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ARTICLE TYPE

Synthesis of new betulinic acid-peptide conjugates and *in vivo* and *in silico* studies of the influence of peptide moieties on the triterpenoid core activity

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

The modification of betulinic acid derivatives bearing an ethynyl group at the C-3 position by different azidopeptides using Cu(I)-catalyzed alkyne-azide cycloaddition has been described. All obtained compounds were tested for their anti-inflammatory activity using the histamine-induced paw edema model. Betulinic acid-peptide conjugates containing histidine, alanine, tryprophan and isoleucine amino acid fragments were found to exhibit high anti-inflammatory activity, comparable with that of indomethacin. It has been shown by the molecular docking that the obtained conjugates are incorporated into the binding site of the protein Keap1 Kelch-domain by their amino acid residues and form more non-covalent bonds, but have lower affinity than the initial triterpenoid core. It has been suggested that peptide moieties can modify the activity of the initial triterpenoid scaffold due to the change in the conformational and thermodynamic characteristics, which influence on the binding of the compound with its molecular target.

Introduction

Betulinic acid [3β-hydroxy-lup-20(19)-en-28-oic acid] (BA), a naturally occurring pentacyclic lupane-type triterpenoid, represents an attractive scaffold for the development of new biologically active compounds, since it exhibits a variety of biological and medicinal properties, such as antiviral, anticancer, anti-bacterial, antimalarial, as well as anti-inflammatory activities.[1]

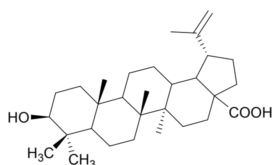

Fig. 1. Betulinic acid (BA)

However, a low water solubility of this compound limits its applications in medicine. A number of structural modifications of BA, mostly at the C-3, C-20 and C-28 positions, were performed in order to increase water solubility of this triterpenoid, as well as to investigate structure-activity relationships and obtain derivatives with improved pharmacological properties.[2] For example, the introduction of sugar moieties at both C-3 and C-28 positions of BA led to a dramatic increase in the anti-cancer activity compared to BA.[3] The introduction of amino acids at C-3 or C-28 positions of BA resulted in improved water solubility, as well as selective cytotoxicity of the obtained conjugates.[4]

The copper(I)-catalyzed azide–alkyne cycloaddition reaction (CuAAC, "click" reaction)[5] has been recognized as a powerful tool for the conjugation and decoration of biologically active molecules.[6] 1,2,3-Triazole moieties are ideal linkers, because they are extremely stable under typical physiological conditions and can form hydrogen bonds.[7] Moreover, 1,2,3-triazole fragment can be considered as a bioisostere of the amide bond.[8]

Recently, we have reported the synthesis of biohybrids of betulonic acid via CuAAC.[9] It was demonstrated that the amides of betulonic acid modified with a 1,2,3-triazole moiety showed anti-inflammatory properties, as well as high antioxidant activity, which exceeded the activity of the reference compound dihydroquercetin by the factor of 1.4.[9a] In continuation of our studies on the modification of plant-derived pentacyclic triterpenoids, we decided to explore biohybrids of betulinic acid as potential anti-inflammatory agents with improved solubility in biological media. Herein we report the synthesis and investigation of pharmacological properties of new betulinic acid derivatives containing 1,2,3-triazole peptide fragments at the C-3 position.

The starting BA derivatives **2a,b** bearing an alkyne group at the C-3 position were obtained *via* the reaction of ethynylmagnesium bromide with betulonic acid **1** (Scheme 1).[10] The reaction furnished the major diastereoisomer **2a** with the axially oriented alkynyl group in 82% yield along with the minor isomer **2b** having the equatorially oriented ethynyl group (11% yield). The absolute stereochemistry for **2a**,**b** was assigned using 2D NMR experiments.[11] The observed stereochemistry of the nucleophilic addition to betulonic acid **1** can be explained by sterical reasons; in this case, axial methyl groups in the 4 and 10 positions control the addition of acetylenic Grignard reagent to the carbonyl group of **1**. As a result, major isomer of the prepared 3-ethynyl substituted betulinic acid has the same orientation of the hydoxy group as a natural betulinic acid.

The starting azidopeptides **3**[12] are accessible by the Ugi reaction[13] of chiral isocyanoazides[14] with carbonyl compounds (formaldehyde or acetone), 2,4-dimethoxybenzyl (DMB) amine,

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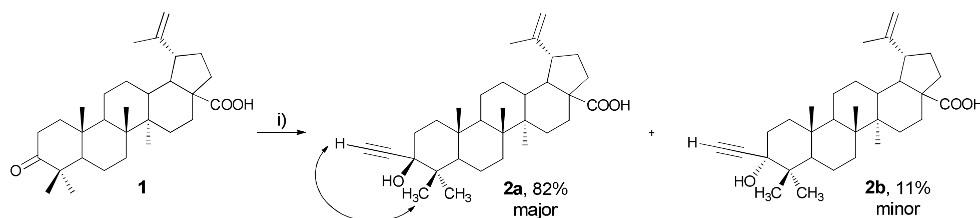
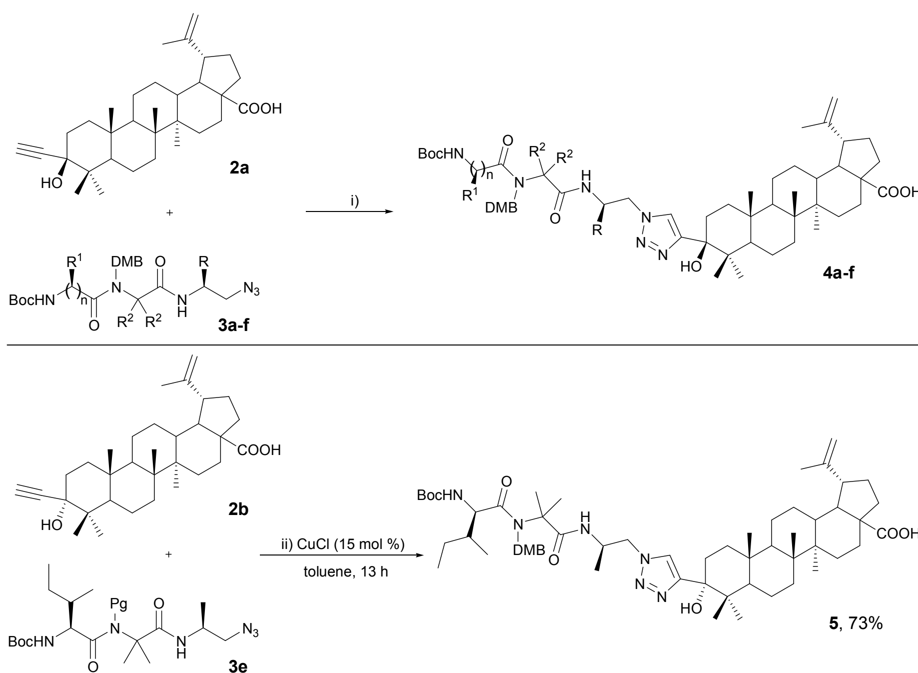
and Boc-protected amino acids. These compounds bearing the azide function can be conjugated with other biologically active molecules *via* CuAAC.



