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Cyclic enediyne–amino acid chimeras as new aminopeptidase N inhibitors

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Abstract Enediyne–peptide conjugates were designed with the aim to inhibit aminopeptidase N, a widespread ectoenzyme with a variety of functions, like protein digestion, inactivation of cytokines in the immune system and endogenous opioid peptides in the central nervous system. Enediyne moiety was embedded within the 12-membered ring with hydrophobic amino acid alanine, valine, leucine or phenylalanine used as carriers. Aromatic part of the enediyne bridging unit and the amino acid side chains were considered as pharmacophores for the binding to the aminopeptidase N (APN) active site. Additionally, the fused enediyne–amino acid "heads" were bound through a flexible linker to the L-lysine, an amino group donor. The synthesis included building the aromatic enediyne core at the C-terminal of amino acids and subsequent intramolecular N-alkylation. APN inhibition test revealed that the alanine-based derivative **9a** inhibits the APN with IC_{50} of 34 ± 11 μM. Enediyne–alanine conjugate **12** missing the flexible linker was much less effective in the APN inhibition. These results show that enediyne-fused amino acids have potential as new pharmacophores in the design of APN inhibitors.

Keywords Aminopeptidase N · Enediyne–peptide conjugate · Enzyme inhibitors · Amino acids

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Introduction

Aminopeptidases catalyze the hydrolysis of the N-terminal peptide bond in peptides and proteins. They are widely distributed in many tissues and cells in animals, plants, bacteria and viruses and have wide substrate specificity (Taylor 1993). Aminopeptidase N (APN; CD13; membrane alanyl aminopeptidase; EC 3.4.11.2) is a zinc-containing metalloenzyme involved in cleavage of neutral or basic amino acids (Ala>Phe>Leu>Arg) from almost all unsubstituted oligopeptides and from amide or aryl amide derivatives of amino acids (Turner 2004). APN is a widespread ectoenzyme, particularly abundant in the brush border membranes of small intestine, kidney and placenta, but its expression range includes also fibroblasts and epithelial cells in the liver, brain and lung, endothelial cells in blood vessels and synaptic membranes (Turner 2004). This transmembrane metallopeptidase has a variety of functions, depending on the cell type and tissue environment. It is involved in the final stages of protein and peptide digestion in intestine, in terminating the actions of certain bioactive peptides and inactivation of cytokines in the immune system. Within the hematopoietic system, APN, also named the thirteenth "cluster of differentiation" antigen or CD13, is accepted as a marker for normal and malignant cells of the myeloid lineage, but has been detected as well on the lymphocytes, following cell activation, inflammation and malignant transformation (Gabrilovac et al. 2005). In addition to being identified as tumor marker of the hematopoietic system, accumulated data suggest that APN plays a pivotal role in solid tumor invasion and angiogenesis (Petrovic et al. 2004; Fukasawa et al. 2006). In tumor vasculature, APN is overexpressed in the endothelium and promotes angiogenesis. Thus, APN inhibition is expected to be useful for cancer treatment. APN is also involved in

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the inactivation of endogenous opioid peptides, the most important analgesia medium in the central nervous system (Chen et al. 1998). Recently, it has been reported that synthetic dual inhibitors of APN and another cell-surface metallopeptidase, neprilysin, can block the degradation of enkephalins and, hence, produce potent physiological analgetic responses (Noble and Roques 2007).

The crystal structure of APN from *Escherichia coli* was first reported in 2006 (Addlagatta et al. 2006; Ito et al. 2006), but the complete crystal structure of human APN has not been determined so far. The active site of APN was determined by X-ray crystallographic studies on the complex of the enzyme and bestatin (also known under the tradename Ubenimex), an aminopeptidase inhibitor currently in the clinical trials for the treatment of acute myelocytic leukemia (Hirayama et al. 2003). Studies revealed that the active site comprises Zn^{2+} ion as a catalytic center of APN, two hydrophobic binding pockets (S1 and S1′) in the vicinity of the Zn^{2+}, and the binding site for the α-amino group of the ligand (bestatin) formed by three glutamic acid residues. Key pharmacophores have been outlined for the potential inhibitor to possess: carbonyl oxygen and hydroxamate or similar group for Zn^{2+} coordination and two hydrophobic groups for the interaction with S1 and S1′ pockets (S1 pocket prefers aromatic rings rather than aliphatic chains). In addition, free α-amino group or a cation that may interact with the glutamic acid residues adjacent to S1 pocket is also advisable (Zhang and Xu 2008). Since the appearance of bestatin, numerous inhibitors have been prepared according to afore-mentioned guidelines as a transition state analogs (Zhang et al. 2011). They mostly belong to peptidomimetics or peptide mimics—the strategy of design used to overcome drawbacks of natural peptides, such as low bioavailability, proteolytic degradation and rapid elimination. The most prominent APN inhibitors are derivatives of bestatin (Gao et al. 2011; Rao et al. 2011; Luan et al. 2011), phosphinic acid (Grzywa and Oleksyszyn 2008; Grzywa et al. 2010), L-*iso*-glutamine (Li et al. 2009), gallic acid (Zhang and Xu 2008), sulfonyl pyrrolidine (Cheng et al. 2008), aminobenzosuberone derivatives (Maiereanu et al. 2011) and compounds containing hydroxamate group (Lee et al. 2005; Flipo et al. 2007).

Here, we propose structurally distinct group of compounds as potential APN inhibitors. These compounds are based on the enediyne chromophore, a key moiety of the enediyne anticancer antibiotics (Nicolaou and Dai 1991). Enediyne group (*Z*-1,5-diyn-3-hexene) is known to undergo the Bergman cyclization (Jones and Bergman 1972) that results in the cleavage of double stranded DNA. However, some recent findings raise questions about DNA as single biological target of enediyne compounds. Generally, poor correlation between their antitumor activity and the observed DNA damage governs scientists to search for new targets and propose proteins as the most probable ones (Nicolaou et al. 1993; Fouad et al. 2005; Dutta et al. 2009). Three independent classes of photoactivated enediynes have been designed to target albumin, histone and estrogen receptor and showed correlation between affinity and protein degrading activity (Fouad et al. 2005). Antibacterial activity of cyclic enediyne–phendioxy conjugates has also been revealed (Joshi et al. 2007). Conjugation of enediyne chromophore with amino acids or peptides was shown to induce novel thermally initiated cyclization–elimination pathway (Gredičak et al. 2010), but also to inhibit certain enzymes, such as topoisomerase I (Lin et al. 2001), proprotein convertase (Basak et al. 2009), chymotrypsin (Dutta et al. 2009) and tyrosine phosphatase (Chandra et al. 2011). In this work, we have designed and synthesized fused enediyne–amino acid "heads", attached them to the L-lysine through a flexible linker and tested for the APN inhibitor activity.

