betulonate (**36**) was introduced into a dry Schlenk flask on the vacuum line, and 15 mL of dry THF was added under inert atmosphere. The flask was cooled to −78 °C and after 30 min of stirring, 11 mL (5.5 mmol) of KHMDS in toluene ($c = 0.5$ mol · L^{-1}) was added dropwise. The yellowish reaction mixture was stirred for 1 h, then 1.5 g (4.2 mmol) of Tf_2NPh was added, and the color changed to red. The reaction mixture was monitored by TLC, after completion in 3 h, the reaction was quenched by water, extracted using EtOAc, organic layers were combined, dried over anhydrous $MgSO_4$, and evaporated. The crude product was purified by flash column chromatography (Hex/Tol 3 : 1) affording 1.76 g (94 %) of the white crystalline product. **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.39–7.30 (m, 5H, arom. Bn group), 5.58–5.52 (m, 1H, H-2), 5.16 (d, $J = 12.3$ Hz, 1H, -CH_2- Bn group), 5.09 (d, $J = 12.3$ Hz, 1H, -CH_2- Bn group), 4.73 (s, 1H, H-29a), 4.60 (s, 1H, H-29b), 3.02 (td, $J_1 = 11.0$ Hz, $J_2 = 4.7$ Hz, 1H, H-19β), 2.29 (dt, $J_1 = 12.4$ Hz, $J_2 = 3.1$ Hz, 1H), 2.23 (td, $J_1 = 12.8$ Hz, $J_2 = 3.6$ Hz, 1H), 2.15 (dd, $J_1 = 17.2$ Hz, $J_2 = 6.9$ Hz, 1H), 1.68 (s, 3H, 30-CH_3), 1.61 (t, $J = 11.4$ Hz, 1H), 1.11 (s, 3H), 1.01 (s, 3H), 0.95 (s, 3H), 0.89 (s, 3H), 0.79 (s, 3H) ppm. Spectral data correspond to the literature.[193]



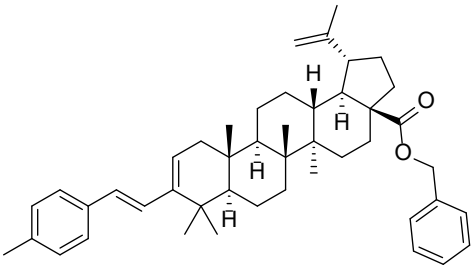
Chemical Formula: $C_{38}H_{51}F_3O_5S$
Exact Mass: 676,3409

General procedure for the Heck reaction: 0.1 g (0.15 mmol) of the triflate (**37**) was dissolved in 3 mL of dry DMF in a dry flask under an inert atmosphere. Subsequently, 24 mg (0.09 mmol) of PPh_3 was added, followed by 0.3 mmol of the corresponding styrene (or the compound with terminal double bond), 30 µL (0.3 mmol) of piperidine, and 5 mg (0.022 mmol) of $Pd(OAc)_2$. The reaction was placed to stir at 85 °C and monitored by TLC. Usually, the reaction color changed from yellow to orange when the temperature reached 85 °C and the reaction started; when it finished in ca 1 h, the reaction turned black. After completion, the product was extracted using EtOAc, organic layers were combined, washed with brine, dried over anhydrous $MgSO_4$, and evaporated. The product was purified by flash column chromatography (Hex/EtOAc, Hex/Tol), affording final conjugates **38a**–**38g** in moderate to high yields.



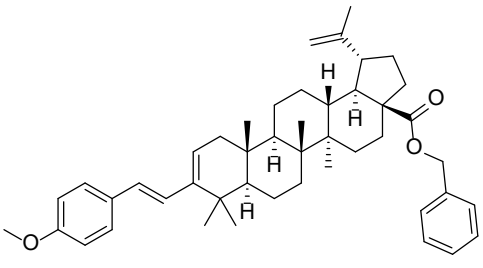
Chemical Formula: $C_{45}H_{58}O_2$
Exact Mass: 630,4437

The compound **38a** was obtained as white solid, 44 mg (47 %) by the general procedure, while the reaction time was 1 h: **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.41–7.28 (m, 9H, arom. ring), 7.22–7.16 (m, 1H, arom. ring), 6.80 (d, J = 15.8 Hz, 1H, double bond H), 6.69 (d, J = 15.8 Hz, 1H, double bond H), 5.87–5.80 (m, 1H, H-2), 5.17 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 5.10 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 4.75 (s, 1H, H-29a), 4.61 (s, 1H, H-29b), 3.05 (td, J_1 = 10.9 Hz, J_2 = 4.7 Hz, 1H, H-19β), 2.29 (dt, J_1 = 12.1 Hz, J_2 = 3.0 Hz, 1H), 2.24 (td, J_1 = 12.7 Hz, J_2 = 3.5 Hz, 1H), 2.11 (dd, J_1 = 17.5 Hz, J_2 = 6.6 Hz, 1H), 1.69 (s, 3H, 30-CH_3), 1.10 (s, 3H), 1.00 (s, 3H), 0.97 (s, 3H), 0.85 (s, 3H), 0.81 (s, 3H) ppm.



Chemical Formula: $C_{46}H_{60}O_2$
Exact Mass: 644,4593

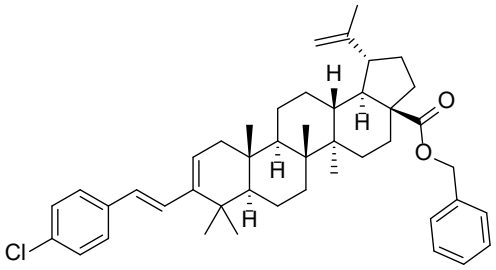
The compound **38b** was obtained as white solid, 75 mg (79 %) by the general procedure, while the reaction time was 1 h: **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.41–7.33 (m, 5H, arom. Bn group), 7.32–7.29 (m, 2H, arom. ring), 7.14–7.11 (m, 2H, arom. ring), 6.76 (d, J = 15.8 Hz, 1H, double bond H), 6.68 (d, J = 15.8 Hz, 1H, double bond H), 5.87–5.81 (m, 1H, H-2), 5.18 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 5.12 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 4.77 (s, 1H, H-29a), 4.63 (s, 1H, H-29b), 3.07 (td, J_1 = 10.9 Hz, J_2 = 4.7 Hz, 1H, H-19β), 2.35 (s, 3H, -CH_3), 2.32 (dt, J_1 = 5.7 Hz, J_2 = 3.2 Hz, 1H), 2.26 (td, J_1 = 12.7 Hz, J_2 = 3.6 Hz, 1H), 2.12 (dd, J_1 = 17.5 Hz, J_2 = 6.5 Hz, 1H), 1.71 (s, 3H, 30-CH_3), 1.65 (t, J = 11.4 Hz, 1H), 1.11 (s, 3H), 1.01 (s, 3H), 0.99 (s, 3H), 0.87 (s, 3H), 0.84 (s, 3H) ppm.



Chemical Formula: $C_{46}H_{60}O_3$
Exact Mass: 660,4542

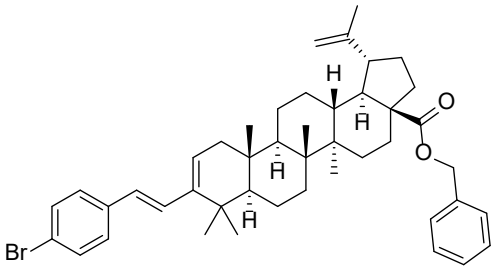
The compound **38c** was obtained as white solid, 75 mg (77 %) by the general procedure, while the reaction time was 1 h: **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.41–7.35 (m, 5H, arom. Bn group), 7.35–7.33 (m, 2H, arom. ring), 6.88–6.84 (m, 2H, arom. ring), 6.71–6.62 (m, 2H, 2 × H double bond), 5.85–5.78 (m, 1H, H-2), 5.18 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 5.12 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 4.76 (s, 1H, H-29a), 4.63 (s, 1H, H-29b), 3.81 (s, 3H, methoxy -CH_3), 3.07 (td, J_1 = 10.9 Hz, J_2 = 4.7 Hz, 1H, H-19β), 2.31 (dt, J_1 = 12.0 Hz, J_2 = 2.9 Hz, 1H), 2.29–2.23 (m, 1H), 2.12 (dd, J_1 = 17.6 Hz, J_2

= 6.6 Hz, 1H), 1.71 (s, 3H, 30-CH_3), 1.65 (t, J = 11.4 Hz, 1H), 1.11 (s, 3H), 1.01 (s, 3H), 0.99 (s, 3H), 0.87 (s, 3H), 0.83 (s, 3H) ppm.



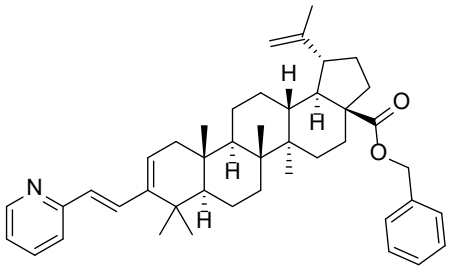
Chemical Formula: $C_{45}H_{57}ClO_2$
Exact Mass: 664,4047

The compound **38d** was obtained as white solid, 65 mg (66 %) by the general procedure, while the reaction time was 3 h: **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.41–7.34 (m, 5H, arom. Bn group), 7.33–7.30 (m, 2H, arom. ring), 7.28–7.25 (m, 2H, arom. ring), 6.77 (d, J = 15.8 Hz, 1H, double bond H), 6.64 (d, J = 15.8 Hz, 1H, double bond H), 5.89–5.81 (m, 1H, H-2), 5.17 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 5.11 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 4.76 (s, 1H, H-29a), 4.62 (s, 1H, H-29b), 3.06 (td, J_1 = 10.8 Hz, J_2 = 4.7 Hz, 1H, H-19β), 2.31 (dt, J_1 = 12.2 Hz, J_2 = 3.1 Hz, 1H), 2.25 (td, J_1 = 12.7 Hz, J_2 = 3.5 Hz, 1H), 2.12 (dd, J_1 = 17.6 Hz, J_2 = 6.6 Hz, 1H), 1.70 (s, 3H, 30-CH_3), 1.10 (s, 3H), 1.00 (s, 3H), 0.98 (s, 3H), 0.86 (s, 3H), 0.83 (s, 3H) ppm.



Chemical Formula: $C_{45}H_{57}BrO_2$
Exact Mass: 708,3542

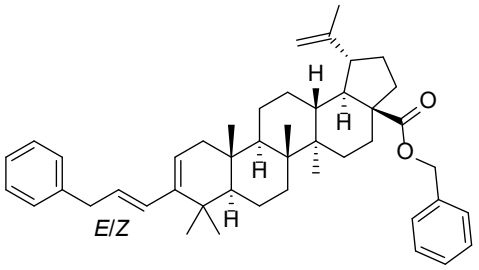
The compound **38e** was obtained as white solid, 26 mg (25 %) by the general procedure, while the reaction time was 4 h: **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.43–7.40 (m, 2H, arom. ring), 7.39–7.31 (m, 5H, arom. Bn group), 7.26–7.24 (m, 2H, arom. ring), 6.78 (d, J = 15.8 Hz, 1H, double bond H), 6.61 (d, J = 15.8 Hz, 1H, double bond H), 5.87–5.82 (m, 1H, H-2), 5.17 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 5.11 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 4.75 (s, 1H, H-29a), 4.61 (s, 1H, H-29b), 3.08–3.01 (m, 1H, H-19β), 2.24 (td, J_1 = 12.8 Hz, H_2 = 3.5 Hz, 1H), 2.11 (dd, J_1 = 17.6 Hz, J_2 = 6.6 Hz, 1H), 1.70 (s, 3H, 30-CH_3), 1.09 (s, 3H), 0.99 (s, 3H), 0.97 (s, 3H), 0.85 (s, 3H), 0.82 (s, 3H) ppm



Chemical Formula: $C_{44}H_{57}NO_2$
Exact Mass: 631,4389

The compound **38f** was obtained as white solid, 67 mg (72 %) by the general procedure, while the reaction time was 1 h: **^{1}H NMR** (500 MHz, $CDCl_3$) δ 8.57–8.50 (m, 1H, H-pyridine), 7.64–7.56 (m, 1H, H-pyridne), 7.39–7.25 (m, 7H, 5 × H of arom. Bn group, 1 × H-pyridine, 1 × H double bond), 7.11–7.04 (m, 1H, H-pyridine), 6.76 (d, J = 15.6 Hz, 1H, double bond H), 5.99–5.91 (m, 1H, H-2), 5.16 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 5.10 (d, J = 12.3 Hz, 1H, -CH_2- Bn group), 4.74 (s, 1H, H-29a), 4.61 (s, 1H, H-29b), 3.04 (td, J_1 = 10.9 Hz, J_2 = 4.7 Hz, 1H, H-19β), 2.29 (dt, J_1 = 12.1 Hz, J_2 = 3.0 Hz, 1H), 2.23

(td, J_1 = 12.8 Hz, J_2 = 3.6 Hz, 1H), 2.13 (dd, J_1 = 17.7 Hz, J_2 = 6.6 Hz, 1H), 1.69 (s, 3H, 30-CH_3), 1.13 (s, 3H), 1.03 (s, 3H), 0.96 (s, 3H), 0.84 (s, 3H), 0.81 (s, 3H) ppm.