Scheme 1 Synthesis of BA derivatives **2a,b**. (i) HC≡CMgBr, THF, rt; then NH_4Cl.



Scheme 2 Synthesis of betulinic acid-peptide conjugates **4** and **5** (see Table 1 for R groups and yields).

Having in hands the set of betulinic acid derivatives **2a,b** and azidopeptides **3**, we investigated the conjugation process. To find the optimal reaction conditions, various reaction conditions have been studied (sodium ascorbate - $CuSO_4$ in CH_2Cl_2-H_2O or THF-H_2O; and CuCl in toluene or butan-1-ol) (Table 1). First, betulinic acid derivative **2a** bearing the axially oriented ethynyl group at the C-3 position was reacted with azidopeptides **3** in the presence of the corresponding Cu(I) source to afford conjugates **4a-f** in 60-88% yields (Scheme 2).[11] As an example, a minor product of ethynylation **2b** was also reacted with peptide **3e** to give the compound **5** in 73% yield (Scheme 2).

Unfortunately, all attempts to remove the 2,4-dimethoxybenzyl protecting groups from **4** and **5** under various conditions were not sucessful. Nevertheless, we evaluated the influence of amino acid fragments on the pharmacological activity of the synthesized conjugates.

The anti-inflammatory activity was chosen as a testing characteristic, since it is an intrinsic property of triterpenoids. It has been studied under the standard model of inflammation in vivo. In addition, we were intrigued to study the influence of amino groups at the C3 position of conjugates on the molecule affinity and its thermodynamic and conformational characteristics in relation to a known molecular target. The Kelch domain of Keap1 protein was selected as a molecular target of triterpenoids.[15,16] It was assumed that the data of docking could explain possible differences in the properties of agents, and thus, would be amplified with the data obtained in vivo. This statement is based on the relationship between the antioxidant and anti-inflammatory activity of tritepenoids observed upon the Nrf2/Keap1/ARE activation. It has been recently demonstrated

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that the activation of this signaling pathway is accompanied not only by the induction of antioxidant factors, but also the suppression of pro-inflammatory enzymes, such as iNOS and iCOX.[15, 17] The testing of compounds **2**, **4** and **5** *in vivo* was carried out using a histamine-induced paw edema model.

Table 1 Yields and conditions for the synthesis of conjugates **4**

Comp.	R	R[1]	R[2]	n	Cond.[a]	Yield (%)
4a	H	(imidazol-2-yl)methyl	H	1	A	77
4b	*i*-Pr	H	Me	2	B	65
4c	Bn	CH_2OH	Me	1	C	71
4d	Bn	(3-indolyl)methyl	Me	1	A	76
4e	Me	*sec*-Bu	Me	1	A	88
4f	Me	Bn	Me	1	D	60

[a] *Reaction conditions* for step (i) in Scheme 2: A = CuCl (15 mol %), toluene; B = sodium ascorbate (40 mol %), $CuSO_4 \cdot 5H_2O$ (10 mol %), CH_2Cl_2-H_2O (10:1), 40 ºC; C = CuCl (15 mol %), butan-1-ol, 115 ºC; D = sodium ascorbate (40 mol %), $CuSO_4 \cdot 5H_2O$ (10 mol %), THF-H_2O (10:1).

It was found that intraperitoneally administered compounds **4b**, **4c**, **4d** and **4a** containing alanine, serine, tryptophan and histidine residues, correspondingly, exhibited a significant anti-inflammatory effect, decreasing histamine-induced edema in mice by 31.5, 23.0, 38.8, and 33.4%, respectively, relative to the control (Table 2).

Table 2 Anti-inflammatory activity of compounds **2**,**4** and **5** in the histamine-induced paw edema model

Agent	Inflammation index (%)	Anti-inflammatory effect (%)	Anti-inflammatory effect of agents relative to the Indomethacin (50 mg/kg, intraperitoneally) (%)
Control	31.7±2.1[###++]	0	-
2a	23.7±2.1*	25.2	60
4a	21.1±1.2***	33.4	79
4b	21.7±2.0**	31.5	75
4c	24.4±2.3*#	23.0	55
4d	19.4±1.2***	38.8	92
4e	21.8±3.2*	31.2	74.3
4f	30.9±2.3[###++]	2.5	6
5	27.1±1.6###	14.5	34.5
Indomethacin intraperitoneally 50 mg/kg	18.4±1.3***	42.0	100
Indomethacin orally 20 mg/kg	21.3±1.7**	32.8	78

*P <0.05, **P <0.01, ***P<0.001 relative to the control.

[#]P <0.05, [##]P <0.01, [###]P <0.001 compared with the group with intraperitoneal injection of indomethacin (50 mg/kg).

[++]P <0.01 compared with the group with intraperitoneal injection of **2a**.

The compound **4e** having isoleucine residue reduced inflammation in the paw of mice by 31.2%, contrary to its epimer **5** having an opposite arrangement of the hydroxyl group and a triazole fragment at the C-3 position, which has demonstated no significant anti-inflammatory effect (14.5%). The conjugate **4f** showed no anti-inflammatory activity at all. The compound **4d** is comparable with indomethacin by the intensity of the effect, while compounds **4a,b,e** are insignificantly (1.2-1.3 times) inferior to indomethacin given intraperitoneally at the same dose. In addition, the activities of the latter are comparable with an activity of indomethacin, when administered orally at a mean effective dose for mice (20 mg / kg). The activity of compound **4c** is 1.5 times lower than that of the reference drug, in the case of an intragastric administration.

It should be noted that the starting 3-ethynyl betulinic acid **2a** exhibits a significant anti-inflammatory effect (25.2%). Based on the obtained data, the contribution of each amino acid residue to the anti-inflammatory activity of this compound could be conventionally evaluated. Taking into account that the activity of the derivative **2a** is 40% less than the activity of indomethacin, when administered intraperitoneally, the introduction of tryptophan, histidine, alanine and isoleucine fragments enhanced its activity by 32.4, 19.5, 15.0 and 14.3%, respectively, while the introduction of a serine fragment resulted in almost the same activity. In the case of compound **5**, the introduction of isoleucine amino acid fragment led to a decrease in the activity of starting compound by 25.5%.