Materials and methods

General

Optical rotations were measured at 20 °C with an Optical Activity LTD automatic AA-10 Polarimeter (some compounds are too colored for an unambiguous determination of optical rotation). Reactions were monitored by TLC on Silica Gel 60 F254 plates (Merck; Darmstadt, Germany) using detection with ninhydrin. Column chromatography was performed on Silica Gel (Merck, 0.040–0.063). RP HPLC analysis was performed on HPLC system coupled with UV detector; C-18 semipreparative (250 × 8 mm, ID 5 μm) column at flow rate of 1 mL/min, or analytical (150 × 4.5 mm, ID 5 μm) column at flow rate of 0.5 mL/min was used under isocratic conditions using different concentration of MeOH in 0.1 % aqueous TFA. UV detection was performed at 254 nm. NMR spectra were recorded on 600 and 300 MHz spectrometers, operating at 150. 92 or 75.47 MHz for ^{13}C and 600.13 or 300.13 MHz for ^{1}H nuclei. TMS was used as an internal standard. Spectra were assigned based on 2D homonuclear (COSY) and heteronuclear (HMQC and HMBC) spectra. Mass spectrometry measurements were performed on HPLC system coupled with triple quadrupole mass spectrometer, operating in positive electrospray ionization (ESI) mode. Spectra were recorded from a 10 μg/mL compound solution in 50 % MeOH/0.1 % FA by injection of 3 μL into the ion source of the instrument by autosampler, at the flow rate of 0.2 mL/min (mobile phase 50 % MeOH/0.1 % FA). HRMS analysis was performed on MALDI-TOF mass spectrometer operating in reflectron mode. Mass spectra

were acquired by accumulating three spectra after 400 laser shots per spectrum. Calibrant and analyte spectra were obtained in positive ion mode. Calibration type was internal with calibrants produced by matrix ionization (monomeric, dimeric and trimeric CHCA), with azithromycin and angiotensin II dissolved in α-cyano-4-hydroxycinnamic acid matrix in the mass range *m/z* 190.0499 to 749.5157 or 1046.5417. Accurately measured spectra were internally calibrated and elemental analysis was performed on Data Explorer v. 4.9 Software with mass accuracy better than 5 ppm. Samples were prepared by mixing 1 μL of analyte methanol solution with 5 μL of saturated (10 mg/mL) solution of α-cyano-4-hydroxycinnamic acid (α-CHCA) and internal calibrants (0.1 mg/mL) dissolved in 50 % acetonitrile/0.1 % TFA. MM2 calculations were performed by a modified version of Allinger's MM2 force field, integrated into the ChemBioOffice 2008 programme. Synthesis of amino acid derivatives **1a–d** has been described previously (Gredičak et al. 2008).

APN activity and inhibition assay

The enzyme activity was assayed using L-Leu-2-naphthylamide (Leu-2NA) as the substrate according to the described procedure (Abramić and Vitale 1992). The enzyme (porcine kidney microsomal aminopeptidase, EC 3.4.11.2, Sigma), in concentration of 1.58×10^{-10} M, was incubated with the selected compound (**6**, **9** or **10**) for 10 min at 23 °C in 50 mM Na-PO_4 buffer pH 7.4, followed by 5 min at 37 °C, in the total volume of 935 μL. The enzyme reaction was then initiated by the addition of 65 μL of 1.36 mM solution of Leu-2NA. After 10-min incubation at 37 °C, the reaction was stopped by adding 0.2 mL of Fast Blue B salt (1.5 mg/mL) solution containing 10 % Tween 80 in 2M sodium acetate buffer pH 4.2. The absorbance at 530 nm was measured after 15 min. The release of the reaction product 2-naphthylamine was followed also continuously, by measuring its fluorescence at 25 °C by Perkin-Elmer luminescence spectrometer LS 50, using excitation and emission wavelength of 332 and 420 nm, respectively (Abramić et al. 2004).

To check if potential inhibitors are hydrolyzed by APN, the incubation of selected compound was performed with the enzyme for 60 min in 0.025 M Na-PO_4 buffer pH 7.4, at 37 °C. The concentration of APN in reaction mixture of 100 μL was 1.58×10^{-9} M, and of the compounds **9a** and **10**, it was 5 and 25 mM, respectively. Control samples did not contain the enzyme. Aliquots (5 μL) of the reaction mixture were analyzed by thin-layer chromatography (TLC) on glass plates precoated with silica gel 60 F254 (Merck, Darmstadt, Germany) using mixture of solvents EtOH–H_2O–NH_4OH (60:25:15, v/v). L-Lysine was analyzed at the same time and visualization was accomplished using ninhydrin (2 % in ethanol). Additionally, samples were analyzed by HPLC–MS. Mobile phase was 0.1 % FA in water (solvent A) and 0.1 % FA in MeOH (solvent B). Gradient was applied as follows: 0 min 100 % A (0 % B); 0–12 min 100 % A (0 % B) – 90 % B (10 % A); 12–15 min 90 % B (10 % A); 15–17 min 90 % B (10 % A) – 100 % A (0 % B). Aliquots (25 μL) of the reaction mixture were diluted with 100 μL of 0.1 % FA in water and 5 μL were injected on the column (Zorbax XDB C18, 3.5 μm, 4.6×75 mm) at the flow rate of 0.5 mL/min.

Synthesis of amino acid derivatives ***2***

CuI (0.161 mmol), $Pd(PPh_3)_4$ (0.016 mmol), piperidine (1.608 mmol) and 1,2-diiodobenzene (2.412 mmol) were dissolved in dry THF under argon and stirred at room temperature for 30 min. A premixed solution of **1** (1.608 mmol) and piperidine (1.608 mmol) in dry THF under argon was added dropwise into the initial reaction mixture. The solution was stirred at room temperature for 3 h. The solvent was evaporated and the product extracted with EtOAc, washed with brine and water and purified by flash chromatography.