Chemical Formula: $C_{46}H_{60}O_2$
Exact Mass: 644,4593

The compound **38g** was obtained as a mixture of inseparable *E*/*Z* isomers in a ratio approximately 1.4 : 1, white solid, 81 mg (85 %) by the general procedure, while the reaction time was 1 h: **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.40–7.13 (m, 17H, arom. ring of both isomers), 6.36 (d, J = 15.8 Hz, 1H, double bond H of isomer A), 6.25–6.17 (m, 1H, double bond H of isomer A), 6.10 (d, J = 15.1 Hz, 0.7H, double bond H of isomer B), 5.92–5.83 (m, 0.7H, double bond H of isomer B), 5.62–5.58 (m, 0.7H, H-2 of isomer B), 5.29–5.25 (m, 1H, H-2 of isomer A), 5.16 (d, J = 12.3 Hz, 1.7H, -CH_2- Bn group of both isomers), 5.10 (d, J = 12.3 Hz, 1.7H, -CH_2- Bn group of both isomers), 4.72 (s, 1.7H, H-29a of both isomers), 4.59 (s, 1.7H, H-29b of both isomers), 3.41 (d, J = 6.9 Hz, 1.4H, -CH_2- of isomer B), 3.03 (td, J_1 = 10.9 Hz, J_2 = 4.7 Hz, 1.7H, H-19β), 2.89 (d, J = 6.6 Hz, 2H, -CH_2- of isomer A), 2.31–2.26 (m, 1.7H, both isomers), 2.22 (td, J_1 = 12.6 Hz, J_2 = 3.2 Hz, 1.7H, both isomers), 1.68 (s, 5.1H, 30-CH_3, both isomers), 1.02 (s, 5.1H, both isomers), 0.95 (s, 5.1H, both isomers), 0.93 (s, 5.1H, both isomers), 0.82 (s, 5.1H, both isomers), 0.80 (s, 5.1H, both isomers) ppm.

7 List of abbreviations

Acm	Acetamidomethyl
AcOH	Acetic acid
AIBN	Azobisisobutyronitrile
BA	Betulinic acid
BINAP	2,2'-Bis(diphenylphosphino)-1,1'-binaphthyl
Bn	Benzyl
BoA	Betulonic acid
BOC	*tert*-Butyloxycarbonyl (protecting group)
CuAAC	Copper(I)-catalyzed azide-alkyne cycloaddition
DCC	*N*,*N*'-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DDQ	2,3-Dichloro-5,6-dicyano-1,4-benzoquinone
DIPEA	N,N-Diisopropylethylamine
DMAP	4-(Dimethylamino)pyridine
DMF	*N*,*N*-Dimethylformamide
EC_{50}	Half maximal effective concentration
ECHO	Enteric cytopathic human orphan virus
eq	Equivalents
Et	Ethyl
EtOAc	Ethylacetate
FMOC	Fluorenylmethyloxycarbonyl (protecting group)
Hex	*n*-Hexane
HRMS	High-resolution mass spectrometry
HSV	Herpes simplex virus
IC_{50}	Half maximal inhibitory concentration
IR	Infrared spectroscopy
KHMDS	Potassium bis(trimethylsilyl)amide
LDA	Lithium diisopropylamide
Me	Methyl
MW	Molecular weight
NBS	*N*-bromosuccinimide
NMR	Nuclear magnetic resonance spectroscopy

OPA	Oxaphosphetane
PA	Platanic acid
PCC	Pyridinium chlorochromate
PEG	Polyethylene glycol
Pom	Pivaloyloxymethyl
PPh_3	Triphenylphosphine
r. t.	Room temperature
SAR	Structure-activity relationship
SI	Selectivity index
TBAF	Tetra-n-butylammonium fluoride
t-BuOH	*tert*-Butyl alcohol
t-BuOK	Potassium *tert*-butoxide
TGR5	G protein-coupled bile acid receptor
THF	Tetrahydrofuran
TLC	Thin layer chromatography
Tol	Toluene
TPA	12-*O*-tetradecanoylphorbol-13-acetate
Ts	Tosyl, toluenesulfonyl

8 List of cell lines

518A2	Melanoma
8505C	Anaplastic thyroid cancer
A253	Head tumor
A2780	Ovarian carcinoma
A375	Melanoma
A431	Zervic cancer
A549	Lung adenocarcinoma
B16 2F2	Mice melanoma
B16	Mice melanoma
B16-F10	Mice melanoma
BEL-7402	Human hepatocellular carcinoma
BJ	Human non-cancer fibroblast
BRISTOL8	Human B-cell lymphoma
Bro	Melanoma
C32	Melanoma
CaOv	Ovarian carcinoma
CCD-18Co	Normal colon human fibroblasts
CCRF-CEM	T-lymphoblastic leukemia
CEM	Human T-lymphoblasts
CEM.CM3	Human lymphoblastic leukemia
CEM-DNR	T-lymphoblastic leukemia resistant against daunorubicin
Colo 38	Melanoma
COR-L105	Lung cancer
DLD-1	Colon cancer
DU145	Human prostate cancer
EPLC-272H	Lung cancer
FaDu	Hypopharyngeal carcinoma
HCT-8	Colon cancer
HCT-15	Colon adenocarcinoma
HCT116	Colon carcinoma
HCT116p53-/-	Colon carcinoma
HeLa	Human cervical adenocarcinoma

HepG2	Human hepatocellular carcinoma
HL-60	Human promyelocytic leukemia
HOP-62	Lung cancer
HT29	Colorectal adenocarcinoma
Jurkat E6.1	Human lymphoblastic leukemia
Jurkat	Human acute T-cell leukemia
K562	Human chronic myelogenous leukemia
K562-TAX	Chronic myelogenous leukemia resistant against paclitaxel
L1210	Murine leukemia
L132	Human lung cancer
Lipo	Liposarcoma
MCF-7	Breast adenocarcinoma
MDA MB-231	Human breast carcinoma
MEL	Murine erythroid progenitor
MIA PaCa-2	Pancreatic cancer
MOLT-4	Human lymphoblastic leukemia
MRC-5	Human non-cancer fibroblasts
NIH 3T3	Non-malignant mouse fibroblasts
PA-1	Human ovary
PC-3	Human prostate adenocarcinoma
SCC2095	Oral squamous cancer
SF-763	Human brain adenocarcinoma
SK-OV-3	Ovary malignant ascites
SK-MEL-2	Skin melanoma
SNB-19	Glioblastoma
SW-480	Colon cancer
SW1736	Anaplastic thyroid cancer
T47D	Breast cancer
U2OS	Osteosarcoma
U937	Human histiocytic lymphoma
WEHI3	Murine leukemia

9 List of author's publications

Publications related to the Thesis:

Pokorný, J.; Olejníková, D.; Frydrych, I.; Lišková, B.; Gurská, S.; Benická, S.; Šarek, J.; Kotulová, J.; Hajdúch, M.; Džubák, P.; Urban, M. Substituted Dienes Prepared from Betulinic Acid – Synthesis, Cytotoxicity, Mechanism of Action, and Pharmacological Parameters. *Eur. J. Med. Chem.* **2021**, 113706.
DOI: https://doi.org/10.1016/j.ejmech.2021.113706

Pokorný, J.; Krajčovičová, S.; Hajdúch, M.; Holoubek, M.; Gurská, S.; Džubák, P.; Volná, T.; Popa, I.; Urban, M., Triterpenic azines, a new class of compounds with selective cytotoxicity to leukemia cells CCRF-CEM, *Future Med. Chem.* **2018**, *10* (5), 483–491.
DOI: https://doi.org/10.4155/fmc-2017-0171

Pokorný, J.; Horka V.; Šidová V.; Urban M., Synthesis and characterization of new conjugates of betulin diacetate and bis(triphenysilyl) betulin with substituted triazoles, *Monatsh. Chem.* **2018**, *149* (4), 839–845.
DOI: https://doi.org/10.1007/s00706-017-2113-7

Pokorný, J.; Borková, L.; Urban, M., Click Reactions in Chemistry of Triterpenes – Advances Towards Development of Potential Therapeutics, *Current Med. Chem.* **2018**, *25* (5), 636–658.
DOI: https://doi.org/10.2174/0929867324666171009122612

Šidová, V.; Zoufalý, P.; **Pokorný, J.**; Džubák, P.; Hajdúch, M.; Popa, I.; Urban, M., Cytotoxic conjugates of betulinic acid and substituted triazoles prepared by Huisgen Cycloaddition from 30-azidoderivatives, *PLoS ONE* **2017**, 12, 2, e0171621.
DOI: https://doi.org/10.1371/journal.pone.0171621

Other publications not included in the Thesis:

de Jong, F.; **Pokorný, J**.; Manshian, B.; Daelemans, B.; Vandaele, J.; Startek, J. B.; Soenen, S.; Van der Auweraer, M.; Dehaen, W.; Rocha, S.; Silveira-Dorta, G., Development and Characterization of BODIPY-Derived Tracers for Fluorescent Labeling of the Endoplasmic Reticulum, *Dyes and Pigments* **2020**, *176* (-), 108200.
DOI: https://doi.org/10.1016/j.dyepig.2020.108200

Hodoň, J.; Borkova, L.; **Pokorný, J.**; Kazakova, A.; Urban, M., Design and synthesis of pentacyclic triterpene conjugates and their use in medicinal research, *Eur. J. Med. Chem.* **2019**, *182* (N/A), Article Number: 111653 (1–25).
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11 Appendices

Due to the copyright of the journals, the original publications are available online only.

Appendix I

Pokorný, J.; Borková, L.; Urban, M., Click Reactions in Chemistry of Triterpenes – Advances Towards Development of Potential Therapeutics, *Current Med. Chem.* **2018**, *25* (5), 636–658.

Available at: https://doi.org/10.2174/0929867324666171009122612

Appendix II

Pokorný, J.; Krajčovičová, S.; Hajdúch, M.; Holoubek, M.; Gurská, S.; Džubák, P.; Volná, T.; Popa, I.; Urban, M., Triterpenic azines, a new class of compounds with selective cytotoxicity to leukemia cells CCRF-CEM, *Future Med. Chem.* **2018**, *10* (5), 483–491.

Available at: https://doi.org/10.4155/fmc-2017-0171

Appendix III

Pokorný, J.; Olejníková, D.; Frydrych, I.; Lišková, B.; Gurská, S.; Benická, S.; Šarek, J.; Kotulová, J.; Hajdúch, M.; Džubák, P.; Urban, M. Substituted Dienes Prepared from Betulinic Acid – Synthesis, Cytotoxicity, Mechanism of Action, and Pharmacological Parameters. *Eur. J. Med. Chem.* **2021**, 113706.

Available at: https://doi.org/10.1016/j.ejmech.2021.113706

Appendix IV

Šidová, V.; Zoufalý, P.; Pokorný, J.; Džubák, P.; Hajdúch, M.; Popa, I.; Urban, M., Cytotoxic conjugates of betulinic acid and substituted triazoles prepared by Huisgen Cycloaddition from 30-azidoderivatives, *PLoS ONE* **2017**, 12, 2, e0171621.

Available at: https://doi.org/10.1371/journal.pone.0171621

Appendix V

Pokorný, J.; Horka V.; Šidová V.; Urban M., Synthesis and characterization of new conjugates of betulin diacetate and bis(triphenysilyl) betulin with substituted triazoles, *Monatsh. Chem.* **2018**, *149* (4), 839–845.

Available at: https://doi.org/10.1007/s00706-017-2113-7

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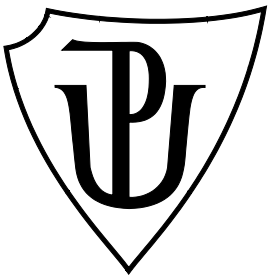
Faculty of Science
Department of Organic Chemistry



Modifications of double bonds of lupane triterpenoids with anticancer activity

Summary of the Ph.D. Thesis

Author: **Mgr. Jan Pokorný**
Supervisor: doc. RNDr. Milan Urban, Ph.D.
The year: 2022

This Ph.D. thesis is based on a research carried out at the Department of Organic Chemistry within the study program P1417 Chemistry, field of study Organic Chemistry, at Faculty of Science, Palacký University Olomouc.

Candidate: **Mgr. Jan Pokorný**
Department of Organic Chemistry, Faculty of Science, Palacký University Olomouc

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Institute of Macromolecular Chemistry of the Czech Academy of Sciences, Prague

The oral defense will take place on ..... in front of the committee for the Ph.D. thesis defense of the study program Chemistry at the Department of Organic Chemistry, Faculty of Science, Palacký University Olomouc, 17. listopadu 12, Olomouc.

The Ph.D. thesis is available at the Department of Organic Chemistry, Faculty of Science, Palacký University Olomouc, 17. listopadu 12, Olomouc, and online at http://stag.upol.cz.

Contents

1 Introduction

Terpenes are one of the largest classes of natural compounds with tens of thousands of representatives to date.[1,2] They are formally oligomers or polymers containing two or more molecules of isoprene unit. Isoprene or 2-methylbuta-1,3-diene (Fig. 1) is an unsaturated organic compound with the molecular formula C_5H_8. Thus, we can write the general formula of terpenes as $(C_5H_8)_n$, where n represents the number of isoprene units of which they are composed. Consequently, there are hemiterpenoids, consisting only of one unit = five carbon atoms (C_5), monoterpenoids (two units, C_{10}, historically considered the smallest terpenic molecules, hence their name[3]), sesquiterpenoids (three units, C_{15}), diterpenoids (four units, C_{20}), sesterterpenoids (C_{25}), triterpenoids (C_{30}), tetraterpenoids (C_{40}), and polyterpenoids. Like the majority of natural processes, the biosynthesis of terpenoids is relatively complex – these natural compounds are not formed by a simple polymerization of isoprene units.[2]

Fig. 1: The structure of isoprene.