Table 3. The nature of interaction of betulinic acid-peptide conjugates with Kelch-domain of protein Keap1

Ligand	Minimum binding energy, kcal/mol	Hydrofobic contacts	Non-covalent bonds	Total number of bonds
Cpd16	-10.6	4	5	9
2a	-9.1	2	1	3
4a	-8.3	5	3	8
5	-8.2	3	1	4
4b	-7.6	5	3	8
4f	-7.5	1	4	5
4c	-7.2	5	1	6
4d	-7.0	6	2	8
4e	-6.8	3	3	6

Besides anti-inflammatory activity, triterpenoids also exhibit a significant antioxidant activity, which is associated with the induction of the cytoprotective signaling pathway Nrf2/Keap1/ARE.[15] The activation of this pathway leads to the ARE-controlled gene expression of enzymes of second phase of biotransformation and other antioxidant molecules. Cysteine residues in Kelch-domain of repressor protein Keap1 represent a well-known molecular target of triterpenoids, binding with which leads to the disintegration of the Keap1-Nrf2 complex and an activation of ARE genes.[16] In order to study the conformational features and the nature of the relationships occurring during the interaction of the initial triterpenoid **2a** and its peptide conjugates with the molecular target of Keap1, their docking at the binding site of Keap1 Kelch-domain model (PDB ID 4IQK) with the ranking of values of the minimum binding energy in comparison with the native ligand **Cpd16** (N,N'-naphthalene-1,4-diylbis (4-methoxybenzenesulfon-amide) has been performed.

The minimum binding energy is inversely proportional to the affinity of the ligand to the binding site of the receptor. According to the docking results, all compounds have lower affinity to a binding site of the 4IQK model than the native ligand **Cpd16** (Table 3). However, the values of minimum binding energy are quite low for all studied ligands that is indicative of a high probability of their interaction with Kelch-domain of Keap1. The analysis of the spatial conformation of the compounds in the binding site of 4IQK revealed different two types of ligand configuration. The compound **2a**, which is embedded in the binding site by a triterpenoid skeleton, is related to the first type of ligand configuration (Fig. 2). Its peptide conjugates, which are embedded in the binding site by the amino acid groups at the C3 position of triterpenoid skeleton as it is shown for example on compound 4a, are related to the second type of ligand configuration (Fig. 3).

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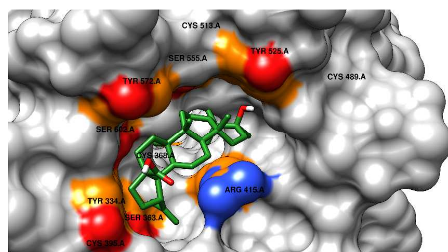

Fig. 2. Docking visualization of **2a** in the Kelch-domain.

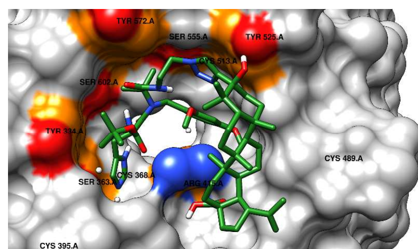

Fig. 3. Docking visualization of **4a** in the Kelch-domain.

The calculation of two-dimensional models of ligand-receptor complexes in the PoseView program made it possible to study in detail the nature of bonds of synthesized compounds with amino acid residues in the binding site of the 4IQK model. Non-covalent interactions between the ligands and amino acid residues at the binding site are represented by the hydrogen bonding and π-interactions with regard to the hydrophobic contacts (Table 2). Stereochemistry of the 4IQK binding site is defined by the presence of the amino acid residues of arginine 415, alanine 556, tyrosine 334, 572 and 525, as well as serines 508, 602 and 555. Moreover, alanine 556 and tyrosine 572 residues form only the hydrophobic surfaces at the binding site. The hydroxyl group of a serine residue 508 forms a hydrogen bond with the ligands, and the remaining amino acid residues are involved both in the hydrophobic contacts and the formation of hydrogen and π-bonds.

In general, in the peptide conjugates under investigation, the π-system of dimethoxyphenyl group typically participates in the formation of π-interaction with the amino acid residues in the binding site. In the compound **4b**, this group interacts with the phenyl groups of tyrosine 334 and phenylalanine 577 residues, and in the compound **4a** - with the guanidine group of arginine 415. The hydroxyl group at the C3 position forms a hydrogen bond with a hydroxyl group of serine 508 in the compound **2a**. In the case of compound **4a**, this group reacts with a hydroxyl group of the tyrosine residue 525. Oxygen atoms with a shifted electron density in the structure of the peptide fragment at the C3 position of betulinic acid play a significant role in the formation of non-covalent interactions between the conjugates and amino acid residues in the binding site of 4IQK.

Thus, the docking data indicates that in spite of the peptide residues at the C3 position of betulinic acid do not increase the affinity of the molecule, but play an essential role in the interaction with they interact with amino acid residues in the binding site of the Kelch-domain of Keap1. Peptide moieties actively enter into non-covalent interactions with the amino acid residues in the binding site and have a conformation, which can be embedded in the geometrical configuration of the pocket of the Keap1 C-terminal domain, due to the presence of polar oxygen atoms and the π-systems of phenyl groups. The higher affinity of triterpenoid **2a,** compared with its derivatives, may be associated with a smaller size of its molecule, and, therefore, with its ability to form more strong links with complementary groups, when it is embedded into the hydrophobic cavity of the site, despite the minimal amount of non-covalent contacts. A decrease in the affinity of the peptide conjugates compared to the starting compound **2a** may result in a shorter retention time of a peptide ligand on the receptor that has no significant influence on its antioxidant effect. This statement is based on the fact that the triterpenoid derivatives have no direct antioxidant activity, but act as pH-sensitive triggers switching the activity of the signaling Nrf2/Keap1/ARE network at the change in the oxidative status of the cell.[17, 18]

Conclusions

In summary, we described the conjugation of betulinic acid derivatives **2a,b** bearing an ethynyl group at the C-3 position with azidopeptides **3** *via* the Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition reaction. The anti-inflammatory activity of obtained compounds **2**,**4** and **5** was evaluated using the histamine-induced paw edema model. Betulinic acid-peptide conjugates **4a,b,d,e** containing histidine, alanine, tryptophan and isoleucine amino acid fragments were found to exhibit high anti-inflammatory activity, which is comparable with that of indomethacin. The introduction of these amino acid residues in the betulinic acid core enhances its anti-inflammatory properties by 19.5, 15.0, 32.4 and 14.3%, respectively. The introduction of the serine amino acid fragment does not significantly affect the activity of starting compound, whereas the incorporation of isoleucine (compound **5**) and phenylalanine fragments reduces its anti-inflammatory activity. The molecular docking of compounds in the binding site of Kelch domain of Keap1 revealed that the peptide conjugates form more noncovalent interactions with amino acid residues in the binding site, but have a lower affinity than their precursor triterpenoid **2a**. At the same time, there are It is obvious these differences among the compound **2a** and its peptide derivatives associated with that, by which part the molecule is embedded in the binding site: triterpenoid core (**2a**) or an amino acid moiety (**4a-f**, **5**). Thus, the peptide fragments modify the activity of the initial triterpenoid core not only by probable increasing the solubility, but presumably changing the conformational and thermodynamic features, which influence on the binding of the compound with its molecular target. The absence of a direct correlation between the docking data and anti-inflammatory activity in vivo indicates a more complex implementation of the anti-inflammatory effect of the conjugates, which may be caused by their effects on the other intracellular factors. The NF-κB signaling pathway may be the most likely target. It is known that betulinic acid and its derivatives inhibit the activity of pro-inflammatory NFκB signaling pathways, interacting with the protein kinase inhibitor of an IKB nuclear factor.[15, 18]