*(S)-N-(3-(2-iodophenyl)prop-2-ynyl)-2-(2-nitrophenylsulfonamido)propanamide (**2a**)* Yield: 41 %. Brown oil. R_f 0.51 (EtOAc–petrol ether 3:2). M_r 513.31. ^{13}C NMR ($CDCl_3$): δ = 19.0 (β Ala), 30.4 (H1 propargyl), 53.5 (α Ala), 85.9, 87.9 (C2,3 propargyl), 100.9 (C2 iodophenyl), 125.7 (C3 oNbs), 128.8 (C1 iodophenyl), 127.8, 129.7, 131.1, 132.7, 133.1, 134.1 (C4,5,6 oNbs, C4,5,6 iodophenyl), 138.7 (C3 iodophenyl), 147.9 (C2 oNbs), 170.3 (CO Ala). ^{1}H NMR ($CDCl_3$): δ = 1.39 (d, 3H, β Ala, $^3J_{\alpha,\beta}$ = 7.2 Hz), 4.05 (q, 1H, α Ala, $^3J_{\alpha,\beta}$ = 7.2 Hz), 4.21–4.27 (m, 2H, H1 propargyl), 5.97 (br s, 1H, NHα), 6.70 (br t, 1H, NH_{amide}), 6.98–7.06, 7.28–7.34, 7.39–7.44 (m, 4H, H3,4,5,6 iodophenyl), 7.69–7.76 (m, 2H, H4,5 oNbs), 7.81–7.91 (m, 1H, H6 oNbs), 8.11–8.17 (m, 1H, H3 oNbs).

*(S)-N-(3-(2-iodophenyl)prop-2-ynyl)-3-methyl-2-(2-nitrophenylsulfonamido)butanamide (**2b**)* Yield: 67 %. Brown oil. R_f 0.40 (petrol ether–EtOAc 2:1). M_r 541.36. ^{13}C NMR ($CDCl_3$): δ = 16.8, 18.7 (γγ′ Val), 29.7 (CH_2 propargyl), 30.5 (β Val), 62.9 (α Val), 85.3 (C2 propargyl), 87.4 (C3 propargyl), 100.2 (C2 iodophenyl), 125.1 (C3 oNbs), 128.4 (C1 iodophenyl), 127.4, 129.2, 130.2, 132.2, 132.4, 133.4 (C4,5,6 iodophenyl, C4,5,6 oNbs), 132.9 (C1 oNbs), 138.2 (C3 iodophenyl), 169.1 (CO Val). ^{1}H NMR ($CDCl_3$): δ = 0.86, 0.96 (d, 6H, γγ′ Val, $^3J_{\beta,\gamma}$ = 6.9 Hz), 2.23–2.29 (m, 1H, β Val), 4.10–4.15 (m, 1H, α Val), 4.18 (t, 2H, CH_2 propargyl, $^3J_{H,NH}$ = 5.0 Hz), 6.03 (d, 1H, NHα, $^3J_{\alpha,NH}$ = 7.2 Hz), 6.50 (br t, 1H, NH_{amide}), 6.99–7.04 (m, 1H, H4

iodophenyl), 7.28–7.32, 7.38–7.41 (m, 2H, H5,6 iodophenyl), 7.64–7.77 (m, 3H, H3 iodophenyl, H4,5 oNbs), 7.83–7.86 (m, 1H, H6 oNbs), 8.08–8.11 (m, 1H, H3 oNbs).

*(S)-N-(3-(2-iodophenyl)prop-2-ynyl)-4-methyl-2-(2-nitrophenylsulfonamido)pentanamide (**2c**)* Yield: 41 %. Brown oil. R_f 0.51 (petrol ether–EtOAc 1:1). M_r 555.39. ^{13}C NMR ($CDCl_3$): $\delta = 20.6$, 22.4 ($\delta\delta'$ Leu), 23.8 (γ Leu), 29.7 (C1 propargyl), 41.4 (β Leu), 56.1 (α Leu), 85.3, 87.4 (C2,3 propargyl), 100.3 (C2 iodophenyl), 125.1 (C3 oNbs), 128.4 (C1 iodophenyl), 127.4, 129.2, 130.4, 132.2, 132.4, 133.4 (C4,5,6 iodophenyl, C4,5,6 oNbs), 132.8 (C1 oNbs), 138.3 (C3 iodophenyl), 147.2 (C2 oNbs), 169.9 (CO Leu). ^{1}H NMR ($CDCl_3$): $\delta = 0.75$, 0.89 (d, 6H, $\delta\delta'$ Leu, $^3J_{\gamma,\delta} = 6.4$ Hz), 1.59–1.70 (m, 3H, β,γ Leu), 3.95–4.01 (m, 1H, α Leu), 4.11–4.20 (m, 2H, H1 propargyl), 5.99 (br s, 1H, NHα), 6.58 (br t, 1H, NH_{amide}), 6.99–7.03, 7.28–7.32, 7.39–7.42 (m, 4H, H3,4,5,6 iodophenyl), 7.64–7.72 (m, 2H, H4,5 oNbs), 7.82–7.87 (m, 1H, H6 oNbs), 8.11–8.16 (m, 1H, H3 oNbs).