Triterpenes are a large subgroup of terpenes, in 2006 approximately 5,000 naturally occurring representatives with more than a hundred different skeleton types were known,[4] and this number is still growing. They may be acyclic as well as they can consist of one to five cycles and these cycles are usually five- or six-membered (see Fig. 2).[5] According to the number of cycles, they can be further divided into groups such as pentacyclic triterpenes, in which lupane, oleanane, ursane, or hopane can be found.[6] However, all triterpenes have one thing in common – their basic skeleton consists of thirty carbon atoms (six isoprene units) due to their biosynthesis from the essential intermediate – squalene.[7]

Triterpenes may occur in nature as free, unsubstituted molecules, as well as bound in esters or glycosides (saponins).[8] They are widespread probably in all living organisms – they were found in both prokaryotes and eukaryotes – in bacteria,[9] animals,[10] humans,[11] plants,[12] algae[13], and fungi.[14] Every year, new triterpenes are discovered, isolated, and described.[15–17] Albeit some terpenoids serve important primary functions as photosynthetic pigments (carotenoids), growth and development

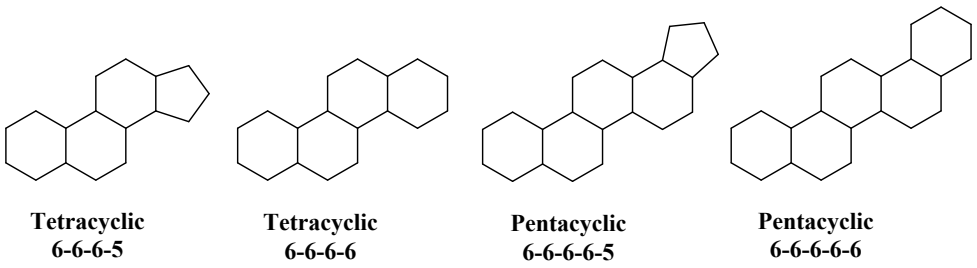


Fig. 2: Examples of basic skeletons of tetracyclic and pentacyclic triterpenes. Sidechains are not shown.

regulators (plant hormones – gibberellins, strigolactones, cytokinins; animal hormones – steroids),[18] or as elements of membrane structure and function (sterols – cholesterol [Fig. 3] forms part of the cellular membrane in animals),[2] *pentacyclic* triterpenoids serve primarily as a plant defense against pathogens, herbivores, pests,[19] and in many cases, their function still remains unknown.

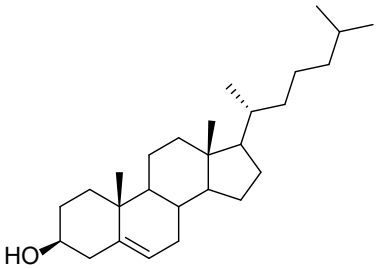


Fig. 3: The structure of cholesterol – tetracyclic triterpenoid.

Pentacyclic triterpenoids represent a highly interesting group of terpenes in terms of their biological activities.[20] Among them, the lupane (**VI**, Fig. 4), oleanane (**VII**), or ursane (**VIII**) analogs are the most important. They can be found mainly in peels, leaves, stems, and bark of numerous plants (e.g. birch and sycamore bark, fruit peels, etc.).[20] It was found that they have antiviral,[21] antibacterial,[22] antifungal,[23] antiulcer,[24] anti-inflammatory,[25] and antidiabetic[26] activities, therefore, some species have been used for centuries in folk or traditional Chinese medicine.[27] Furthermore, they exhibit antioxidative,[28] hepatoprotective,[29] cardioprotective,[30] neuroprotective,[31] antimalarial,[32,33] anti-HIV,[34,35] and anticancer[36–38] activity. It is more than obvious that due to such a palette of biological activities and structures, triterpenes are extremely interesting for detailed research.

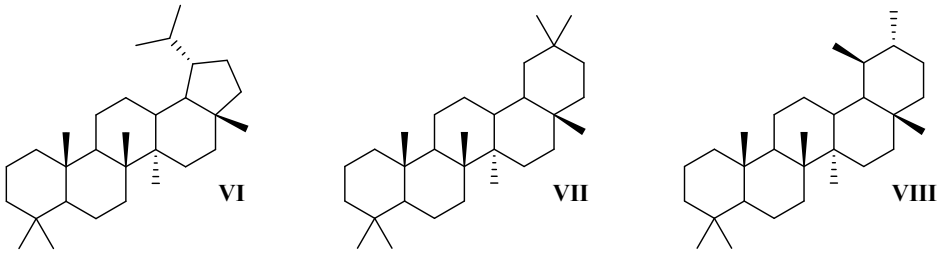


Fig. 4: Structures of lupane (**VI**), oleanane (**VII**), and ursane (**VIII**).

Not to mention only the good properties, pentacyclic triterpenes also have some disadvantages. It results from the nature of their structure that they are not readily soluble in water. This makes it difficult to ensure their delivery to the desired targets in living organisms and specific cells, which affects their bioavailability.[39,40] Furthermore, the effective concentration of the drug is often not low enough to be useful as a therapeutic, sometimes their selectivity is low. Finally, triterpenes have many mechanisms of action,[41] some of them still remain unknown, making their studies complicated. Hence, many scientists focus their forces on the deep exploration, development, and improvement of these unfavorable triterpenoid properties. They are looking for possible solutions to the above-mentioned problems by modifications and derivatization of the parent active structures. Every year, hundreds of new triterpenes are synthesized/isolated from natural sources and tested for various biological activities; all studies are periodically summarized in the literature.[42] Appropriately modified pentacyclic triterpenes can undoubtedly be a useful tool in the fight against diseases of civilization, such as cancer.

The theoretical part of the Ph.D. thesis is focused on the lupane-type triterpenoids only. The lupane structure (**VI**, Fig. 5, see the numbering) is composed of four six-membered rings A–D and one five-membered ring E, bearing an isopropyl group. Six methyl groups (numbered 23–28) are part of the basic skeleton. All other compounds of this type are derived from the structure below.

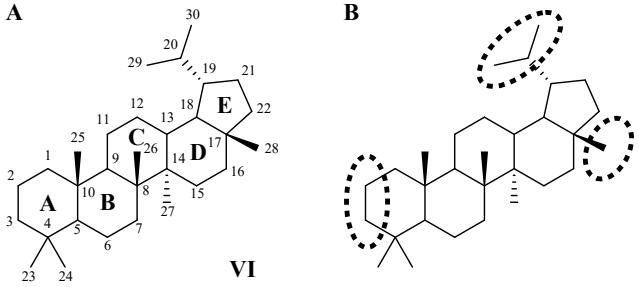


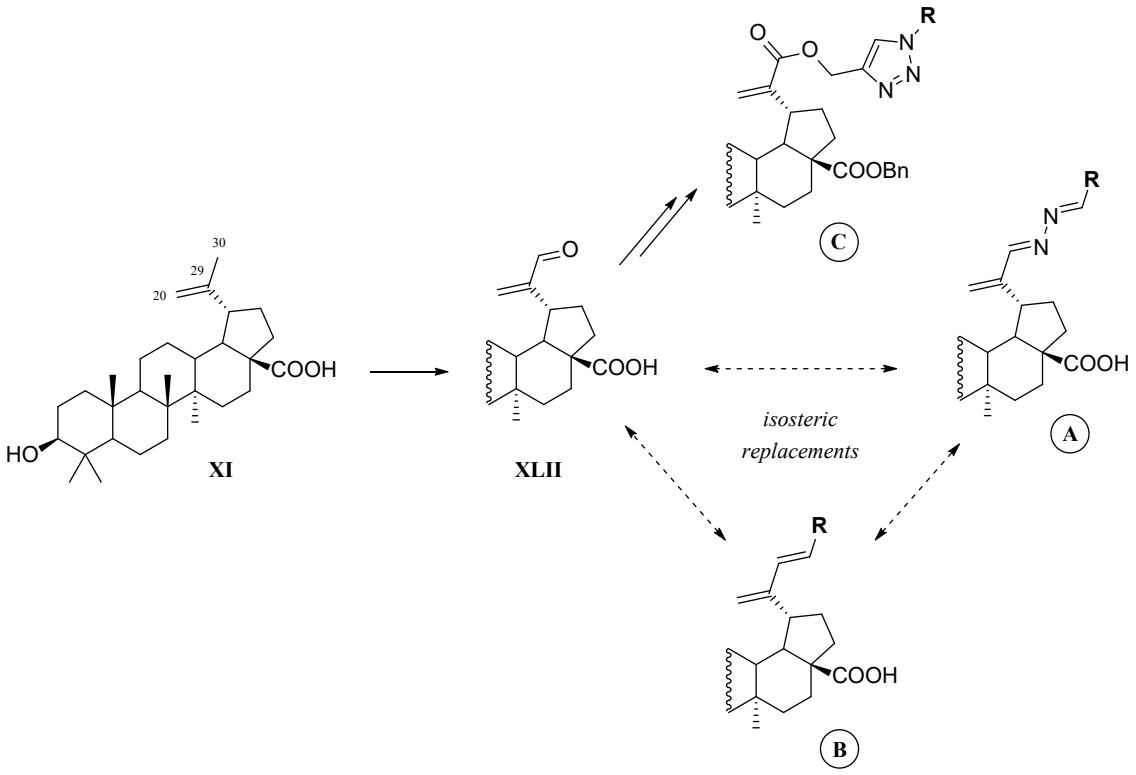
Fig. 5: A) Numbering of the lupane (**VI**) structure and cycles;
B) typical positions for modification.

The theoretical part includes a comprehensive review focusing on the methods for possible modification of the double bond C-20(29) at the isopropenyl moiety of lupene and other possibilities for introducing a new double bond at the position C-30 in the context of biological, in particular cytotoxic activity. Cyclizations of the whole isopropenyl unit considering the double bond are also discussed. Detailed literature research of the modifications at this position was performed since the vast majority of the practical work of this Ph.D. thesis is devoted to the modification of the C-30 position. Then, an overview of possible positions of double bonds at the A-ring of the lupane skeleton is discussed. Next chapters are focused on the Huisgen cycloaddition reaction, its copper(I)-catalyzed variant, Wittig reactions and Heck reactions, which were all experimentally used to modify the triterpenoid structure.

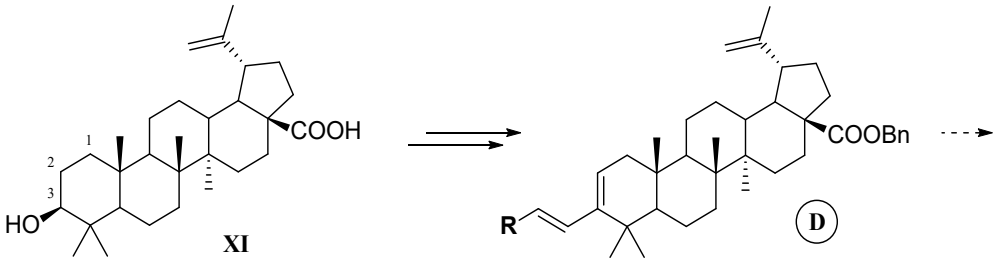
2 Aims of the work

The aims of the Ph.D. Thesis are the following:

- theoretical overview focused on the modifications of double bonds of lupane-type triterpenoids and their biological activity
- synthesis, purification, and characterization of new betulinic acid derivatives modified at the position C-30 according to Scheme 1
- development of the synthetic route for the preparation of new compounds with a double bond using Heck reaction according to Scheme 2
- cytotoxic testing of the prepared final compounds (performed by the biological department at the Institute of Molecular and Translational Medicine, Faculty of Medicine, Palacký University Olomouc)



Scheme 1: Derivatives of betulinic acid (**XI**) modified at the C-30 position: A) aromatic azines, B) Wittig products, C) triazoles.



Scheme 2: D) Derivatives of betulinic acid (**XI**) modified at the C-3 position using the Heck reaction.

Based on the literature review, it is clear that betulinic acid (**XI**) still has a lot to offer due to its wide scope of biological activities. There are many unexplored options for further modifications and improvement of its undesirable properties. In particular, 30-oxobetulinic acid (**XLII**) with its low IC_{50} values could potentially be a hot candidate for cancer treatment, but the major drawback of this compound is the low selectivity between cancer and healthy cells caused by the presence of the Michael acceptor. Therefore, this thesis aimed to find synthetic approaches leading to new betulinic acid (**XI**) derivatives with anticancer activity modified especially at the C-30 position, which tried to overcome the problem of low selectivity of the parent compound **XLII** with the preservation of the 30–C=X double bond.

Nitrogen-containing substituents in both C-30 and C-3 positions, such as variously substituted imines, hydrazones, or oximes, significantly contributed to the biological activity of the prepared lupane compounds.[43–47] In the first project, analogous approach was applied for masking the aldehyde moiety in 30-oxobetulinic acid (**XLII**), and novel hydrazone conjugates bearing variously substituted aromatic rings were prepared (A, Scheme 1). These derivatives were used to verify whether such modification of **XLII** could help to improve the selectivity while retaining the activity against cancer cells. After this study was finished, the improvement in selectivity was successfully confirmed, however, limited stability of the hydrazone moiety became apparent which led to a second aim of the thesis – to modify the aldehyde group at the C-30 position using Wittig reactions, which represent a bioisosteric replacement of the azine bridge with a carbon atom. The new double bond 30–C=C was expected to act similarly as the original 30–C=N in living cells, but its stability could be much higher (B, Scheme 1).