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Experimental

General Information

Melting points were determined with a Kofler apparatus. Column

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chromatography was performed on SiO_2 ('Aldrich'). Analytical TLC was performed with Merck silica gel 60 F_{254} plates. Visualization was accomplished by UV light and spraying by aqueous potassium permanganate ($KMnO_4$). The IR spectra were recorded on a UR-20 spectrometer in nujol. The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer (400 and 100 MHz respectively) and Bruker AVANCE III spectrometer (600.30 MHz and 150.96 MHz, respectively), in $CDCl_3$ with TMS as internal standard. The high-resolution ESI mass spectra were recorded on a Bruker micrOTOF II spectrometer. Chemical shifts were given in (δ in ppm) relative to the residual signals of $CHCl_3$ (δH 7.24 ppm and δC 76.90 ppm). Coupling constants (*J* in Hz) were accurate to ±0.2 Hz. Due to the complexity of signals in the ^{1}H NMR spectra for the betulonic acid derivatives,[19] only the characteristic signals are assigned. Most protons of the triterpenoid skeleton resonate between 0.8 and 2.7 ppm.

The assignment of signals in ^{1}H and ^{13}C NMR spectra based on data obtained in 2D COSY and NOESY experiments, and ^{1}H—^{13}C -correlations HSQC and HMBC. The figures show characteristic interactions observed in the NOESY experiment (the spectrum was recordered with a τm= 310 and a recovery delay of 2.0 s. 1K data points was collected for 256 increments of 16 scans, using TPPI f1 quadrature detection. Data were processed with a squared cosine-bell window in both dimensions with a single zero-fill in f1). The choice in favor of the axial location of ethinyl group in the 2a was made on the basis of NOE lack for the axial 23-CH_3 because of the largest distance between them (~6,4Å). The signals of a solvent (δH = 7.26 ppm, δC = 76.9 ppm) were used as internal standard.

General procedure for the synthesis of peptides 3

The corresponding amine (5 mmol) and acetone (10 mmol) were dissolved in 5 mL of MeOH and *N*-Boc protected amino acid (5 mmol) and isocyanide (5 mmol) were added at room temperature. The mixture was stirred for 24 h. The solvent was removed in vacuo and the residue was purified by column chromatography (hexane–ethyl acetate 2:1).

***N*-(tert-Butoxycarbonyl)-L-histidyl-*N*^{1}-(2-azidoethyl)-*N*^{2}-(2,4-dimethoxybenzyl)-2-methylalaninamide (3a)**. According to the general procedure for the synthesis of peptides, **3a** was obtained from 2,4-dimethoxybenzylamine, CH_2O, *N*-Boc-L-histidine and 1-azido-2-isocyanoethane. White solid, yield 0.58 g (49%), mp 59-62°C. IR (neat, ν/cm^{-1}) 1707, 1760 (C=O); 2110 (N_3), 3350 (NH). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.22 (s, 3H), 1.39 (s, 3H), 1.44 (s, 9H), 2.80-2.86 (m, 1H), 3.03-3.07 (m, 1H), 3.33-3.50 (m, 4H), 3.70 (s, 3H), 3.77 (s, 3H), 3.99-4.18 (m, 2H), 4.86-4.90 (m, 1H), 5.84 (d, 1H, *J* = 4 Hz), 6.38 (s, 2H), 6.95-6.97 (d, 1H, *J* = 4 Hz), 7.30 (s, 1H), 8.12 (s, 1H), 8.86 (br.s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.8, 25.1, 27.9, 32.5, 38.8, 43.4, 50.3, 52.3, 54.7, 54.9, 62.9, 79.1, 98.2, 103.5, 115.3, 116.8, 129.0, 136.1, 138.4, 154.4, 157.9, 160.2, 171.8, 175.3. HRMS (ESI) calcd for $C_{26}H_{38}O_6N_8$ 558.2909, found $[M]^+$ 558.2906.

Synthesis of betulinic acid derivatives 2a,b

3-Ethynyl-3-hydroxy-20(29)-lupen-28-oic acid (2). A solution of ethylmagnesium bromide was prepared from 1.2 g. (50 mmol) of magnesium turnings, 6.0 g (55 mmol) of ethyl bromide in 40 ml of tetrahydrofuran. The warm solution (40-50°C) of ethylmagnesium bromide was added dropwise to acetylene was passed through the tetrahydrofuran. After adding of the last portion of ethylmagnesium bromide and evolved the entire ethane, a solution of 1.0 g (2.2 mmol) betulonic acid 1 in 10 ml of THF was added, immediately in the flask a precipitate formed. The reaction mixture was stirred at room temperature for 3 h and then 50 ml of a saturated solution of NH_4Cl was added. The organic layer was separated, and the aqueous fraction was extracted with diethyl ether 3 × 50 ml. The combined organic layers were dried over Na_2SO_4 and filtered. The solvent was removed in vacuo, precipitate was purified by column chromatography on silica gel (toluene-ethyl acetate 15:1) to give the two diastereomers in 93% total yield (982 mg). Yield 82%, mp 282-284°C (major isomer) and 11%, mp 212-217°C (minor isomer).

Major isomer 2a. IR (neat, ν/cm^{-1}) 1695 (C=O); 2660 (OH); 3300 (CH). ^{1}H NMR ($CDCl_3$, 600.30 MHz) δ 0.83 (H25), 0.85 (H23), 0.93 (H26), 1.00 (H27), 1.05 (H24), 1.07 (H12e), 1.15 (H5),1.19 (H15e), 1.26 (H11a), 1.30 (H1a), 1.35 and 1.49 (H6), 1.36 and 1.42 (H7), 1.36 (H9), 1.40 (H21β),1.41 (H16a), 1.44 (H11e), 1.49 (H22β), 1.54 (H15a), 1.63 (H1e), 1.63 (H18), 1.68 (H2e), 1.69 (CH_3-C20), 1.69 (H12e), 1.93 (H2a), 1.96 (H22α), 2.00 (H21α), 2.18 (H13), 2.27 (H16e), 2.48 (H30), 3.00 (H19), 4.61 (=CH_2-trans), 4.74 (=CH_2-cis). ^{13}C NMR δ 14.8 (C27), 15.9 (C26), 16.4 (C25), 17.3 (C23), 18.4 (C6), 19.3 (CH3-C20), 20.7 (C11), 25.36 (C12), 25.57 (C24), 29.66 (C15), 30.48 (C21), 32.07 (C16), 32.5 (C2), 34.1 (C7), 36.9 (C10), 37.1 (C22), 37.8 (C1), 38.4 (C13), 40.6 (C8), 41.2 (C4), 42.3 (C14), 46.8 (C19), 49.2 (C18), 50.4 (C9), 53.0 (C5), 56.3 (C17), 73.4 (C30), 75.6 (C3), 87.0 (C29), 109.6 (CH_2=C20), 150.3 (C20), 181.8 (COOH). HRMS (ESI) calcd for $C_{32}H_{48}O_3+Na^+$ 503.3496, found $[M+Na]^+$ 503.3496.