*(S)-N-(3-(2-iodophenyl)prop-2-ynyl)-2-(2-nitrophenylsulfonamido)-3-phenylpropanamide (**2d**)* Yield: 53 %. Brown oil. R_f 0.67 (petrol ether–EtOAc 2:1). M_r 589.40. ^{13}C NMR ($CDCl_3$): $\delta = 30.4$ (C1 propargyl), 38.5 (β Phe), 59.7 (α Phe), 86.0 (C3 propargyl), 88.2 (C2 propargyl), 101.2 (C2 iodophenyl), 126.2 (C6 oNbs), 127.5, 128.0, 128.9, 129.3, 129.9 (δ,ε,ζ Phe, C4,5 iodophenyl), 129.2 (C1 iodophenyl), 131.1 (C3 oNbs), 133.0 (C1 oNbs), 132.9, 133.3, 133.9 (C4,5 oNbs, C6 iodophenyl), 135.2 (γ Phe), 138.9 (C3 iodophenyl), 147.3 (C2 oNbs), 169.9 (CO Phe). ^{1}H NMR ($CDCl_3$): $\delta = 2.95$, 3.26 (dd, 2H, β Phe, $^2J_{\beta,\beta'} = 14.1$ Hz, $^3J_{\alpha,\beta} = 5.2$ Hz, $^3J_{\alpha,\beta'} = 8.9$ Hz), 4.15–4.18 (m, 1H, α Phe), 4.24, 4.36 (dd, 2H, H1 propargyl, $^3J_{NH,H1} = 5.7$ Hz, $^3J_{NH,H1'} = 4.9$ Hz, $^2J_{H1,1'} = 17.7$ Hz), 6.83 (br t, 1H, NHα), 6.99–7.04 (m, 5H, δ,ε,ζ Phe), 7.28–7.32 (m, 2H, H4,5 iodophenyl), 7.41–7.44, 7.62–7.68 (m, 3H, H4,5 oNbs, H6 iodophenyl), 7.74–7.76 (m, 1H, H6 oNbs), 7.82–7.85 (m, 1H, H3 iodophenyl), 7.95–7.98 (m, 1H, H3 oNbs).

*Synthesis of amino acid derivatives **3***

Compound **2** (1.294 mmol), $Pd(PPh_3)_4$ (0.013 mmol), CuI (0.129 mmol) and piperidine (2.588 mmol) were dissolved in dry THF under argon and stirred at room temperature for 30 min. Propargyl alcohol (3.883 mmol) was added and the reaction mixture was stirred at room temperature overnight. The solvent was evaporated and the product extracted with EtOAc, washed with brine and water and purified by flash chromatography.

*(S)-N-(3-(2-(3-hydroxyprop-1-ynyl)phenyl)prop-2-ynyl)-2-(2-nitrophenylsulfonamido)propanamide (**3a**)* Yield: 75 %. Yellow oil. R_f 0.40 (EtOAc–petrol ether 2:1). $[\alpha]_D$ −40.0° (c 1.0 MeOH). M_r 441.46. ^{13}C NMR ($CDCl_3$): $\delta = 19.1$ (β Ala), 30.5 (C1 propargyl), 51.8 (C1′ propargyl), 53.6 (α Ala), 82.4, 84.3 (C2,3 propargyl), 88.7 (C3′ propargyl), 92.1 (C2′ propargyl), 125.5, 126.0 (C1,2 enediyne aromatic ring), 125.8 (C3 oNbs), 128.3, 128.4, 131.2, 131.4, 132.4, 133.3, 134.2 (C4,5,6 oNbs, C3,4,5,6 enediyne aromatic ring), 133.5 (C1 oNbs), 147.9 (C2 oNbs), 171.2 (CO Ala). ^{1}H NMR ($CDCl_3$): $\delta = 1.36$ (d, 3H, β Ala, $^3J_{\alpha,\beta} = 7.0$ Hz), 4.02–4.31 (m, 3H, H1 propargyl, α Ala), 4.58 (s, 2H, H1′ propargyl), 7.16 (br t, 1H, NH_{amide}), 7.24–7.49 (m, 4H, H3,4,5,6 enediyne aromatic ring), 7.60–7.67 (m, 2H, H4,5 oNbs), 7.81–7.87 (H6 oNbs), 8.08–8.15 (H3 oNbs).

*(S)-N-(3-(2-(3-hydroxyprop-1-ynyl)phenyl)prop-2-ynyl)-3-methyl-2-(2-nitrophenylsulfonamido)-butanamide (**3b**)* Yield: 73 %. Yellow oil. R_f 0.47 (EtOAc–petrol ether 2:1). $[\alpha]_D$ −63.0° (c 1.0 MeOH). M_r 469.51. ^{13}C NMR (CD_3OD): $\delta = 15.7$, 16.6 ($\gamma\gamma'$ Val), 21.2 (β Val), 29.9 (C1 propargyl), 48.4 (C1′ propargyl), 61.3 (α Val), 79.2 (C3 propargyl), 81.1 (C2 propargyl), 86.6 (C3′ propargyl), 90.3 (C2′ propargyl), 123.3 (C3 oNbs), 123.5, 124.0 (C1,2 enediyne aromatic ring), 126.3, 126.5, 128.7, 130.0, 130.2, 130.8, 132.1 (C3,4,5,6 enediyne aromatic ring, C4,5,6 oNbs), 131.9 (C1 oNbs), 146.3 (C2 oNbs), 169.3 (CO Val). ^{1}H NMR (CD_3OD): $\delta = 0.95$, 0.98 (d, 6H, $\gamma\gamma'$ Val, $^3J_{\beta,\gamma} = 6.7$ Hz), 1.94–2.04 (m, 1H, β Val), 3.73 (d, 1H, α Val, $^3J_{\alpha,\beta} = 7.4$ Hz), 3.92, 3.99 (d, 2H, H1 propargyl, $^2J_{H,H'} = 17.5$ Hz), 4.45 (s, 2H, H1′ propargyl), 7.29–7.46 (m, 4H, H3,4,5,6 enediyne aromatic ring), 7.58–7.74 (m, 2H, H4,5 oNbs), 7.76–7.80 (m, 1H, H6 oNbs), 8.05–8.09 (m, 1H, H3 oNbs).