The next goal was to prepare new betulinic acid derivatives using Cu(I)-catalyzed azide-alkyne cycloaddition in order to build on the previous research in this area (C, Scheme 1).

Finally, the last target was to optimize the use of the Heck reaction as a modern cross-coupling method for introducing a new double bond attached to the A–ring of the lupane skeleton (D, Scheme 2). The scope of this reaction has not been well explored in the chemistry of triterpenes yet. Therefore, its use was evaluated in terms of double bonds at the A-ring at the position C-3 where similar cross-coupling reactions on triterpenoids were performed previously.

3 Results and discussion

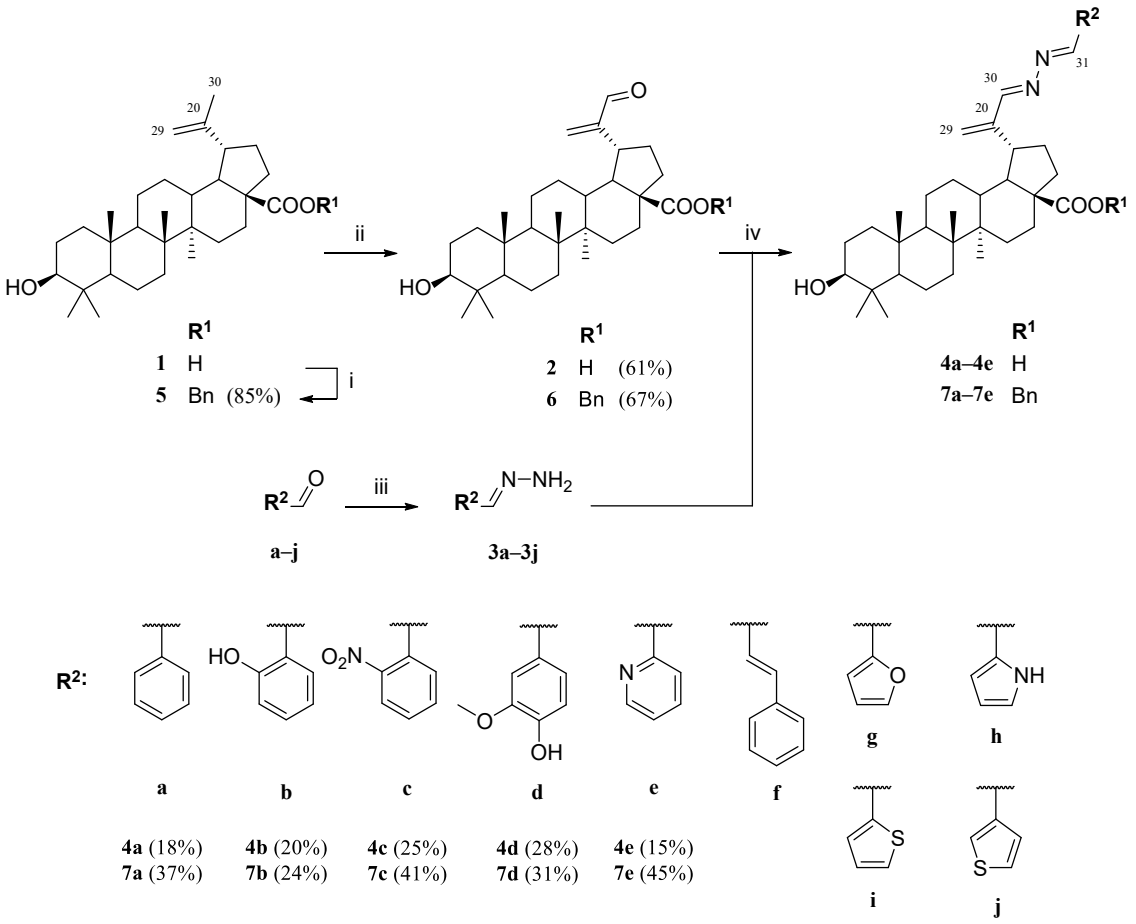
3.1 Modifications of betulinic acid at the isopropenyl moiety

3.1.1 Azines

This part was published in the Future Medicinal Chemistry journal in 2018:

Pokorný, J.; Krajčovičová, S.; Hajdúch, M.; Holoubek, M.; Gurská, S.; Džubák, P.; Volná, T.; Popa, I.; Urban, M., Triterpenic azines, a new class of compounds with selective cytotoxicity to leukemia cells CCRF-CEM, *Future Med. Chem.* **2018**, *10* (5), 483–491.

In order to improve the low selectivity of the 30-oxobetulinic acid (**2**, Scheme 3), condensation reactions with substituted aromatic hydrazones were performed to mask the Michael acceptor present in the parent compound. New derivatives containing fully conjugated azine bridge showed promising anticancer properties with high selectivity towards the CCRF-CEM cancer cell line. The disadvantages were low yields of the desired compounds and limited stability in water. It was sufficient for the *in vitro* tests but would likely be insufficient in anticancer drugs. Ten new derivatives of betulinic acid (**1**, Scheme 21) were prepared, five as free acids at the position C-28 (**4a**–**4e**), and five with protection by benzyl ester at C-28 carboxyl function (**7a**–**7e**). All compounds were fully characterized and tested for their cytotoxic activities.



Scheme 3: Preparation of the azine derivatives **4a**–**4e** and **7a**–**7e**. Reagents and conditions: i) Benzyl bromide, K_2CO_3, THF, reflux, 4 h; ii) SeO_2, 2-methoxyethanol, 110 °C, 4 h; iii) $N_2H_4 \cdot H_2O$, EtOH, reflux, 15 min; iv) **3a**–**3j** or phenylhydrazine, EtOH, reflux, 2–6 h.

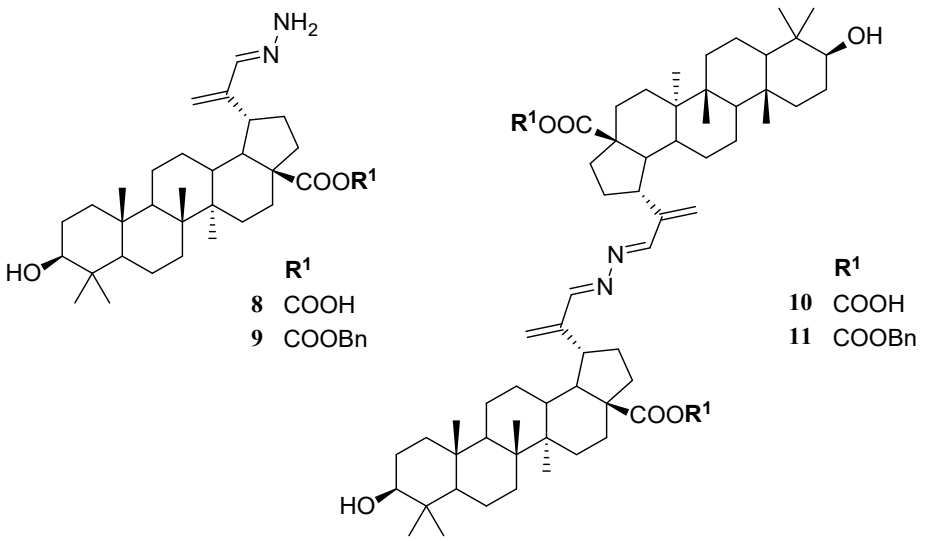


Fig. 6: Sideproducts of the reactions.

Prepared azines **4a**–**4e** and **7a**–**7e**, together with parent compounds – betulinic acid (**1**) and 30-oxobetulinic acid (**2**) were evaluated for their cytotoxic activity *in vitro* using MTS assay against a panel of eight cancer cell lines (CCRF-CEM, CEM-DNR, HCT116, HCT116 $p53^{-/-}$, K562, K562-TAX, A549, U2OS), and two human normal fibroblasts (BJ, MRC-5). Results of the IC_{50} values are displayed in Tab. 1.

Tab. 1: Cytotoxic activities of prepared azine derivatives **4a**–**4e** and **7a**–**7e** in eight tumor and two normal fibroblast cell lines. The most active derivative **4d** is highlighted.

Comp.	IC_{50} ($\mu mol \cdot L^{-1}$)[a]										
	CCRF-CEM	CEM-DNR	HCT 116	HCT116 $p53^{-/-}$	K562	K562-TAX	A549	U2OS	BJ	MRC-5	SI[b]
1[c]	45.5	45.4	38.0	>50	40.0	43.1	43.4	>50	37.6	32.9	0.7
2[c]	**3.1**	**3.7**	**2.5**	**3.0**	7.6	**3.3**	**3.8**	**3.5**	6.1	**4.1**	1.6
4a	**4.5**	30.9	21.5	16.7	34.9	28.3	25.2	29.9	>50	>50	>11.1
4b	5.6	26.8	29.4	20.8	39.8	26.8	34.2	23.4	>50	40.4	>8.1
4c	8.8	31.9	39.0	36.3	47.8	46.3	33.0	37.1	>50	>50	>5.7
4d	**3.4**	27.1	11.4	11.5	15.6	28.8	29.7	14.1	>50	48.9	>14.7
4e	**3.9**	27.9	9.6	16.6	20.7	28.7	28.1	10.5	45.2	33.1	10.0
7a	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	-
7b	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	-
7c	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	-
7d	21.7	>50	45.9	37.9	34.5	>50	47.0	33.5	32.1	36.9	1.6
7e	18.8	>50	>50	30.3	25.6	>50	48.5	30.5	45.7	45.3	2.4

[a] The lowest concentration that kills 50 % of cells. The maximum standard deviation in cytotoxicity assays is typically 15% of the average value. [b] Selectivity index is calculated for the IC_{50} of the CCRF-CEM line vs. average IC_{50} values of both fibroblasts (BJ and MRC-5). [c] Parent compound is used as a standard.

Results showed that all derivatives **4a**–**4e** with free carboxyl group were active against the CCRF-CEM line in a low micromolar range, compound **4d** was the most active and selective with the selectivity index SI > 14.7, meaning significant improvement in comparison with the parent compounds **1** and **2** which suffered from general toxicity. Cell cycle analysis and nucleic acid synthesis in CCRF-CEM cell lines were further evaluated to show that compounds trigger selective apoptosis *via* intrinsic pathway.

3.1.2 Wittig reactions

Results from this project were published in the European Journal of Medicinal Chemistry in 2021:

Pokorný, J.; Olejníková, D.; Frydrych, I.; Lišková, B.; Gurská, S.; Benická, S.; Šarek, J.; Kotulová, J.; Hajdúch, M.; Džubák, P.; Urban, M., Substituted Dienes Prepared from Betulinic Acid – Synthesis, Cytotoxicity, Mechanism of Action, and Pharmacological Parameters, *Eur. J. Med. Chem.* **2021**, *224*, 113706.

In the previous project, triterpenic azines were proved to be selective against the CCRF-CEM cancer cell line in comparison with the parent compound, 30-oxobetulinic acid (**2**, Scheme 3). However, they also exhibited low stability and their preparation suffered from poor yields. Therefore, it was decided to modify the same substrate using Wittig reactions to obtain isosteric analogs with conjugated double bonds and variously substituted aromatic ring. In total, forty-nine triphenylphosphonium salts **12.1**–**12.51** (Fig. 7) were prepared and two purchased to react in the Wittig reaction with 30-oxobetulinic acid (**2**). The reaction was optimized and forty new triterpenoid conjugates **13.1**–**13.49** (Scheme 4) were obtained, fully characterized, and tested for cytotoxic activity in eight tumor cell lines and two non-cancer fibroblasts. During the optimizations, both *E*/*Z* isomers were often found in various ratios in NMR spectra of the resulting Wittig products after the purification. From NMR analyses, it was found out that the reaction stereochemistry outcome is dependent on the reaction temperature. When the Wittig reaction was performed at a higher temperature, the amount of the *Z* isomer was usually higher (up to 3 : 1 for *E*/*Z* in refluxing toluene, depending on the nature of the ylide used), while at room temperature the ratio was lower but still significant (up to 5 : 1 for *E*/*Z* in toluene, NMR analysis). Unfortunately, the two isomers were not visible on TLC as two spots, and they were inseparable using standard column chromatography. Finally, the reaction temperature at 0 °C during the addition of the aldehyde **2** into the prepared ylide markedly helped to decrease the amount of the *Z* isomer, while the formation of the *E* isomer was predominant (at least 15 : 1 for *E*/*Z* at 0 °C in THF). Using these optimized conditions, most of the salts **12.1**–**12.51** were successful in the Wittig reaction giving the pure *E* isomer in moderate – high yield.