Minor isomer 2b. IR (neat, ν/cm^{-1}) 1687 (C=O); 2108 (C≡C); 2869 (OH); 3306 (CH). ^{1}H NMR ($CDCl_3$, 600.30 MHz) δ 0.85 (H25), 0.93 (H26), 0.97 (H23), 0.99 (H27), 1.03 (H12e), 1.04 (H24), 1.19 (H15e), 1.24 (H11a), 1.26 (H1a), 1.28 (H5), 1.35 and 1.42 (H7), 1.36 and 1.41 (H6), 1.39 (H21β), 1.39 (H9),1.41 (H16a), 1.42 (H11e), 1.42 (H1e), 1.48 (H22β), 1.53 (H15a), 1.62 (H18), 1.69 (CH_3-C20), 1.69 (H12e), 1.75 (H2e), 1.96 (H22α), 1.98 (H21α), 2.10 (H2a), 2.17 (H13), 2.27 (H16e), 2.41 (H30), 3.00 (H19), 4.61 (=CH_2-trans), 4.74 (=CH_2-cis). ^{13}C NMR δ 14.7 (C27), 15.8 (C25), 15.9 (C26), 18.7 (C6), 19.3 (CH3-C20), 20.6 (C11), 21.3 (C23), 25.2 (C24), 25.4 (C24), 29.6 (C15), 30.5 (C21), 31.9 (C2), 32.1 (C16), 31.9 (C2), 32.1 (C16), 34.0 (C1), 34.1 (C7), 36.9 (C10), 37.0 (C22), 38.3 (C13), 40.4 (C4), 40.6 (C8), 42.4 (C14), 46.9 (C19), 49.0 (C5), 49.2 (C18), 50.1 (C9), 56.3 (C17), 71.9 (C3), 74.1 (C3), 87.5 (C29), 109.6 (CH_2=C20), 150.3 (C20), 181.5 (COOH). HRMS (ESI) calcd for $C_{32}H_{48}O_3$ 480.3598, found $[M]^+$ 480.3605.

Synthesis of betulinic acid-peptide conjugates 4 and 5

***N*-[(1,1-dimethylethoxy)carbonyl]-L-histidyl-*N*^{1}-[2-[4-[3,28-dihydroxy-28-oxolup-20(29)-en-3-yl]-1*H*-1,2,3-triazol-1-yl]ethyl]-*N*^{2}-[(2,4-dimethoxyphenyl)methyl]-2-methyl-alaninamide (4a)**. A mixture of 103 mg (0.21 mmol) of alkyne **2a**, 120 mg (0.21 mmol) of azide **3a** and 3 mg of CuCl in 7 ml of toluene was kept for 11 h. After the reaction was completed, the reaction mixture was transferred to a separatory funnel and

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washed with aqueous ammonia to remove the copper salts. The organic layer was separated, dried over Na_2SO_4 and filtered. The solvent was removed in vacuo. Hexane was added and the resulting precipitate was filtered off to afford 170 mg (77%) of the triazole **4a** as a white solid, mp 150-153°C. IR (neat, ν/cm^{-1}) 1710, 1758 (C=O); 3423 (NH). ^{1}H NMR (CDCl$_3$, 400 MHz) δ 0.58 (s, 3H), 0.93-0.95 (m, 6H), 1.00-1.02 (m, 6H), 1.44 (s, 9H), 1.68 (s, 3H), 2.94-3.05 (m, 2H), 3.69 (s, 3H), 3.76 (s, 3H), 3.91-3.95 (m, 1H), 4.10-4.14 (m, 1H), 4.54-4.58 (m, 3H), 4.73 (s, 1H), 4.87-4.90 (m, 1H), 5.97 (d, 1H, J = 8.0 Hz), 6.36-6.38 (m, 2H), 6.94 (d, 1H, J = 8.0 Hz), 7.25 (s, 1H), 7.67 (s, 1H), 8.02 (s, 1H), 9.02 (br. s, 1H). ^{13}C NMR (CDCl$_3$, 100 MHz) δ 14.8, 15.5, 16.1, 18.2, 18.9, 18.9, 20.4, 21.6, 25.1, 25.3, 28.0, 29.4, 30.1, 31.8, 32.4, 33.8, 36.1, 36.6, 36.7, 38.0, 39.5, 40.2, 41.0, 42.0, 43.4, 46.5, 48.5, 48.8, 50.1, 51.0, 52.2, 54.9, 54.7, 55.9, 63.0, 75.6, 79.2, 98.2, 103.5, 109.2, 115.3, 116.6, 122.5, 129.0, 136.3, 138.0, 146.3, 150.0, 152.5, 154.4, 157.9, 160.2, 171.8, 175.9, 180.6. HRMS (ESI) calcd for $C_{58}H_{86}N_8O_9$+H$^+$ 1039.6596, found [M+H]$^+$ 1039.6568.