*(S)-N-(3-(2-(3-hydroxyprop-1-ynyl)phenyl)prop-2-ynyl)-4-methyl-2-(2-nitrophenylsulfonamido)pentanamide (**3c**)* Yield: 63 %. Yellow oil. R_f 0.22 (EtOAc–petrol ether 1:1). $[\alpha]_D$ −61.0° (c 1.0 MeOH). M_r 483.54. ^{13}C NMR (CD_3OD): $\delta = 18.7$, 20.4 ($\delta\delta'$ Leu), 22.6 (γ Leu), 27.4 (C1 propargyl), 40.3 (β Leu), 48.4 (C1′ propargyl), 54.2 (α Leu), 79.3 (C3 propargyl), 81.1 (C2 propargyl), 86.7 (C3′ propargyl), 90.3 (C2′ propargyl), 123.2 (C3 oNbs), 123.6, 123.9 (C1,2 enediyne aromatic ring), 126.3, 126.5, 128.7, 130.0, 130.2, 130.8, 132.2 (C3,4,5,6 enediyne aromatic ring, C4,5,6 oNbs), 131.8 (C1 oNbs), 146.3 (C2 oNbs), 170.4 (CO Leu). ^{1}H NMR (CD_3OD): $\delta = 0.87$, 0.94 (d, 6H, $\delta\delta'$ Leu, $^3J_{\gamma,\delta} = 6.8$ Hz), 1.43–1.82 (m, 3H, β,γ Leu), 3.99 (s, 2H, H1 propargyl), 4.02–4.09 (m, 1H, α Leu), 4.47 (s, 2H, H1′ propargyl), 7.27–7.49 (m, 4H, H3,4,5,6 enediyne aromatic ring), 7.59–7.82 (m, 3H, H4,5,6 oNbs), 8.05–8.13 (m, 1H, H3 oNbs).

(S)-N-(3-(2-(3-hydroxyprop-1-ynyl)phenyl)prop-2-ynyl)-2-(2-nitrophenylsulfonamido)-3-phenylpropanamide ***(3d)*** Yield: 61 %. Brown oil. R_f 0.38 (EtOAc–petrol ether 1:1). M_r 517.55. ^{13}C NMR ($CDCl_3$): $\delta = 30.4$ (C1 propargyl), 38.4 (β Phe), 51.5 (C1′ propargyl), 59.6 (α Phe), 82.1 (C3′ propargyl), 84.0 (C3 propargyl), 88.5 (C2 propargyl), 92.1 (C2′ propargyl), 125.3, 125.9 (C1,2 enediyne aromatic ring), 125.8 (C6 oNbs), 127.2, 128.0, 128.2, 128.5, 129.2 (δ,ε,ζ Phe, C4,5 enediyne aromatic ring), 130.8 (C3 oNbs), 131.3, 131.4 (C4,5 oNbs), 133.0 (C1 oNbs), 133.1, 133.6 (C3,6 enediyne aromatic ring), 135.2 (γ Phe), 147.1 (C2 oNbs), 170.3 (CO Phe). ^1H NMR ($CDCl_3$): $\delta = 2.95$, 3.20 (dd, 2H, β Phe, $^3J_{\alpha,\beta} = 5.3$ Hz, $^3J_{\alpha,\beta'} = 8.5$ Hz, $^2J_{\beta,\beta'} = 14.0$ Hz), 4.10, 4.34 (dd, 2H, H1 propargyl, $^2J_{H,H'} = 17.6$ Hz, $^3J_{NH,H} = 6.2$ Hz, $^3J_{NH,H'} = 4.8$ Hz), 4.18–4.25 (m, 1H, α Phe), 4.57 (s, 2H, H1′ propargyl), 6.96–7.06 (m, 5H, δ,ε,ζ Phe), 7.18 (br t, 1H, NH_{amide}), 7.24–7.30 (m, 2H, H4, 5 enediyne aromatic ring), 7.36–7.43 (m, 2H, H3,6 enediyne aromatic ring), 7.55–7.61 (m, 2H, H4,5 oNbs), 7.70–7.75 (m, 1H, H6 oNbs), 7.91–7.96 (m, 1H, H3 oNbs).

Synthesis of amino acid derivatives ***4***

Compound **3** (0.917 mmol) was dissolved in dry DCM and PBr_3 (1.834 mmol) was added. The reaction mixture was stirred for 2 h at room temperature. The reaction was quenched with an ice cold 10 % $NaHCO_3$ solution. The solvent was evaporated and the product extracted with EtOAc, washed with brine and water and purified by flash chromatography.

(S)-N-(3-(2-(3-bromoprop-1-ynyl)phenyl)prop-2-ynyl)-2-(2-nitrophenylsulfonamido)propanamide ***(4a)*** Yield: 79 %. Yellow oil. R_f 0.60 (EtOAc–petrol ether 2:1). $[\alpha]_D$ −27.0° (*c* 1, MeOH). M_r 504.35. ^{13}C NMR ($CDCl_3$): $\delta = 15.1$ (C1′ propargyl), 18.6 (β Ala), 29.9 (C1 propargyl), 53.0 (α Ala), 81.6 (C3 propargyl), 83.4 (C2 propargyl), 84.8 (C3′ propargyl), 87.8 (C2′ propargyl), 124.6, 124.7 (C1,2 enediyne aromatic ring), 125.2 (C3 oNbs), 127.8, 128.1 (C4,5 enediyne aromatic ring), 130.5 (C6 oNbs), 131.4. 131.5 (C3,6 enediyne aromatic ring), 132.6 (C4 oNbs), 132.7 (C1 oNbs), 133.6 (C5 oNbs), 147.3 (C2 oNbs), 169.8 (CO Ala). ^1H NMR ($CDCl_3$): $\delta = 1.39$ (d, 3H, β Ala, $^3J_{\alpha,\beta} = 7.2$ Hz), 4.06 (m, 1H, α Ala), 4.24, 4.28 (dd, 2H, H1 propargyl, $^3J_{H,NH} = 4.8$ Hz, $^2J_{H,H'} = 17.8$ Hz), 4.26 (s, 2H, H1′ propargyl), 5.98 (br d, 1H, NHα), 6.64 (br t, 1H, NH_{amide}), 7.27–7.31 (m, 2H, H4,5 enediyne aromatic ring), 7.40–7.45 (m, 2H, H3,6 enediyne aromatic ring), 7.66–7.73 (m, 2H, H4,5 oNbs), 7.85–7.88 (H6 oNbs), 8.11–8.15 (H3 oNbs).