All prepared derivatives **13.1**–**13.49** with the parent aldehyde **2** were tested for their cytotoxic activities *in vitro* using standard MTS assay against a panel of eight cancer cell lines (CCRF-CEM, CEM-DNR, HCT116, HCT116 $p53^{-/-}$, K562, K562-TAX, A549, U2OS), and two human normal fibroblasts (BJ, MRC-5). Results of the IC_{50} values are displayed in Tab. 2. Derivatives are considered inactive when $IC_{50} > 50\ \mu mol \cdot L^{-1}$. Compounds **13.22** and **13.39** showed the best results in primary screening with the highest cytotoxic activity against CCRF-CEM ($IC_{50} = 3.2$ μmol/L for **13.22**, and $IC_{50} = 3.6$ μmol/L for **13.39**, respectively) and favorable

selectivity index (SI > 13.0 for **13.22**), therefore, they were subjected for further biological testing (cell cycle analysis, synthesis of DNA and RNA, protein expression, cell death, pharmacological parameters) to evaluate their mechanism of action. It was found that both compounds trigger selective apoptosis in cancer cells *via* intrinsic pathway. Pharmacological parameters of **13.22** were better than for **13.39**, therefore, the derivative **13.22** was the finally selected candidate for further preclinical development as anticancer drug

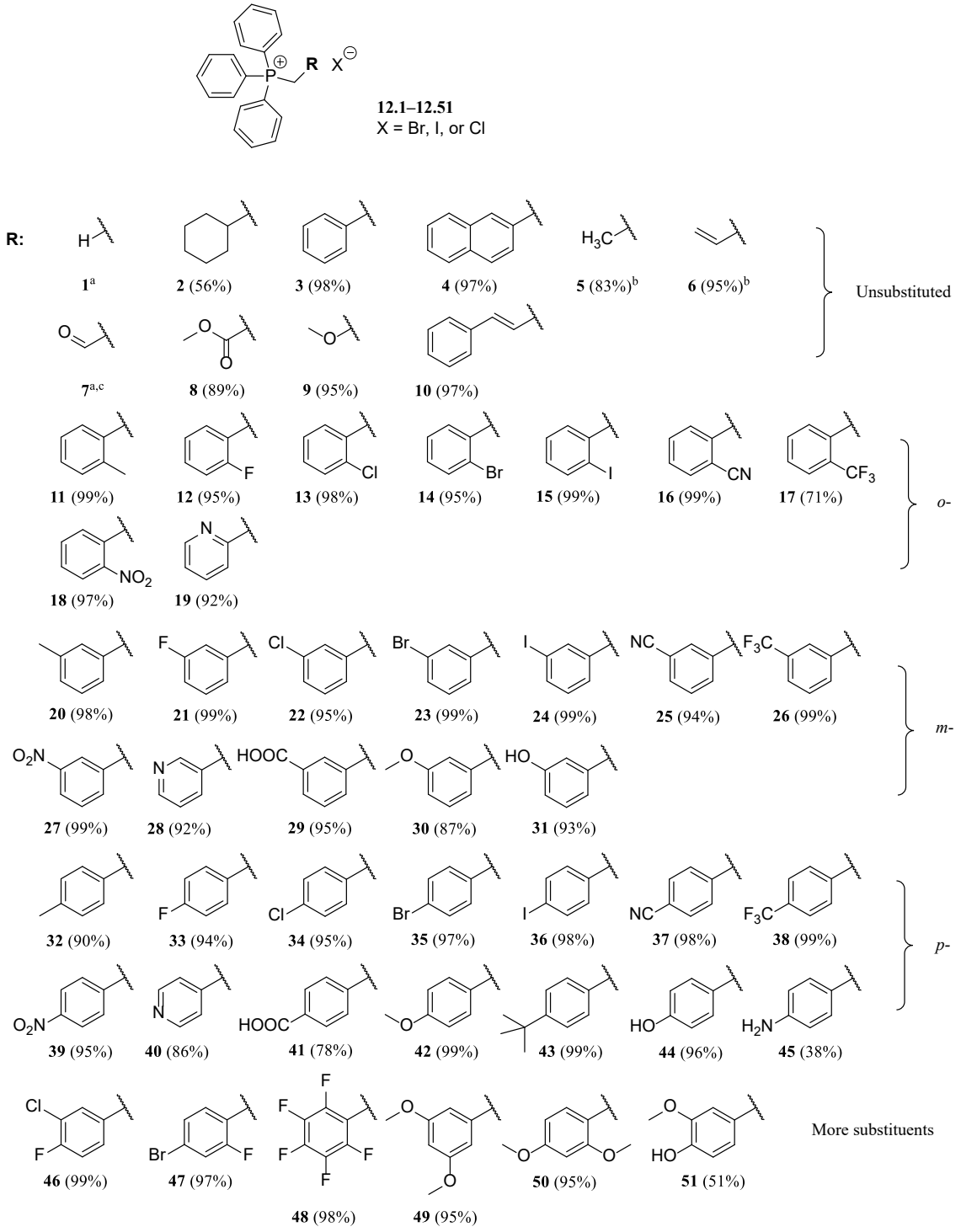
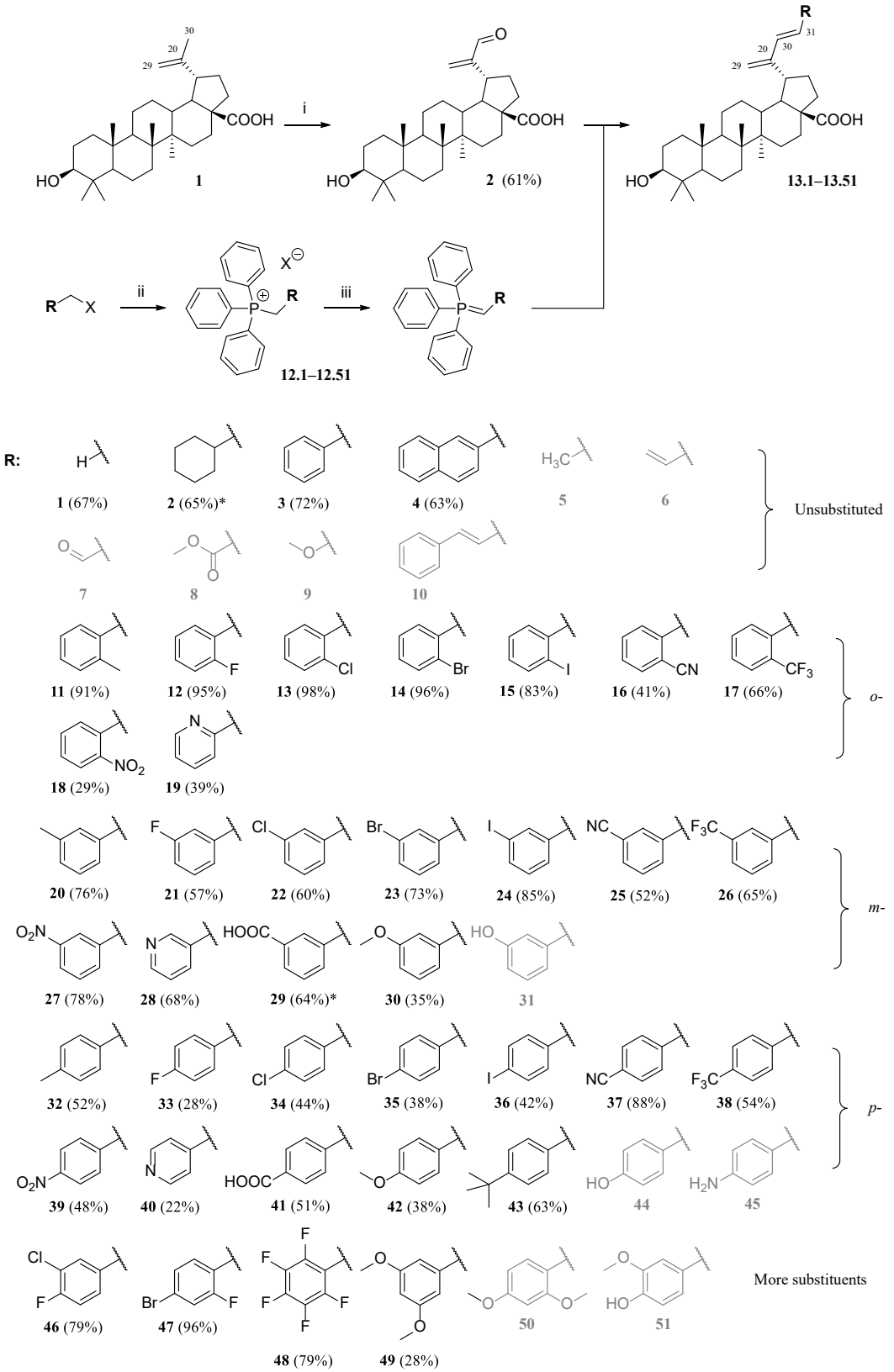


Fig. 7: List of triphenylphosphonium salts prepared in this project with corresponding yields. [a] Purchased salt; [b] Iodide salt; [c] Chloride salt.



Scheme 4: Wittig reactions with 30-oxobetulinic acid (**2**). Reagents and conditions: i) SeO_2, 2-methoxyethanol, 110 °C, 4 h; ii) PPh_3, toluene/acetonitrile, reflux, overnight; iii) *t*-BuOK, THF, r. t., 30 min; iv) THF, 0 °C, then r. t., 2–18 h. *Yields for compounds **12.2** and **12.29** are given for a mixture of both *E*/*Z* isomers. Derivatives **12.5**–**12.10**, **12.31**, **12.44**, **12.45**, **12.50**, and **12.51** were not obtained.

Tab. 2: Cytotoxic activities of prepared derivatives **13.1**–**13.49** and the parent compound **2** in eight tumor and two normal fibroblast cell lines. The most active derivatives **13.22** and **13.39** are highlighted.

Comp.	IC_{50} ($\mu mol \cdot L^{-1}$)[a]										
	CCRF -CEM	CEM- DNR	HCT 116	HCT116 $p53^{-/-}$	K562	K562- TAX	A549	U2OS	BJ	MRC- 5	SI[b]
2[c]	**0.8**	**3.8**	**1.9**	**1.9**	**3.4**	**1.3**	>50	**2.2**	6.7	**2.6**	5.7
13.1	11.1	25.1	24.1	24.3	15.7	26.2	23.9	24.1	44.6	24.3	3.1
13.2[d]	8.0	45.7	15.2	>50	>50	43.6	>50	>50	>50	>50	>6.3
13.3	6.5	30.2	35.5	39.5	40.0	25.3	41.8	24.2	>50	>50	>7.7
13.4	11.8	47.0	30.2	26.3	>50	45.8	32.9	27.2	>50	43.1	>3.9
13.11	19.1	22.8	29.7	25.9	28.1	25.9	26.8	28.0	33.0	31.5	1.7
13.12	15.4	23.2	24.5	22.6	21.8	25.1	29.7	27.7	42.1	31.7	2.4
13.13	18.7	42.1	26.6	24.8	23.9	39.3	29.4	28.1	42.3	32.6	2.0
13.14	16.8	49.1	27.2	24.5	21.6	47.9	28.6	27.8	46.7	35.3	2.4
13.15	20.6	>50	29.6	29.5	25.1	>50	29.7	28.5	>50	29.5	>1.9
13.16	23.2	26.8	36.6	47.0	>50	24.8	>50	40.4	>50	>50	>2.2
13.17	21.8	>50	33.0	36.1	48.7	>50	37.0	29.6	>50	>50	>2.3
13.18	23.7	30.6	34.8	39.3	>50	29.8	45.2	33.1	>50	>50	>2.1
13.19	19.7	27.7	>50	>50	>50	29.2	>50	>50	>50	>50	>2.5
13.20	9.5	21.4	24.7	22.3	24.9	23.6	36.5	18.2	>50	42.2	>4.9
13.21	11.7	23.1	23.0	21.0	21.6	21.9	28.7	27.7	41.5	32.1	3.1
13.22	**3.2**	25.5	16.4	15.2	27.7	25.6	29.2	17.3	>50	32.6	>13.0
13.23	8.8	35.9	25.3	26.8	23.6	32.8	33.1	27.6	>50	>50	>5.7
13.24	11.6	>50	27.3	29.5	25.2	48.4	38.7	28.4	>50	>50	>4.3
13.25	12.1	21.6	35.1	29.4	>50	22.7	>50	>50	>50	>50	>4.1
13.26	8.0	28.1	23.1	24.8	26.5	25.1	35.1	24.6	>50	42.9	>5.8
13.27	12.4	25.3	37.5	35.5	>50	24.3	48.8	29.3	>50	45.1	>3.8
13.28	11.3	17.4	30.9	32.8	>50	14.2	26.2	30.9	>50	>50	>4.4
13.29[d]	20.1	33.9	43.4	43.1	>50	36.7	>50	>50	>50	>50	>2.5
13.30	**4.6**	30.6	34.6	39.6	41.4	25.6	40.1	25.2	>50	44.1	>10.3
13.32	5.9	26.1	21.4	18.2	>50	26.3	>50	>50	>50	>50	>8.5
13.33	**4.9**	31.4	35.4	41.0	36.2	29.8	40.6	23.9	44.6	38.3	8.5
13.34	5.4	33.1	39.5	38.5	46.3	29.2	45.4	25.8	>50	>50	>9.2
13.35	6.5	28.9	33.2	31.8	>50	29.3	>50	27.0	>50	35.2	>6.6
13.36	16.0	>50	30.7	33.3	>50	>50	>50	32.2	>50	41.2	>2.9
13.37	23.8	27.0	39.1	43.3	>50	27.2	>50	44.1	>50	49.7	>2.1
13.38	21.5	40.9	40.9	31.9	40.5	45.6	41.4	28.2	49.5	42.4	2.1
13.39	**3.6**	22.9	18.6	15.7	11.5	23.4	14.3	19.5	24.3	19.2	6.1
13.40	5.9	20.5	35.0	30.6	43.4	20.6	46.0	29.7	>50	>50	>8.4
13.41	17.5	32.0	39.0	42.5	>50	39.2	>50	49.0	>50	>50	>2.9
13.42	20.9	>50	42.4	48.9	>50	48.6	>50	48.6	>50	>50	>2.4
13.43	45.6	>50	>50	>50	>50	>50	>50	45.1	>50	>50	>1.1
13.46	5.5	27.8	21.8	20.8	40.6	25.2	45.5	26.5	>50	32.4	>7.5
13.47	22.4	>50	33.5	33.6	26.0	>50	32.5	29.7	43.0	33.7	1.7
13.48	22.1	>50	32.2	36.3	29.8	>50	32.2	30.2	>50	43.2	>2.1
13.49	10.0	13.7	17.3	20.8	22.2	16.0	19.0	22.3	47.6	29.2	3.8

[a] The lowest concentration that kills 50 % of cells. The maximum standard deviation in cytotoxicity assays is typically 15 % of the average value. Compounds with $IC_{50} > 50$ μmol/L were considered inactive. [b] Selectivity index is calculated for IC_{50} of CCRF-CEM line vs. the average IC_{50} values of both fibroblasts (BJ and MRC-5). [c] Parent compound is used as a standard. [d] IC_{50} value was measured for a mixture of both *E*/*Z* isomers.