***N*-[(1,1-dimethylethoxy)carbonyl]-β-alanyl-*N*^{1}-[(1*S*)-1-[[4-[3,28-dihydroxy-28-oxolup-20(29)-en-3-yl]-1*H*-1,2,3-triazol-1-yl]methyl]-2-methylpropyl]-*N*^{2}-[(2,4-dimethoxyphenyl) methyl]-2-methyl-alaninamide (4b)**. A mixture of 100 mg (0.21 mmol) of alkyne **2a**, 111 mg (0.21 mmol) of azide **3b**, 5 mg of $CuSO_4 \cdot 5H_2O$ and 16 mg of sodium ascorbate in 10 ml of the mixture of CH_2Cl_2/H_2O (10:1 ratio) was stirred at 40°C for 7 h. Then mixture of CH_2Cl_2/H_2O was replaced by aqueous ethanol (4:1). The reaction mixture was kept for additional 7 h. After the reaction was completed, the reaction mixture was transferred to a separatory funnel and washed with aqueous ammonia to remove the copper salts. The organic layer was separated, dried over Na_2SO_4 and filtered. The solvent was removed in vacuo. Hexane was added and the resulting precipitate was filtered off to afford 140 mg (65%) of the triazole **4b** as a white solid, mp 135-137°C. IR (neat, ν/cm^{-1}) 1647, 1708 (C=O), 3390 (NH). ^{1}H NMR (CDCl$_3$, 400 MHz) δ 0.62 (s, 3H), 0.90-1.07 (m, 18H), 1.41 (s, 9H), 1.68 (s, 3H), 2.98-3.05 (m, 1H), 3.29 (br. s, 2H), 3.79, 3.80 (s, 6H), 4.48-4.54 (m, 4H), 4.60 (s, 1H), 4.73 (s, 1H), 5.55 (m, 1H), 6.00 (d, 1H, J = 8.0 Hz), 6.44 (d, 1H, J = 4.0 Hz), 6.49 (d, 1H, J = 8.0 Hz), 7.42 (d, 1H, J = 8.0 Hz), 7.85 (s, 1H). ^{13}C NMR (CDCl$_3$, 100 MHz) δ 13.8, 14.7, 15.5, 16.1, 18.1, 18.4, 19.0, 19.2, 20.4, 23.5, 25.1, 28.0, 29.1, 29.4, 30.2, 31.8, 32.7, 33.8, 34.2, 36.0, 36.6, 36.7, 38.0, 40.2, 40.9, 42.0, 46.5, 48.8, 49.9, 50.2, 50.7, 54.2, 54.8, 54.9, 55.9, 60.0, 62.2, 75.6, 78.5, 98.1, 103.8, 109.2, 117.7, 123.3, 127.5, 150.1, 153.6, 155.62, 156.7, 159.8, 173.0, 174.6, 180.8. HRMS (ESI) calcd for $C_{58}H_{90}N_6O_9$+H$^+$ 1015.6848, found [M+H]$^+$ 1015.6842.

***N*-[(1,1-dimethylethoxy)carbonyl]-L-seryl-*N*^{1}-[(1*S*)-2-[4-[3,28-dihydroxy-28-oxolup-20(29)-en-3-yl]-1*H*-1,2,3-triazol-1-yl]-1-(phenylmethyl)ethyl]-*N*^{2}-[(2,4-dimethoxyphenyl)methyl]-2-methyl-alaninamide (4c)**. 120 mg (0.25 mmol) of the compound **2a**, 149 mg (0.25 mmol) of azide **3c**, 3 mg CuCl were added to 5 mL of butan-1-ol. The reaction mixture was kept at 115°C for 7 h, diluted with 10 mL of toluene and then washed with aqueous ammonia. The organic layer was separated, dried over Na_2SO_4 and filtered. The solvent was removed in vacuo. Hexane was added and the resulting precipitate was filtered off to afford 193 mg (71%) of the triazole **4c** as a white solid, mp 130-132°C. IR (neat, ν/cm^{-1}) 1654, 1710 (C=O); 3360 (NH, OH). ^{1}H NMR (CDCl$_3$, 400 MHz) δ 0.64 (s, 3H), 0.93-0.94 (m, 6H), 1.00-1.01 (m, 6H), 1.43 (s, 9H), 1.70 (s, 3H), 2.99-3.05 (m, 1H), 3.61-3.65 (m, 1H), 3.74, 3.78 (s, 6H), 3.88-3.91 (m, 1H), 4.42-4.48 (m, 3H), 4.61-4.77 (m, 4H), 4.93 (br. s, 1H), 6.00 (d, 1H, J = 8.0 Hz), 6.41-6.43 (m, 2H), 7.11 (d, 1H, J = 8.0 Hz), 7.18-7.31 (m, 5H), 7.71 (s, 1H). ^{13}C NMR (CDCl$_3$, 100 MHz) δ 13.8, 14.6, 15.5, 16.2, 18.1, 18.9, 19.0, 21.5, 24.8, 25.2, 27.9, 29.4, 30.2, 31.1, 31.8, 32.6, 33.7, 36.1, 36.6, 38.0, 40.2, 41.0, 42.0, 43.7, 46.5, 48.8, 49.9, 50.8, 50.9, 51.2, 53.1, 54.7, 54.9, 55.9, 60.0, 63.2, 76.0, 79.4, 98.2, 103.6, 109.2, 117.0, 123.7, 126.4, 128.2, 128.8, 136.8, 150.0, 152.8, 154.8, 157.7, 160.2, 172.3, 175.1, 180.9. HRMS (ESI) calcd for $C_{62}H_{90}N_6O_{10}$+H$^+$ 1079.6797, found [M+H]$^+$ 1079.6791.

***N*-[(1,1-dimethylethoxy)carbonyl]-3-(1*H*-indol-2-yl)alanyl-*N*^{1}-[(1*S*)-2-[4-[3,28-dihydroxy-28-oxolup-20(29)-en-3-yl]-1*H*-1,2,3-triazol-1-yl]-1-(phenylmethyl)ethyl]-*N*^{2}-[(2,4-dimethoxyphenyl)methyl]-2-methyl-alaninamide (4d)**. A mixture of 140 mg (0.29 mmol) of alkyne **2a**, 202 mg (0.29 mmol) of azide **3d**, and 5 mg CuCl in 7 ml of toluene was kept for 9 h. After the reaction was completed, the reaction mixture was transferred to a separatory funnel and washed with aqueous ammonia to remove the copper salts. The organic layer was separated, dried over Na_2SO_4 and filtered. The solvent was removed in vacuo. Hexane was added and the resulting precipitate was filtered off to afford 260 mg (76%) of the triazole **4d** as a white solid, mp 142-144°C. IR (neat, ν/cm^{-1}) 1697, 1702 (C=O), 3423 (NH). ^{1}H NMR ((CD$_3$)$_2$SO, 400.13 MHz) δ 0.48 (s, 3H), 0.84-0.87 (m, 12H), 1.24-1.28 (m, 15H), 1.64 (s, 3H), 2.64-2.82 (m, 2H), 2.93-3.02 (m, 3H), 3.73 (s, 3H), 3.77 (s, 3H), 4.21-4.42 (m, 5H), 4.55 (s, 1H), 4.65-4.68 (m, 2H), 4.83 (d, 1H, J = 20.0 Hz), 6.38-6.44 (m, 1H), 6.59 (s, 1H), 6.74-6.83 (m, 2H), 6.94-7.09 (m, 2H), 7.23-7.26 (m, 3H), 7.32-7.34 (m, 4H), 7.60-7.62 (m, 1H), 7.76-7.80 (m, 1H), 10.73 (s, 1H), 12.06 (s, 1H). ^{13}C NMR ((CD$_3$)$_2$SO, 100 MHz) δ 14.0, 14.8, 15.7, 16.4, 18.1, 18.9, 19.7, 20.5, 22.1, 24.2, 25.4, 27.2, 28.1, 29.4, 30.2, 31.0, 31.8, 32.6, 33.9, 35.5, 36.7, 37.7, 38.2, 40.9, 40.9, 41.9, 42.0, 46.7, 48.6, 49.7, 49.8, 51.0, 53.3, 55.2, 55.4, 55.4, 61.8, 74.5, 78.0, 98.1, 104.3, 109.6, 109.9, 111.1, 118.0, 118.1, 119.0, 120.7, 123.9, 124.5, 126.3, 127.1, 128.4, 129.3, 136.0, 138.2, 150.4, 154.8, 155.5, 156.8, 159.7, 173.3, 174.0, 177.3. HRMS (ESI) calcd for $C_{70}H_{95}N_7O_9$ +H$^+$ 1178.7270, found [M+H]$^+$ 1178.7264.