(S)-N-(3-(2-(3-bromoprop-1-ynyl)phenyl)prop-2-ynyl)-3-methyl-2-(2-nitrophenylsulfonamido) butanamide ***(4b)*** Yield: 82 %. Colorless oil. R_f 0.44 (EtOAc–petrol ether 1:1). $[\alpha]_D$ −69.0° (*c* 1.0 MeOH). M_r 532.41. ^{13}C NMR ($CDCl_3$): $\delta = 15.81$ (C1′ propargyl), 17.56, 19.41 ($\gamma\gamma'$ Val), 30.39 (C1 propargyl), 31.38 (β Val), 63.64 (α Val), 82.22, 85.49 (C3,3′ propargyl), 88.46, 88.52 (C2,2′ propargyl), 125.06, 125.48 (C1,2 enediyne aromatic ring), 125.75 (C3 oNbs), 128.53, 128.84, 130.92, 132.13, 132.23, 133.12, 134.05 (C4,5,6 oNbs, C3,4,5,6 enediyne aromatic ring), 133.66 (C1 oNbs), 147.96 (C2 oNbs), 169.85 (CO Val). ^1H NMR ($CDCl_3$): $\delta = 0.88$, 0.96 (d, 6H, $\gamma\gamma'$ Val, $^3J_{\beta,\gamma} = 6.7$ Hz), 2.20–2.27 (m, 1H, β Val), 3.79–3.83 (m, 1H, α Val), 4.15, 4.20 (dd, 2H, H1 propargyl, $^3J_{NH,H} = 5.6$ Hz, $^2J_{H,H'} = 17.8$ Hz), 4.25, 4.28 (d, 2H, H1′ propargyl, $^2J_{H,H'} = 14.1$ Hz), 6.07 (d, 1H, NHα, $^3J_{NH,\alpha} = 8.3$ Hz), 6.50 (br t, 1H, NH_{amide}), 7.28–7.31 (m, 2H, H4,5 enediyne aromatic ring), 7.38–7.45 (m, 2H, H3,6 enediyne aromatic ring), 7.59–7.67 (m, 2H, H4,5 oNbs), 7.81–7.84 (m, 1H, H6 oNbs), 8.08–8.11 (m, 1H, H3 oNbs).

(S)-N-(3-(2-(3-bromoprop-1-ynyl)phenyl)prop-2-ynyl)-4-methyl-2-(2-nitrophenylsulfonamido)pentanamide ***(4c)*** Yield: 82 %. Colorless oil. R_f 0.57 (EtOAc–petrol ether 1:1). $[\alpha]_D$ −70.0° (*c* 1.0 MeOH). M_r 545.45. ^{13}C NMR ($CDCl_3$): $\delta = 15.7$ (C1′ propargyl), 21.1, 23.0 ($\delta\delta'$ Leu), 24.3 (γ Leu), 30.2 (C1 propargyl), 41.9 (β Leu), 56.6 (α Leu), 82.0, 82.9 (C3,3′ propargyl), 85.3, 88.3 (C2,2′ propargyl), 124.8, 125.3 (C1,2 enediyne aromatic ring), 125.6 (C3 oNbs), 128.4, 128.7, 130.9, 131.9, 132.0, 133.0, 133.9 (C4,5,6 oNbs, C3,4,5,6 enediyne aromatic ring), 133.3 (C1 oNbs), 147.7 (C2 oNbs), 170.5 (CO Leu). ^1H NMR ($CDCl_3$): $\delta = 0.76$, 0.89 (d, 6H, $\delta\delta'$ Leu, $^3J_{\gamma,\delta} = 6.1$ Hz), 1.58–1.76 (m, 3H, β,γ Leu), 3.96–4.05 (m, 1H, α Leu), 4.13–4.20 (m, 2H, H1 propargyl), 4.25, 4.31 (d, 2H, H1′ propargyl, $^2J_{H,H'} = 14.2$ Hz), 6.01 (d, 1H, NHα, $^3J_{\alpha,NH} = 7.7$ Hz), 6.55 (t, 1H, NH_{amide}, $^3J_{H,NH} = 4.9$ Hz), 7.25–7.33, 7.38–7.47 (m, 4H, H3,4,5,6 enediyne aromatic ring), 7.58–7.70 (m, 2H, H4,5 oNbs), 7.81–7.87 (m, 1H, H6 oNbs), 8.10–8.16 (m, 1H, H3 oNbs).

(S)-N-(3-(2-(3-bromoprop-1-ynyl)phenyl)prop-2-ynyl)-2-(2-nitrophenylsulfonamido)-3-phenylpropanamide ***(4d)*** Yield: 78 %. Yellow oil. R_f 0.53 (EtOAc–petrol ether 1:1). $[\alpha]_D$ −3.0° (*c* 0.5, MeOH). M_r 580.45. ^{13}C NMR ($CDCl_3$): $\delta = 15.1$ (C1′ propargyl), 29.9 (C1 propargyl), 38.0 (β Phe), 59.2 (α Phe), 81.5 (C1′ propargyl), 84.7 (C3 propargyl), 87.8, 87.9 (C2,2′ propargyl), 124.4, 124.8 (C1,2 enediyne aromatic ring), 125.4 (C6 oNbs), 126.8, 127.8, 128.1, 128.2, 128.6, 130.4, 131.5, 132.6, 133.2 (δ,ε,ζ Phe, C4,5 oNbs, C3,4,5,6 enediyne aromatic ring), 131.5 (C3 oNbs), 132.3 (C1 oNbs), 138.2 (γ Phe), 146.6 (C2 oNbs),

169.2 (CO Phe). ^{1}H NMR ($CDCl_3$): $\delta = 2.96$, 3.25 (dd, 2H, β Phe, $^2J_{\beta,\beta'} = 14.6$ Hz, $^3J_{\alpha,\beta} = 5.1$ Hz), 4.17–4.20 (m, 1H, α Phe), 4.23, 4.37 (dd, 2H, H1 propargyl, $^2J_{H,H'} = 17.8$ Hz, $^3J_{NH,H} = 5.9$ Hz), 4.25 (s, 2H, H1′ propargyl), 6.10 (d, 1H, NHα, $^3J_{\alpha,NH} = 6.2$ Hz), 6.79 (br t, 1H, NH_{amide}), 6.98–7.06 (m, 5H, δ,ε,ζ Phe), 7.26–7.31 (m, 2H, H4,5 enediyne aromatic ring), 7.40–7.45 (m, 2H, H3,6 enediyne aromatic ring), 7.59–7.65 (m, 2H, H4,5 oNbs), 7.72–7.75 (m, 1H, H6 oNbs), 7.95–7.98 (m, 1H, H3 oNbs).