3.1.3 CuAAC reactions at the position C-30

The background of this project was described in the review article:

Pokorný, J.; Borková, L.; Urban, M., Click Reactions in Chemistry of Triterpenes – Advances Towards Development of Potential Therapeutics, *Current Med. Chem.* **2018**, *25* (5), 636–658.

The project follows these publications:

Šidová, V.; Zoufalý, P.; **Pokorný, J.**; Džubák, P.; Hajdúch, M.; Popa, I.; Urban, M., Cytotoxic conjugates of betulinic acid and substituted triazoles prepared by Huisgen Cycloaddition from 30-azidoderivatives, *PLoS ONE* **2017**, 12, 2, e0171621.

and

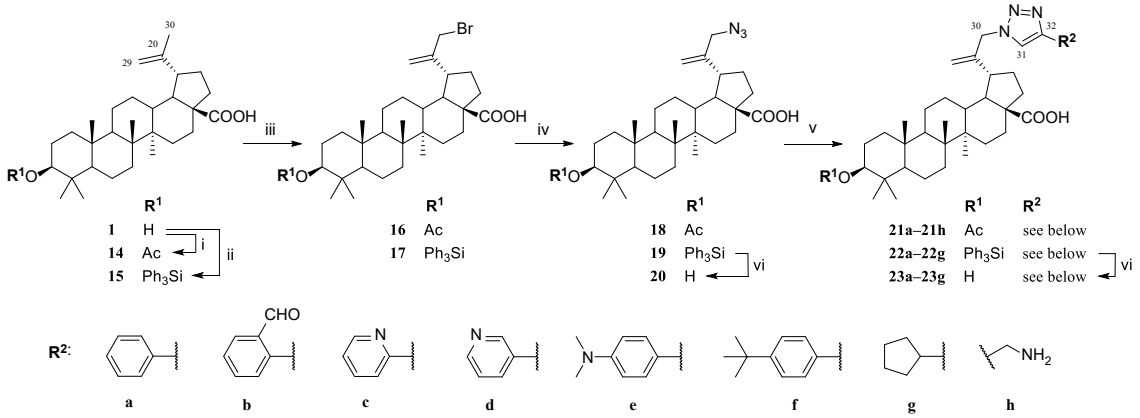
Pokorný, J.; Horka V.; Šidová V.; Urban M., Synthesis and characterization of new conjugates of betulin diacetate and bis(triphenysilyl) betulin with substituted triazoles, *Monatsh. Chem.* **2018**, *149* (4), 839–845.

In these two studies, three sets of betulinic acid derivatives with the free C-28 carboxyl group **21**–**23** (Scheme 5) and variously protected/not protected C-3 hydroxyl group, followed by two sets of both OH-protected betulin derivatives **31**–**32** (Scheme 6) were prepared using CuAAC leading to new conjugates, containing a variously substituted triazole ring at the position C-30. Most of the compounds in both series were synthetized by students under my supervision. All derivatives were then subjected to cytotoxicity testing against 8 tumor cell lines and 2 non-cancer fibroblasts.

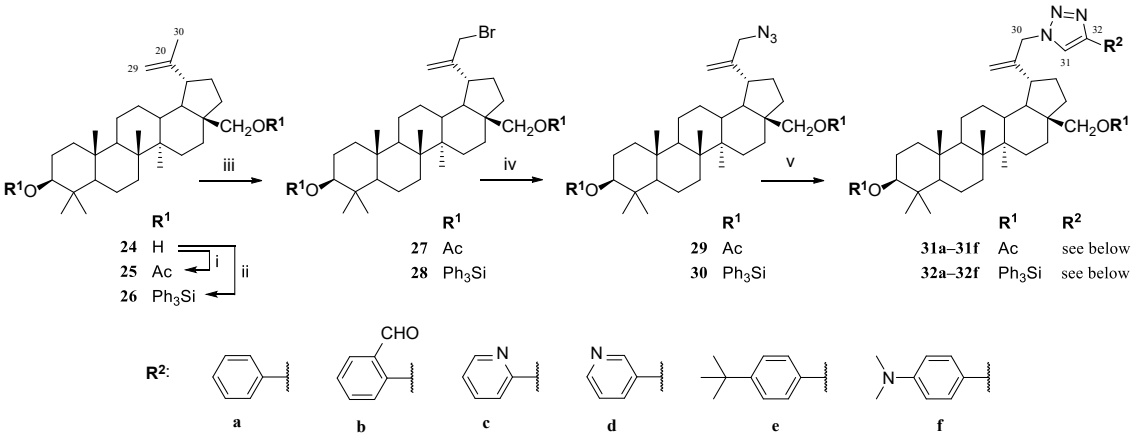
In the first study[48], 22 final derivatives **21a**–**21h** and **22a**–**22g** (Scheme 5) with the protection at C-3 were prepared by CuAAC using alkynes **a**–**h** in moderate to high yields. Unprotected derivatives **23a**–**23g** were then prepared from their silylated analogs. Cytotoxicity testing revealed that acetates **21a**–**21h** were highly active on multiple cancer cell lines with IC_{50} values in low micromolar ranges, which was in contrast with their less active unprotected analogs **23a**–**23g** with the free 3β-OH, which we expected to have the potential highest activity. Moreover, the 3β-*O*-acetate precursors **16** and **18** showed significant cytotoxicity. The most active derivative **21b** with benzaldehyde connected to the triazole ring had IC_{50} = 3.3 $\mu mol \cdot L^{-1}$ against the CCRF-CEM cell line. Results suggested that both the acetate and triazole probably positively influence the cellular uptake while the triazole may also become part of the pharmacophore.

In the second article[49], twelve new final conjugates – diacetates **31a**–**31f** and bis(triphenylsilyl) derivatives **32a**–**32f** (Scheme 6) were synthesized in moderate-high yields for the SAR study to extend the previously prepared library of betulinic acid

derivatives. Unfortunately, the solubility of the final compounds was not sufficient to perform cytotoxicity assays. In addition, attempts to deprotect the derivatives that could help to improve their solubility in water-based media used in biological tests were unsuccessful, and therefore only their synthesis was published, and the compounds were not further developed.



Scheme 5: Derivatives of betulinic acid (**1**) prepared by CuAAC reaction. Reagents and conditions: i) Ac_2O, pyridine, r. t., 16 h; ii) Ph_3SiCl, imidazole, DMF, r. t., 36 h; iii) NBS, AIBN, CCl_4, 75 °C for 1 h, then 50 °C for 3 h; iv) NaN_3, DMSO, r. t., 36 h; v) $CuSO_4 \cdot 5H_2O$, sodium L-ascorbate, alkynes **a**–**h**, DMF, r. t. or 50 °C; vi) TBAF, THF, r. t. 18–32 h, or HCl, DCM, r. t., 5–11 h.[48]

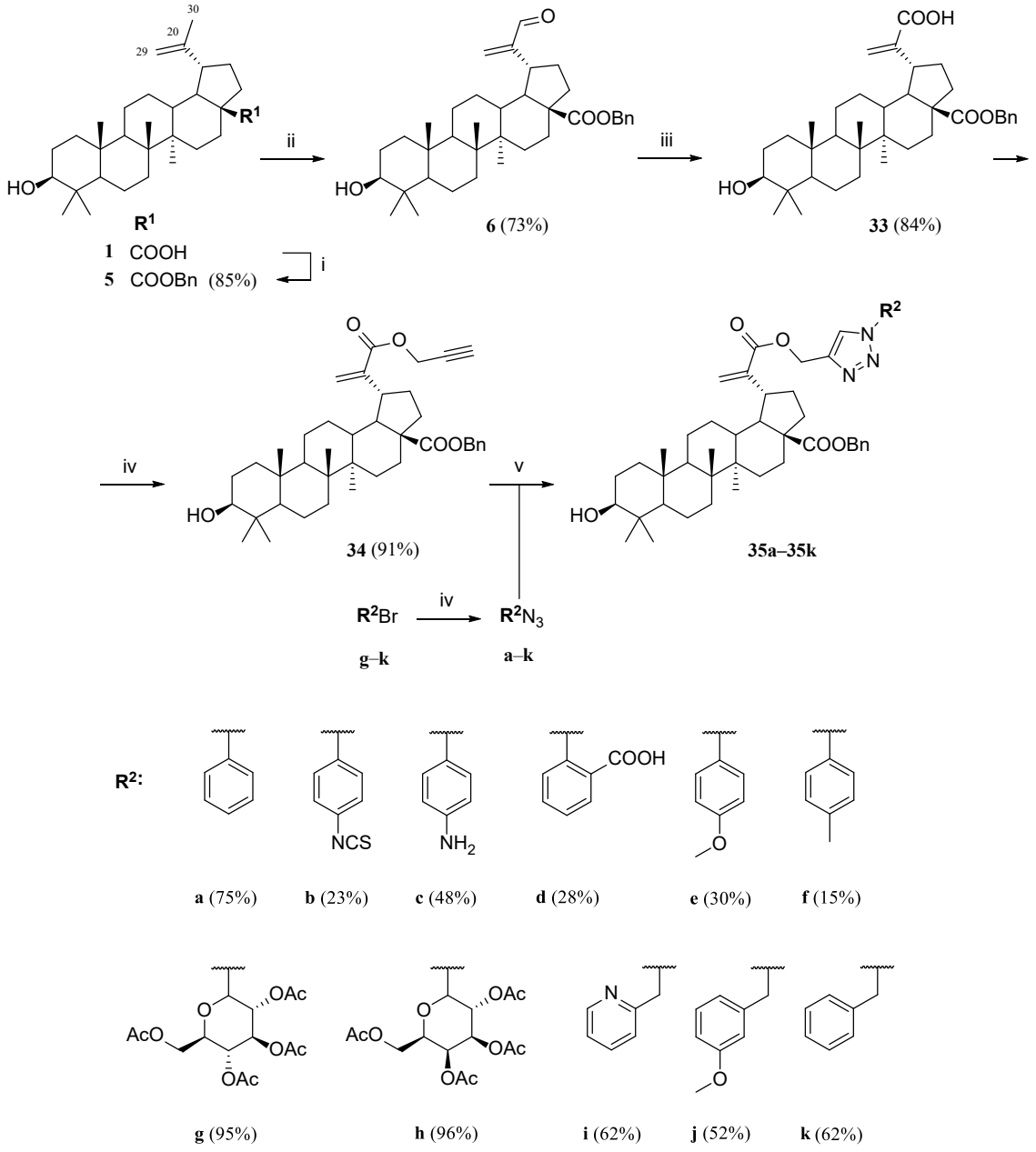


Scheme 6: Derivatives of betulin (**24**) prepared by CuAAC reaction. Reagents and conditions: i) Ac_2O, pyridine, r. t., 16 h; ii) Ph_3SiCl, imidazole, DMF, 50 °C, 36 h; iii) NBS, AIBN, CCl_4, reflux; iv) NaN_3, DMSO, r. t., 36 h; v) $CuSO_4 \cdot 5H_2O$, sodium L-ascorbate, alkynes **a**–**h**, *t*-BuOH/H_2O 2 : 1, 50 °C, 16 h.[49]

In order to further extend existing biological information from the previous SAR study in the articles above, a new set of eleven triterpenic triazoles **35a**–**35k** (Scheme 7) was prepared from betulinic acid (**1**) using CuAAC. In this project, the connection between the terpene and the triazole ring was represented by an ester function, which could be easily cleaved by nonspecific esterases in a cell, and therefore, such compound could possibly serve as a prodrug. Moreover, some compounds from previous studies with the same type of connection between the terpene and

the triazole ring showed strong neuroprotective activity.[50] Therefore, this new set of derivatives will be evaluated in neurodegeneration models. Following results have not yet been published.