***N*-[(1,1-dimethylethoxy)carbonyl]-L-isoleucyl-*N*^{1}-[(1*S*)-2-[4-[3,28-dihydroxy-28-oxolup-20(29)-en-3-yl]-1*H*-1,2,3-triazol-1-yl]-1-methylethyl]-*N*^{2}-[(2,4-dimethoxyphenyl) methyl]-2-methyl-alaninamide (4e)**. 140 mg (0.29 mmol) of the compound **2a**, 160 mg (0.29 mmol) of azide **3e**, 5 mg of CuCl were added to 7 mL of toluene. The reaction mixture was kept at 110°C for 24 h, diluted with 10 mL of toluene and then washed with aqueous ammonia. The organic layer was separated, dried over Na_2SO_4 and filtered. The solvent was removed in vacuo. Hexane was added and the resulting precipitate was filtered off to afford 264 mg (88%) of the triazole **4e** as a white solid, mp 148-150°C. IR (neat, ν/cm^{-1}) 1708, 1714 (C=O), 3434 (NH). ^{1}H NMR (CDCl$_3$, 400 MHz) δ 0.58 (s, 3H), 0.80-0.87 (m, 6H), 0.93-1.02 (m, 12H), 1.41 (m, 15H), 1.69 (s, 3H), 2.98-3.05 (m, 1H), 3.79 (s, 6H), 4.30-4.68 (m, 7H), 4.73 (s, 1H), 5.36 (d, 1H, J = 8.0 Hz), 5.81-5.83 (m, 1H), 6.44-6.49 (m, 2H), 7.37 (d, 1H, J = 8.0 Hz), 7.62

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(m, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 11.0, 14.7, 15.2, 15.5, 16.1, 17.2, 18.1, 18.9, 20.4, 23.1, 23.4, 25.1, 27.9, 29.4, 30.2, 31.8, 32.6, 33.8, 36.0, 36.6, 36.7, 38.0, 38.2, 40.2, 40.9, 42.0, 42.2, 45.5, 46.5, 48.9, 50.0, 50.9, 53.3, 54.9, 55.4, 55.9, 62.6, 75.6, 78.9, 98.3, 103.8, 109.2, 117.8, 122.4, 128.4, 150.1, 153.4, 155.0, 156.9, 159.9, 173.5, 174.4, 180.7. HRMS (ESI) calcd for $C_{59}H_{92}N_6O_9$ +H^+ 1029.7004, found [M+H]+ 1029.6999.

***N*-[(1,1-dimethylethoxy)carbonyl]-L-phenylalanyl-*N*^{1}-[(1*S*)-2-[4-[3,28-dihydroxy-28-oxolup-20(29)-en-3-yl]-1*H*-1,2,3-triazol-1-yl]-1-methylethyl]-*N*^{2}-[(2,4-dimethoxyphenyl) methyl]-2-methyl-alaninamide (4f)**. A mixture of 120 mg (0.25 mmol) of alkyne **2a**, 145 mg (0.25 mmol) of azide **3f**, 5 mg of $CuSO_4 \cdot 5H_2O$ and 7 mg of sodium ascorbate in 10 ml of the mixture of THF/H_2O (1:1 ratio) was kept for 7 h. After the reaction was completed, the reaction mixture was transferred to a separatory funnel and washed with aqueous ammonia to remove the copper salts. The organic layer was separated, dried over Na_2SO_4 and filtered. The solvent was removed in vacuo. Hexane was added and the resulting precipitate was filtered off to afford 160 mg (60%) of the triazole **4f** as a white solid, mp 157-159°C. IR (neat, ν/cm^{-1}) 1654, 1708 (C=O), 3429 (NH, OH). ^1H NMR ($CDCl_3$, 400 MHz) δ 0.61 (s, 3H), 0.93-1.03 (m, 12H), 1.35 (s, 9H), 1.69 (s, 3H), 2.78-2.83 (m, 1H), 2.95-3.03 (m, 2H), 3.78 (s, 6H), 4.24-4.65 (m, 7H), 4.74 (s, 1H), 5.39 (d, 1H, *J* = 8.0 Hz), 5.87 (d, 1H, *J* = 8.0 Hz), 6.43 (s, 2H), 7.06 (s, 2H), 7.21 (m, 4H), 7.69 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.7, 14.7, 15.5, 16.1, 17.3, 18.1, 18.9, 19.0, 23.2, 23.4, 25.1, 27.9, 29.4, 30.2, 31.2, 32.7, 33.8, 36.0, 36.7, 38.0, 39.3, 40.2, 40.9, 42.0, 45.5, 46.5, 48.9, 49.9, 50.8, 52.7, 53.2, 54.8, 54.9, 55.9, 60.0, 62.4, 75.7, 79.1, 98.2, 103.9, 109.2, 117.9, 122.7, 126.3, 128.0, 129.2, 131.7, 136.2, 150.1, 153.5, 154.6, 156.8, 159.8, 173.1, 174.3, 180.8. HRMS (ESI) calcd for $C_{62}H_{90}N_6O_9$+H^+ 1063.6848, found [M+H]$^+$ 1063.6842.