Synthesis of amino acids derivatives ***5***

Compound **4** (0.079 mmol) was dissolved in 10 mL DMF and added dropwise via a syringe pump into the solution of K_2CO_3 (0.158 mmol) in 10 mL DMF (flow rate 0.49 mL/h). The solvent was evaporated and the product extracted with EtOAc, washed with brine and water and purified by flash chromatography.

*(5S)-5-methyl-4-(2-nitrophenylsulfonyl)-benz-4,7-diazacyclododeca-1,9-diyn-6-one (**5a**)* Yield: 33 %. Colorless oil. R_f 0.62 (EtOAc–petrol ether 3:1). $[\alpha]_D$ +55.0° (*c* 0.5 MeOH). M_r 423.44. ^{13}C NMR ($CDCl_3$): $\delta = 13.4$ (β Ala), 30.0 (C1 propargyl), 33.7 (C1′ propargyl), 53.9 (α Ala), 82.4, 82.6 (C2,2′ propargyl), 87.2, 90.2 (C3,3′ propargyl), 123.8 (C3 oNbs), 124.9, 126.3 (C1,2 enediyne aromatic ring), 127.2, 127.7 (C4,5 enediyne aromatic ring), 128.4 (C6 oNbs), 130.4 (C4 oNbs), 131.3, 131.5 (C3,6 enediyne aromatic ring), 132.0 (C1 oNbs), 133.7 (C5 oNbs), 168.6 (CO Ala). ^{1}H NMR ($CDCl_3$): $\delta = 1.34$ (d, 3H, β Ala, $^3J_{\alpha,\beta} = 7.0$ Hz), 3.71, 4.65 (d, 2H, H1′ propargyl, $^2J_{H,H'} = 18.5$ Hz), 4.30, 4.71 (dd, 2H, H1 propargyl, $^3J_{H,NH} = 4.1$ Hz, $^3J_{H',NH} = 8.6$ Hz, $^2J_{H,H'} = 17.5$ Hz), 4.53 (q, 1H, $^3J_{\alpha,\beta} = 7.0$ Hz), 7.20–7.35 (m, 4H, H3,4,5,6 enediyne aromatic ring), 7.56–7.70 (m, 3H, H4,5,6 oNbs), 8.18–8.21 (m, 1H, H3 oNbs).

*(5S)-5-isopropyl-4-(2-nitrophenylsulfonyl)-benz-4,7-diazacyclododeca-1,9-diyn-6-one (**5b**)* Yield: 55 %. Colorless oil. R_f 0.44 (EtOAc–petrol ether 2:1). $[\alpha]_D$ +89.0° (*c* 0.5 MeOH). M_r 451.49. ^{13}C NMR ($CDCl_3$): $\delta = 18.4$, 19.6 ($\gamma\gamma'$ Val), 26.5 (β Val), 30.5 (C1 propargyl), 35.0 (C1′ propargyl), 65.3 (α Val), 82.0, 83.7 (C2,2′ propargyl), 88.7, 90.2 (C3,3′ propargyl), 123.5 (C3 oNbs), 126.1, 126.8 (C1,2 enediyne aromatic ring), 127.9, 128.3 (C4,5 enediyne aromatic ring), 129.4 (C6 oNbs), 131.1, 131.4, 132.4, 133.9 (C3,6 enediyne aromatic ring, C4,5 oNbs), 133.3 (C1 oNbs), 147.8 (C2 oNbs), 168.5 (CO Val). ^{1}H NMR ($CDCl_3$): $\delta = 0.80$, 0.93 (d, 6H, $\gamma\gamma'$ Val, $^3J_{\beta,\gamma} = 6.9$ Hz), 2.34–2.44 (m, 1H, β Val), 3.55, 3.90 (d, 2H, H1′ propargyl, $^2J_{H,H'} = 17.4$ Hz), 4.33, 4.76 (d, 2H, H1 propargyl, $^2J_{H,H'} = 18.3$ Hz), 4.54 (dd, 1H, α Val, $^3J_{NH,\alpha} = 9.8$ Hz, $^3J_{\alpha,\beta} = 7.5$ Hz), 7.18–7.25 (m, 4H, H3,4,5,6 enediyne aromatic ring), 7.31–7.35 (m, 2H, H4,5 oNbs), 7.49–7.51 (m, 2H, H3,6 oNbs).

*(5S)-5-(2-methylpropyl)-4-(2-nitrophenylsulfonyl)-benz-4,7-diazacyclododeca-1,9-diyn-6-one (**5c**)* Yield: 43 %. Colorless oil. R_f 0.55 (EtOAc–petrol ether 1:1). $[\alpha]_D$ +67.0° (*c* 1.0 MeOH). M_r 465.52. ^{13}C NMR ($CDCl_3$): $\delta = 22.1$, 22.7 ($\delta\delta'$ Leu), 24.4 (γ Leu), 30.4 (C1 propargyl), 34.6 (C1′ propargyl), 37.6 (β Leu), 56.9 (α Leu), 82.4, 83.1 (C3,3′ propargyl), 88.3, 90.3 (C2,2′ propargyl), 123.9 (C3 oNbs), 125.6, 126.7 (C1,2 enediyne aromatic ring), 127.7, 128.2, 129.1, 130.9, 131.6, 132.3, 134.1 (C4,5,6 oNbs, C3,4,5,6 enediyne aromatic ring), 132.7 (C1 oNbs), 147.7 (C2 oNbs), 168.5 (CO Leu). ^{1}H NMR ($CDCl_3$): $\delta = 0.79$, 0.81 (d, 6H, $\delta\delta'$ Leu, $^3J_{\gamma,\delta} = 2.1$ Hz), 1.24–1.35, 1.99–2.10 (m, 2H, $\beta\beta'$ Leu), 1.42–1.54 (m, 1H, γ Leu), 3.62, 4.58 (dd, 2H, H1 propargyl, $^2J_{H,H'} = 17.5$ Hz, $^3J_{H,NH} = 4.5$ Hz, $^3J_{H',NH} = 8.1$ Hz), 4.31–4.36 (m, 1H, α Leu), 4.34, 4.74 (d, 2H, H1′ propargyl, $^2J_{H,H'} = 18.7$ Hz), 7.15–7.45, 7.58–7.64 (m, 7H, H3,4,5,6 enediyne aromatic ring, H4,5,6 oNbs), 8.18–8.26 (m, 1H, H3 oNbs).