During the synthesis, it was found that the purity of the used azide significantly affects the yield of the cycloaddition. Only moderate–low yields were achieved with commercially available azides **b**–**f**, while fresh purified azides (**g**, **h**) afforded products in almost quantitative yields with 100% conversion of the starting material as is typical for the click chemistry. This fact is supported by the observations of our



Scheme 7: CuAAC reactions on betulinic acid (**1**). Reagents and conditions: i) BnBr, K_2CO_3, THF, reflux, 3 h; ii) SeO_2, 2-methoxyethanol, 110 °C, 4 h; iii) $NaClO_2$, KH_2PO_4, 2-methyl-2-butene, *t*-BuOH/H_2O 1 : 1, r. t. 5 h; iv) propargyl bromide, K_2CO_3, THF, reflux, 4 h; v) corresponding azide, $CuSO_4 \cdot 5H_2O$, sodium ascorbate, *t*-BuOH/H_2O 2 : 1, 50 °C, 18 h; iv) NaN_3, DMF, r. t., overnight.

reactions with azides, which were freshly prepared but not purified (**i**–**k**), and then gave only medium yields of about 60%, while the conversion was still not complete. Moreover, the addition of the azide and other reactants did not help to complete the reaction, instead, unknown sideproducts were formed in minor amounts. Thus, the use of CuAAC in this case cannot be considered as the typical example of click chemistry due to lower yields, longer reaction times and higher reaction temperatures.

Prepared triazole derivatives **35a**–**35k** were tested for their cytotoxic activity *in vitro* using standard MTS assay against a panel of eight cancer cell lines (CCRF-CEM, CEM-DNR, HCT116, HCT116 $p53^{-/-}$, K562, K562-TAX, A549, U2OS), and two human normal fibroblasts (BJ, MRC-5). Results of the IC_{50} values are displayed in Tab. 3. Derivatives are considered inactive when $IC_{50} > 50\ \mu mol \cdot L^{-1}$.

Tab. 3: Cytotoxic activities of prepared triazole derivatives **35a**–**35k** in eight tumor and two normal fibroblast cell lines. The most active derivative **35i** is highlighted.

Comp.	IC_{50} ($\mu mol \cdot L^{-1}$)[a]										
	CCRF-CEM	CEM-DNR	HCT 116	HCT116 $p53^{-/-}$	K562	K562-TAX	A549	U2OS	BJ	MRC-5	SI[b]
1[c]	8.1	14.0	15.8	16.0	**4.3**	14.1	9.4	20.8	24.2	28.2	3.2
35a	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	-
35b	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	-
35c	30.4	>50	>50	49.3	>50	14.9	>50	15.2	>50	>50	>1.6
35d	14.6	29.8	29.5	28.8	27.1	21.7	29.1	23.2	29.3	25.7	1.9
35e	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	-
35f	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	-
35g	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	-
35h	44.1	>50	>50	>50	>50	>50	>50	>50	>50	>50	>1.1
35i	5.9	23.9	>50	>50	>50	8.7	>50	>50	>50	>50	>8.5
35j	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	-
35k	19.6	>50	>50	>50	>50	42.3	>50	>50	>50	>50	>2.6

[a] The lowest concentration that kills 50 % of cells. The maximum standard deviation in cytotoxicity assays is typically 15% of the average value. [b] Selectivity index is calculated for the IC_{50} of the CCRF-CEM line vs. average IC_{50} values of both fibroblasts (BJ and MRC-5). [c] Parent compound is used as a standard.

Results showed that only five derivatives **35c**, **35d**, **35h**, **35i**, and **35k** were active, however, this was expected due to the protection of the C-28 carboxyl group. Among them, compound **35d** showed general cytotoxicity in all tested cell lines in the moderate IC_{50} range of 14.6–29.8 $\mu mol \cdot L^{-1}$. The rest of the active compounds showed selective cytotoxicity against CCRF-CEM and K562-TAX cell lines, while they were inactive in human normal fibroblasts. The best candidate, derivative **35i**, was the most active in a low micromolar range in CCRF-CEM (IC_{50} = 5.9 $\mu mol \cdot L^{-1}$) and K562-TAX (IC_{50} = 8.7 $\mu mol \cdot L^{-1}$) cell lines with high selectivity (the selectivity index SI > 8.5), meaning significant improvement in comparison

with the parent compound, betulinic acid **1**, which suffer from general toxicity (SI = 3.2). The cell cycle analysis and nucleic acid synthesis in the CCRF-CEM cell lines were evaluated for the compound **35i**, results are summarized in Tab. 4.

Tab. 4: Cell cycle analysis and influence on DNA and RNA synthesis in CCRF-CEM cancer cells treated with 1 × IC_{50} and 5 × IC_{50} of compound **35i**.

		Used conc. ($\mu mol \cdot L^{-1}$)	**Sub G1** (%)	**G0/G1** (%)	**S** (%)	**G2/M** (%)	**pH3^{Ser10}** (%)	**DNA synthesis** (%)	**RNA synthesis** (%)
Control		0	2.22	40.73	41.22	18.06	1.92	36.39	32.41
35i	1 × IC_{50}	5.86	6.52	38.84	41.28	19.89	1.72	32.07	19.48
	5 × IC_{50}	29.30	9.75	44.77	38.44	16.80	1.09	27.87	23.91

The influence of the compound **35i** on cell cycle and DNA/RNA synthesis is not significant, however, inhibition of DNA and RNA synthesis is obvious at 5 × IC_{50} concentration. Compound **35i** led to the accumulation of the cells in the G0/G1 cell cycle phase at 5 × IC_{50}.

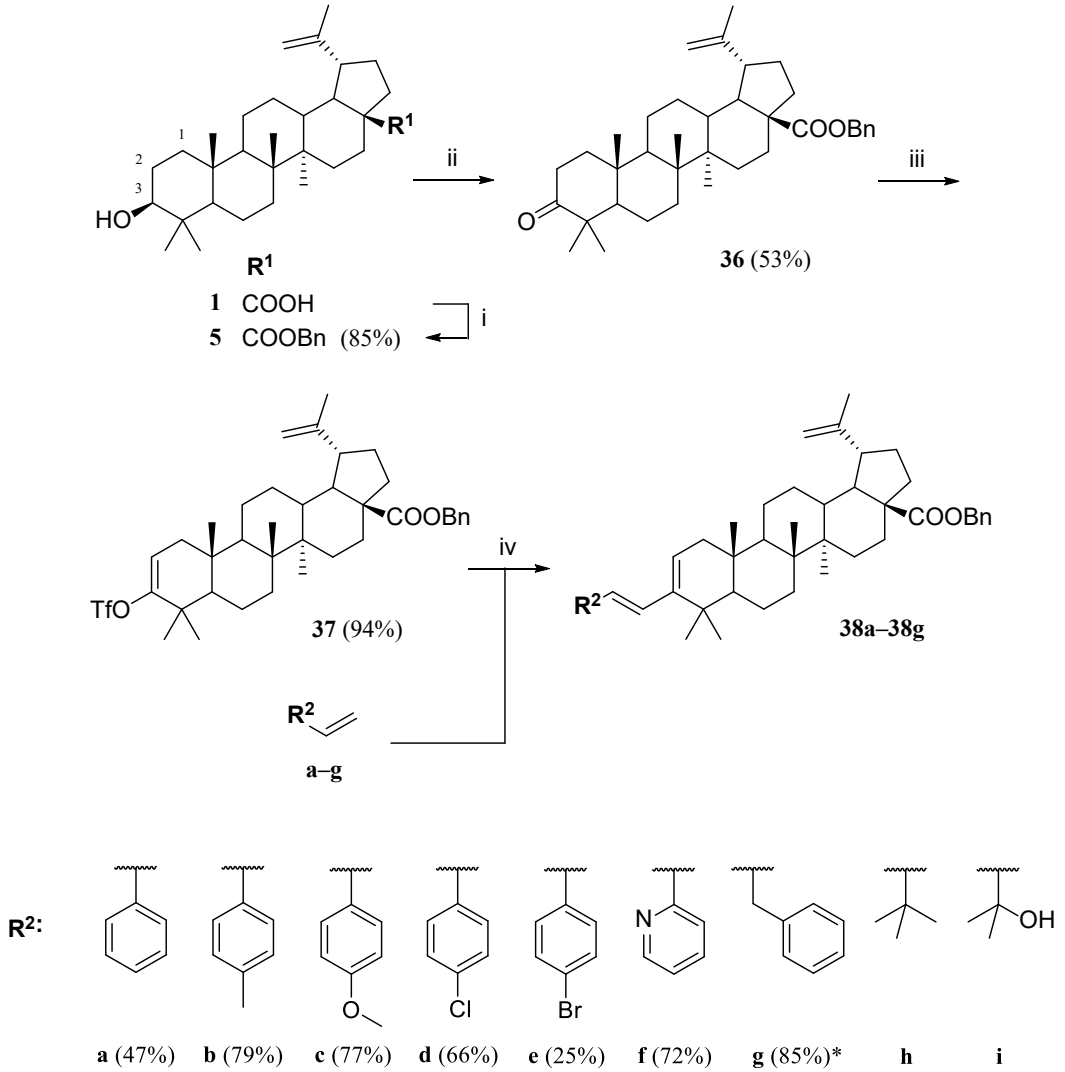
3.2 Modifications of the double bonds at the A-ring

Unsuccessful attempts to derivatize the isopropenyl unit by the Heck reaction were performed in the study of Zhu et al.[51] with very low yields, problems with conversion, stereoselectivity, and a special $Fe(OTf)_3$ catalyst. We decided to verify the standard Heck procedure in a less hindered position of the lupane skeleton. From the literature,[52,53] it is known that the Suzuki-Miyaura cross-coupling reaction works at the position C-3 at the A-ring. Therefore, this approach was chosen to determine if the Heck reaction is suitable for an introduction of a new double bond into the molecule next to the A-ring. This work was focused on the scope and limitations of this reaction since it is promising for the potential synthesis of numerous new compounds in the future.

3.2.1 Exploration of the Heck reaction at the position C-3

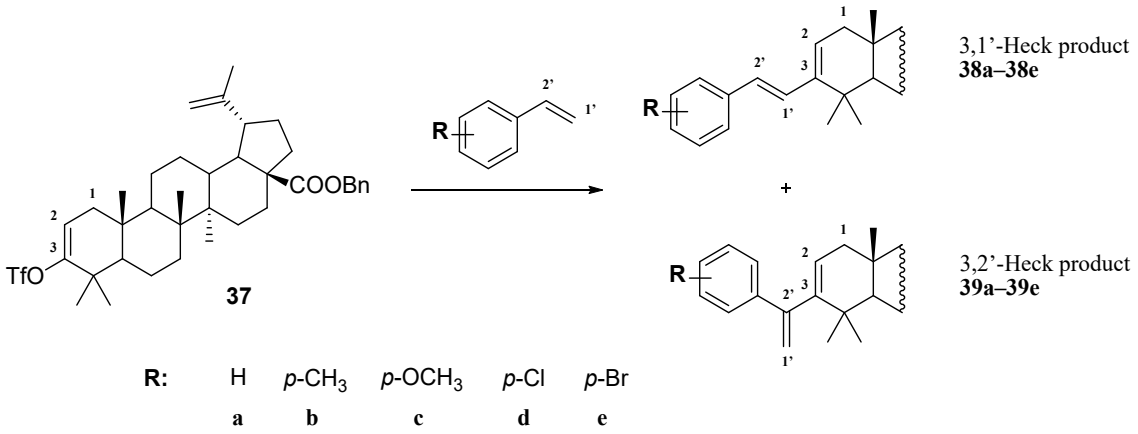
For the exploration of the Heck reaction at the position C-3 of betulinic acid (**1**), the triflate **37** (Scheme 8) was prepared according to the literature[54] with an excel-

lent yield of 94 %. Having the starting triterpene substrate, searching for the optimal reaction conditions started with styrene (**a**, 200 mol %), palladium acetate as a Pd catalyst (15 mol %), together with triphenylphosphine (30 mol %), K_2CO_3 as a base (200 mol %), DMF as a solvent, and reaction temperature at 70 °C. After several test reactions on a small scale, the reaction seemed to proceed with one major and some minor products according to the TLC analysis, indicating a potential Heck product. After purification by column chromatography and subsequent ^{1}H NMR analysis the structure of the product **38a** was proved with unique *E* stereoselectivity of the new double bond as is typical for the Heck reaction. The reaction was further optimized using various bases. It was found that piperidine is the best choice in comparison with Et_3N and K_2CO_3, giving full conversion of the starting triterpene in 1–4 h with a minimum amount of sideproducts.



Scheme 8: Heck reactions at the position C-3. Reagents and conditions: i) BnBr, K_2CO_3, THF, reflux, 3 h; ii) $Na_2Cr_2O_7 \cdot 2H_2O$, AcOH, Ac_2O, NaOAc $\cdot$ $3H_2O$, r. t., 18 h; iii) Tf_2NPh, KHMDS, THF, −78 °C, 4 h; iv) styrene **a**–**f** or allylbenzene **g**, PPh_3, $Pd(OAc)_2$, piperidine, DMF, 85 °C, 1–4 h. *The product **38g** was obtained as a mixture of *E* and *Z* isomers.

During NMR analyses, it was found that in some cases different Heck product (**39a**–**39e**, Scheme 9) was formed in a smaller amount. The possible result of the reaction is shown in Scheme 9, where the major 3,1'-regioisomer was formed, but also the 3,2'-product was observed meaning that about 10 % of the mass is a different compound, which was not directly visible as another spot on TLC, as it has almost identical R_f. From this point of view, it is clear that there is still a lot of space to optimize the stereoselectivity of the reaction, which was found to be unspecific under these conditions. This will be the subject of further study, where different ligands will be used.