***N*-[(1,1-dimethylethoxy)carbonyl]-L-isoleucyl-*N*^{1}-[(1*S*)-2-[4-[3,28-dihydroxy-28-oxolup-20(29)-en-3-yl]-1*H*-1,2,3-triazol-1-yl]-1-methylethyl]-*N*^{2}-[(2,4-dimethoxyphenyl) methyl]-2-methyl- alaninamide (5)**. A mixture of 70 mg (0.146 mmol) of alkyne **2b**, 80 mg (0.146 mmol) of azide **3e** and 3 mg of CuCl in 5 ml of toluene was kept for 13 h. After the reaction was completed, the reaction mixture was transferred to a separatory funnel and washed with aqueous ammonia to remove the copper salts. The organic layer was separated, dried over Na_2SO_4 and filtered. The solvent was removed in vacuo. Hexane was added and the resulting precipitate was filtered off to afford 110 mg (73%) of the triazole **5** as a white solid, mp 151-153°C. IR (neat, ν/cm^{-1}) 1643, 1714 (C=O), 3435 (NH). ^1H NMR ($CDCl_3$, 400 MHz) δ 0.61 (s, 3H), 0.79-0.85 (m, 12H), 0.92 (s, 3H), 0.99 (s, 3H), 1.37 (s, 12H), 1.67 (s, 3H), 2.96-3.03 (m, 1H), 3.76-3.77 (m, 6H), 4.24-4.58 (m, 7H), 4.72 (s, 1H), 5.19 (d, 1H, *J* = 8.0 Hz), 5.66 (d, 1H, *J* = 8.0 Hz), 6.41-6.46 (m, 2H), 7.41 (d, 1H, *J* = 8.0 Hz), 7.76 (s, 1H). ^{13}C NMR δ 11.3, 14.7, 15.5, 15.8, 15.9, 17.4, 18.7, 19.2, 20.6, 21.2, 23.0, 23.7, 23.9, 25.3, 28.2, 29.6, 30.4, 31.0, 32.0, 34.1, 34.4, 37.0, 37.1, 38.2, 38.8, 40.6, 42.3, 42.4, 45.6, 46.8, 49.1, 50.1, 50.2, 53.4, 55.2, 55.6, 56.2, 60.3, 62.7, 74.9, 79.2, 98.5, 104.1, 109.5, 118.1, 122.6, 128.5, 150.5, 152.6, 155.2, 157.0, 160.2, 173.6, 174.4, 180.9. Anal. calcd for $C_{59}H_{92}N_6O_9$: C, 68.84; H, 9.01; N, 8.16. Found: C, 68.54; H, 8.99; N, 7.92.

Pharmacological experiments

The experiments were carried out using outbred male mice housed in standard environmental conditions. The animals were given standard granulated food and water ad libitum. All experimental procedures were approved by the Bio-Ethical Committee of Medicine Chemistry Department of Novosibirsk Institute of Organic Chemistry SB RAS in accordance with European Communities Council Directive 86/609/EEC.

Histamine-induced edema model

Inflammatory edema was induced by subplanar injection of 0.05 ml 0.1% 1 histamine in water solution into the hind paw of male mice. The test compounds were administered intraperitoneally at a dose of 50 mg/kg b.w. as aqueous-Tween-80 suspension (water:Tween-80 100:1, V/v) 1 h before the histamine injection. This route of administration was selected to exclude possible metabolic transformation of conjugates in the gastrointestinal tract. The reference agent indometacin ('Fluka BioChemica') was administered at two doses and regimens, one of which corresponded to the dose and regimen of agents administration (50 mg / kg i.p.), and the second - to a routine administration and dosage of indomethacin in mice - 20 mg/kg orally.

Hardware and software

4-Core AMD Phenom II X4 925 based on the configuration running Linux 3.0.0-14 OS was used for calculations. All structures were visualized using UCSF Chimera 1.8 program.[20] Docking was performed using AutoDock Vina 1.1.2 algorithms.[21] PyRx 0.9 and MGL Tools 1.5.6 GUIs were utilized for the convenient docking preparation. Visualization of ligand-receptor complexes was carried out using PoseView web interface.[22] The ProFit web interface [Martin, A.C.R. and Porter, C.T., http://www.bioinf.org.uk/software/profit] was applied to calculate the root mean square deviation (RMSD) of the ligands' coordinates. To minimize the energy of structures of novel derivatives and to record them different chemical formats OpenBabel 2.3.2 program was used.[23]

Models

4IQK is the X-ray crystallographic model of Keap1 Kelch-domain.[24] In the binding site of 4IQK the Cpd16 molecule is coordinated, the structure of which was obtained by the authors of the model as a result of high-throughput screening of MLPCN chemical database. The models of new betulinic acid derivatives were obtained by mmff94 energy minimization algorithm.

Receptor and ligands preparation

Model of Keap1 Kelch-domain is available in the Protein Data Bank (www.pdb.com), PDB ID 4IQK. Model represents a symmetric subunit of a cylindrical shape with a distinct binding site, in which the Cpd16 molecule is coordinated. For the realization of docking it was necessary to adapt 4IQK model for UCSF Chimera 1.8 program. The Cpd16 molecule was removed from binding sites, which was stored separately without changing the coordinates and conformation for docking validation and accurate mapping of the binding site by re-docking into the adapted model 4IQK. Adapted model 4IQK and all ligands were prepared for docking in AutoDock Vina by conversion to pdbqt

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format, which contains the coordinates of the atoms, charges, solubility data and descriptions of rigid and flexible parts of the molecules.

Algorithms validation, search space optimization and docking

Docking procedure was carried out for the unchanged conformation of the receptor and flexible ligand molecules. The standard parameters of the AutoDock Vina program were used. Re-docking of Cpd16 into the adapted model of 4IQK was done to validate the docking algorithms of AutoDock Vina. The docking algorithm is adequate upon the condition that the standard deviation (RMSD) between the coordinates of native ligand and the best configuration obtained with re-docking satisfies RMSD <2 Å. AutoDock Vina search area was configured exactly to the coordinates of the native ligand and it presents a required minimum that significantly increases the accuracy of the result. The docking of all studied ligands in the binding site of the adapted model of 4IQK was performed after the docking algorithms validation was carried out. The results of ligands docking were compared with a minimum energy of binding of Cpd16 obtained as a result of re-docking.

Acknowledgements

This work was supported by the Interdisciplinary Grant №41 of SB of the Russian Academy of Sciences (2012-2014), the Chemical Service Centre of SB RAS and Russian Foundation for Basic Research (Grants No. 12-03-31582, 12-03-00292a, and 13-03-00129a).

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† Electronic Supplementary Information (ESI) available: [Synthetic procedures and characterization data for all obtained compounds, biochemical experiments]. See DOI: 10.1039/b000000x/

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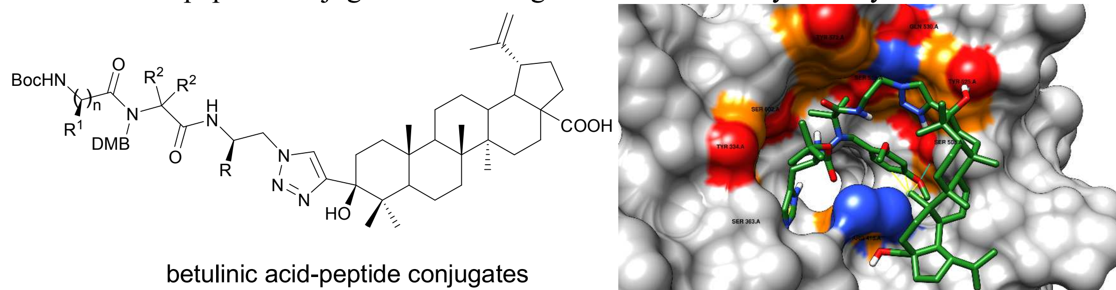

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