Scheme 9: Two regioisomers 3,1' and 3,2' as products of the Heck reaction.
Reagents and conditions: substituted styrene, PPh_3, $Pd(OAc)_2$, piperidine, DMF, 85 °C, 1–4 h.

4 Conclusions and future directions

The main aim of this Ph.D. thesis was to synthesize new derivatives of betulinic acid (**1**) with anticancer activity. Within the thesis, a theoretical review focused on modifications of the lupane-type triterpenoids containing double bonds with an emphasis on the biological activity of these compounds was performed. Synthesis, purification, and characterization of new derivatives were done according to the set goals:

1) The low selectivity of the 30-oxobetulinic acid (**2**) was successfully solved by the C-30 isosteric replacement approach:

   A. A small set of ten new highly cytotoxic triterpenic azines with the free carboxyl group **4a**–**4e**, and protected derivatives **7a**–**7e** (Scheme 3) was prepared in the first project, which was focused on masking of the Michael acceptor of the parent aldehyde **2** by condensation reactions with variously substituted hydrazones. Nonprotected compounds **4a**–**4e** showed great selectivity towards the CCRF-CEM cancer cell line while the toxicity decreased significantly towards other cell lines including nonmalignant fibroblasts, meaning that the goal was achieved. The most active derivative **4d** had comparable cytotoxic activity to the parent 30-oxobetulinic acid (**2**). However, poor stability was the biggest drawback of this class of derivatives, together with low yields and sideproducts. This work was published in 2018 in the Future Medicinal Chemistry Journal.

   B. A large library of forty new triterpenoid dienes **13.1**–**13.51** (Scheme 4) was prepared from the same substrate **2** using Wittig reactions. For this purpose, a wide palette of variously substituted triphenylphosphonium salts **12.1**–**12.51** (Fig. 7) was prepared. The main interest was focused on the improvement of inappropriate properties of previously prepared azines. An isosteric replacement of C=N by C=C bond yielded stable compounds which showed comparable anticancer activities and high selectivity compared to azines. Therefore, the lack of selectivity of the parent compound, 30-oxobetulinic acid (**2**), was diminished and stability was achieved. The most active derivatives were further tested for DNA and RNA synthesis,

cell cycle analysis, protein expression, and pharmacological parameters to find out the mechanism of action. The compounds cause selective cell apoptosis particularly by the mitochondrial pathway. The derivative **13.22** was considered a potential candidate for further preclinical development. The work was published in 2021 in the European Journal of Medicinal Chemistry.

2) Copper(I)-catalyzed azide-alkyne cycloadditions were employed in the third project focusing on the preparation of triterpenoid conjugates with a triazole ring. In previous studies, it was found that 3β-*O*-acetyl derivatives of betulinic acid (**1**) with free C-28 carboxyl group (**21a**–**21h**, Scheme 5) were more active than their nonprotected analogs (**23a**–**23g**). Unfortunately, derivatives of betulin (**24**) with protection on both -OH groups (**31a**–**31f**, and **32a**–**32f**, Scheme 6) were not sufficiently soluble to perform cytotoxicity assays. Within my work, eleven new compounds **35a**–**35k** (Scheme 7) were prepared, fully characterized, and tested *in vitro* for their cytotoxic activity. The best derivative **35i** showed higher cytotoxicity against the CCRF-CEM cell line than the parent compound, betulinic acid (**1**); however, most of the compounds were inactive. In contrast, these compounds may be effective as neurodegenerative agents, which will be verified in further studies.

3) The Heck reaction was introduced to the chemistry of triterpenes in the last project focused on the exploration of possibilities to use this cross-coupling reaction for a modification at the A-ring of betulinic acid (**1**). Seven new derivatives **38a**–**38g** (Scheme 8) containing a new double bond have been prepared to date, however, further investigation to optimize the regioisomers formation is needed. This could be solved particularly by a change in the used ligand since appropriately selected ligands can sterically block the formation of unwanted isomers. Reducing the amount of catalyst used in the reaction will be also further optimized. This project will be followed up with a different protecting group to obtain final derivatives with the free C-28 carboxyl group which is desirable for higher cytotoxic activity.

In conclusion, four different synthetic targets dealing with modifications of betulinic acid (**1**) are part of this Ph.D. thesis. In summary, 68 new final triterpenoid compounds were synthesized, fully characterized by physical and spectral data, and most of them were tested for cytotoxic activity against eight cancer cell lines and two normal fibroblasts, providing valuable SAR information. Some of the most promising derivatives were further evaluated in the following biological assays. Cytotoxicity tests and biological assays were performed by other researchers at IMTM (Soňa Gurská, Ivo Frydrych, Barbora Lišková).

Future directions:

- The isosteric replacement at the position C-30 was successful in the improvement of selectivity of new compounds. This approach may be generally used in future projects.
- Newly synthesized dienes opened the triterpenoid skeleton for many new transformations – dienes are good substrates for Diels-Alder reactions which are already successfully investigated in our research group.
- The Heck reaction may be used for the preparation of a number of new compounds. In order to use these compounds in biology, it is necessary to find better protecting groups for the position C-28 that can be easily deprotected.

5 List of cell lines

A549	Lung adenocarcinoma
BJ	Human non-cancer fibroblast
CCRF-CEM	T-lymphoblastic leukemia
CEM-DNR	T-lymphoblastic leukemia resistant against daunorubicin
HCT116	Colon carcinoma
HCT116p53-/-	Colon carcinoma
K562	Human chronic myelogenous leukemia
K562-TAX	Chronic myelogenous leukemia resistant against paclitaxel
MRC-5	Human non-cancer fibroblasts
U2OS	Osteosarcoma

6 List of author's publications

Publications related to the Thesis:

Pokorný, J.; Olejníková, D.; Frydrych, I.; Lišková, B.; Gurská, S.; Benická, S.; Šarek, J.; Kotulová, J.; Hajdúch, M.; Džubák, P.; Urban, M. Substituted Dienes Prepared from Betulinic Acid – Synthesis, Cytotoxicity, Mechanism of Action, and Pharmacological Parameters. *Eur. J. Med. Chem.* **2021**, 113706.

DOI: https://doi.org/10.1016/j.ejmech.2021.113706

Pokorný, J.; Krajčovičová, S.; Hajdúch, M.; Holoubek, M.; Gurská, S.; Džubák, P.; Volná, T.; Popa, I.; Urban, M., Triterpenic azines, a new class of compounds with selective cytotoxicity to leukemia cells CCRF-CEM, *Future Med. Chem.* **2018**, *10* (5), 483–491.

DOI: https://doi.org/10.4155/fmc-2017-0171

Pokorný, J.; Horka V.; Šidová V.; Urban M., Synthesis and characterization of new conjugates of betulin diacetate and bis(triphenysilyl) betulin with substituted triazoles, *Monatsh. Chem.* **2018**, *149* (4), 839–845.

DOI: https://doi.org/10.1007/s00706-017-2113-7

Pokorný, J.; Borková, L.; Urban, M., Click Reactions in Chemistry of Triterpenes – Advances Towards Development of Potential Therapeutics, *Current Med. Chem.* **2018**, *25* (5), 636–658.

DOI: https://doi.org/10.2174/0929867324666171009122612

Šidová, V.; Zoufalý, P.; **Pokorný, J.**; Džubák, P.; Hajdúch, M.; Popa, I.; Urban, M., Cytotoxic conjugates of betulinic acid and substituted triazoles prepared by Huisgen Cycloaddition from 30-azidoderivatives, *PLoS ONE* **2017**, 12, 2, e0171621.

DOI: https://doi.org/10.1371/journal.pone.0171621

Other publications not included in the Thesis:

de Jong, F.; **Pokorný, J**.; Manshian, B.; Daelemans, B.; Vandaele, J.; Startek, J. B.; Soenen, S.; Van der Auweraer, M.; Dehaen, W.; Rocha, S.; Silveira-Dorta, G., Development and Characterization of BODIPY-Derived Tracers for Fluorescent Labeling of the Endoplasmic Reticulum, *Dyes and Pigments* **2020**, *176* (-), 108200.

DOI: https://doi.org/10.1016/j.dyepig.2020.108200

Hodoň, J.; Borkova, L.; **Pokorný, J.**; Kazakova, A.; Urban, M., Design and synthesis of pentacyclic triterpene conjugates and their use in medicinal research, *Eur. J. Med. Chem.* **2019**, *182* (N/A), Article Number: 111653 (1-25).

DOI: https://doi.org/10.1016/j.ejmech.2019.111653

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8 Summary

Pentacyclic triterpenoids are a large group of natural substances with a broad palette of biological activities. Many researchers are interested in their synthetic modifications in order to exploit their promising potential in drug development. One of the best-known compounds from the group of triterpenes is betulinic acid, which is known for its strong anticancer properties. Oxidation of betulinic acid at the position C-30 results in 30-oxobetulinic acid that had IC_{50} values in the low micromolar range against multiple cancer cell lines. However, this molecule suffered from the general toxicity caused by the presence of the Michael acceptor in its structure, resulting in low selectivity. In this Ph.D. thesis, three different synthetic approaches to modify the 30-oxobetulinic acid at the position C-30 are described in order to overcome its low selectivity and to improve other properties such as low solubility in water. An isosteric replacement was chosen in the first two projects, where the –CH=O formation of the parent compound was replaced with the –CH=N– or –CH=CH– to mask the Michael acceptor. Within the first project, a small series of triterpenic azines was prepared by condensation of variously substituted aromatic hydrazones and 30-oxobetulinic acid. Triterpenic azines had anticancer activities against the lymphoblastic leukemia cell line comparable to 30-oxobetulinic acid, but selectivity was significantly improved. On the other hand, they were unstable and hard to prepare in higher yields. The Wittig reaction was employed in the second project, where both problems were successfully resolved and a large group of forty new dienes was prepared. Two representatives were selected due to their high cytotoxicity to study their pharmacological parameters and mechanism of action. Both disrupt mitochondrial permeability and activate selective apoptosis *via* an intrinsic pathway. One derivative was chosen as a potential candidate for further preclinical development. In the third project, 30-oxobetulinic acid was further oxidized to carboxylic acid and transformed to propargyl ester that was able to react in the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC). The connection of the terpene with a rather polar triazole ring may improve the solubility of the molecule in water-based media and the bioavailability. The Heck reaction was optimized for use at the position C-3 in the fourth project. Throughout the work, the effect of double bonds in different parts of the triterpene structure was studied and reviewed in the theoretical part of this thesis.

9 Souhrn

Pentacyklické triterpenoidy představují velkou skupinou přírodních látek se širokou paletou biologických aktivit. Mnoho výzkumných skupin se zajímá o jejich syntetické modifikace s cílem využít jejich slibný potenciál při vývoji léčiv. Jednou z nejznámějších sloučenin ze skupiny triterpenoidů je kyselina betulinová, známá svými silnými protinádorovými účinky. Oxidací kyseliny betulinové v poloze C-30 lze připravit kyselinu 30-oxobetulinovou, která vykazuje hodnoty IC_{50} v nízkém mikromolárním rozmezí proti řadě nádorových buněčných linií. Její nevýhodou je však obecně vysoká toxicita, způsobená přítomností Michaelova akceptoru ve struktuře, což má za následek nízkou selektivitu této sloučeniny. V předložené dizertační práci jsou popsány tři různé syntetické přístupy modifikace kyseliny 30-oxobetulinové v poloze C-30 s cílem překonat její nízkou selektivitu a zlepšit další vlastnosti, jako je např. nízká rozpustnost ve vodě. U prvních dvou projektů byla zvolena cesta isosterické záměny, při níž byla formace –CH=O výchozí sloučeniny nahrazena za –CH=N– nebo –CH=CH– za účelem zamaskování Michaelova akceptoru. V rámci prvního projektu tak byla připravena malá série triterpenických azinů kondenzací různě substituovaných aromatických hydrazonů a kyseliny 30-oxobetulinové. Připravené aziny vykázaly protirakovinnou aktivitu vůči buněčné linii lymfoblastické leukemie CCRF-CEM srovnatelnou s kyselinou 30-oxobetulinovou, selektivita však byla výrazně zvýšena. Bohužel byly tyto látky nestabilní a obtížně se připravovaly ve vyšších výtěžcích. Wittigova reakce byla použita ve druhém projektu, kde se podařilo oba problémy minimalizovat a připravit knihovnu čtyřiceti nových dienů s konjugovanými vazbami. Dva nejaktivnější zástupci byli podrobeni studiu farmakologických parametrů a mechanismu účinku. Obě látky narušují mitochondriální permeabilitu a aktivují selektivní apoptózu mitochondriální dráhou. Jeden derivát byl vybrán jako potenciální kandidát pro další preklinický vývoj. Ve třetím projektu byla kyselina 30-oxobetulinová dále oxidována v poloze C-30 na kyselinu a alkylována na propargylester, který byl schopen reagovat v mědí(I) katalyzované azido-alkynové cykloadici (CuAAC). Spojením terpenu s poměrně polárním triazolovým kruhem lze zlepšit rozpustnost molekuly ve vodném prostředí a biologickou dostupnost. Heckova reakce byla optimalizována pro použití v poloze C-3 ve čtvrtém projektu. V průběhu celé práce byl studován vliv dvojných vazeb v různých částech struktury triterpenu na biologickou aktivitu, který byl shrnut v teoretické části této práce.