*(5S)-5-benzyl-4-(2-nitrophenylsulfonyl)-benz-4,7-diazacyclododeca-1,9-diyn-6-one (**5d**)* Yield: 28 %. Colorless oil. R_f 0.49 (EtOAc–petrol ether 1:1). $[\alpha]_D$ +60.0° (*c* 0.5 MeOH). M_r 499.54. ^{13}C NMR ($CDCl_3$): $\delta = 30.8$ (C1 propargyl), 35.1, 35.1 (C1′ propargyl, β Phe), 60.6 (α Phe), 82.9, 83.2 (C2,2′ propargyl), 88.4, 90.5 (C3,3′ propargyl), 124.6 (C3 oNbs), 125.8, 127.1 (C1,2 enediyne aromatic ring), 127.0, 127.9, 128.3, 128.6, 129.2, 129.6, 131.2, 132.0, 132.2, 134.1 (δ,ε,ζ Phe, C4,5,6 oNbs, C3,4,5,6 enediyne aromatic ring), 133.0 (C1 oNbs), 136.2 (γ Phe), 147.8 (C2 oNbs), 168.2 (CO Phe). ^{1}H NMR ($CDCl_3$): $\delta = 2.89$, 3.39 (dd, 2H, β Phe, $^3J_{\alpha,\beta} = 6.8$ Hz, $^3J_{\alpha,\beta'} = 7.9$ Hz, $^2J_{\beta,\beta'} = 14.1$ Hz), 3.60, 3.63 (dd, 2H, H1 propargyl, $^2J_{H,H'} = 17.5$ Hz, $^3J_{NH,H} = 4.2$ Hz, $^3J_{NH,H'} = 13.0$ Hz), 4.51, 4.83 (d, 2H, H1′ propargyl, $^2J_{H,H'} = 18.6$ Hz), 4.55–4.60 (m, 1H, α Phe), 7.01–7.10 (m, 5H, δ,ε,ζ Phe), 7.20–7.25, 7.33–7.36, 7.41–7.44, 7.53–7.59 (m, 7H, H4,5,6 oNbs, H3,4,5,6 enediyne aromatic ring), 8.00–8.04 (m, 1H, H3 oNbs).

Synthesis of amino acid derivatives ***6***

Compound **5** (0.026 mmol), K_2CO_3 (0.052 mmol) and PhSH (0.052 mmol) were dissolved in dry DMF and stirred for 1 h at room temperature. The solvent was evaporated and the product extracted with EtOAc, washed with brine and water and purified by flash chromatography.

*(5S)-5-methylbenz-4,7-diazacyclododeca-1,9-diyn-6-one (**6a**)* Yield: 67 %. Colorless oil. R_f 0.13 (EtOAc–petrol ether 3:1). $[\alpha]_D$ +33.0° (*c* 0.25 MeOH). M_r 238.28. ^{13}C

NMR ($CDCl_3$): $\delta = 20.7$ (β Ala), 30.1 (C1 propargyl), 40.1 (C1′ propargyl), 60.9 (α Ala), 82.4, 84.1 (C2,2′ propargyl), 90.8, 92.6 (C3,3′ propargyl), 124.7, 125.6 (C1,2 enediyne aromatic ring), 127.6, 127.7, 128.7, 131.0 (C3,4,5,6 enediyne aromatic ring), 176.4 (CO Ala). ^{1}H NMR ($CDCl_3$): $\delta = 1.37$ (d, 3H, β Ala, $^3J_{\alpha,\beta} = 7.2$ Hz), 3.23 (q, 1H, α Ala, $^3J_{\alpha,\beta} = 7.1$ Hz), 3.67, 4.04 (d, 2H, H1′ propargyl, $^2J_{H,H'} = 17.4$ Hz), 3.88, 4.56 (dd, 2H, H1 propargyl, $^3J_{H,NH} = 3.5$ Hz, $^3J_{H',NH} = 9.3$ Hz, $^2J_{H,H'} = 17.6$ Hz), 7.17–7.37 (m, 4H, H3,4,5,6 enediyne aromatic ring).

(5S)-5-isopropylbenz-4,7-diazacyclododeca-1,9-diyn-6-one ***(6b)*** Yield: 67 %. Colorless oil. R_f 0.44 (EtOAc–petrol ether 2:1). $[\alpha]_D$ +64.0° (*c* 0.5 EtOAc). M_r 266.34. ^{13}C NMR ($CDCl_3$): $\delta = 18.1$, 19.8 ($\gamma\gamma'$ Val), 30.4 (C1 propargyl), 31.8 (β Val), 41.1 (C1′ propargyl), 71.2 (α Val), 82.5, 84.1 (C2,2′ propargyl), 91.5, 92.8 (C3,3′ propargyl), 125.0, 128.0 (C1,2 enediyne aromatic ring), 127.8, 127.9, 129.0, 131.3 (C3,4,5,6 enediyne aromatic ring), 175.5 (CO Val). ^{1}H NMR ($CDCl_3$): $\delta = 0.96$, 1.05 (d, 6H, $\gamma\gamma'$ Val, $^3J_{\beta,\gamma} = 7.1$ Hz), 2.12–2.18 (m, 1H, β Val), 2.96 (d, 1H, α Val, $^3J_{\alpha,\beta} = 5.0$ Hz), 3.59, 4.12 (d, 2H, H1′ propargyl, $^2J_{H,H'} = 17.5$ Hz), 3.98, 4.56 (dd, 2H, H1 propargyl, $^3J_{H,NH} = 3.8$ Hz, $^3J_{H',NH} = 9.0$ Hz, $^2J_{H,H'} = 17.7$ Hz), 7.23–7.26 (m, 2H, H4,5 enediyne aromatic ring), 7.29–7.32, 7.37–7.39 (m, 2H, H3,6 enediyne aromatic ring).

(5S)-5-(2-methylpropyl)-benz-4,7-diazacyclododeca-1,9-diyn-6-one ***(6c)*** Yield: 61 %. Colorless oil. R_f 0.52 (EtOAc–petrol ether 1:1). $[\alpha]_D$ +72.0° (*c* 0.5 MeOH). M_r 280.16. ^{13}C NMR ($CDCl_3$): $\delta = 21.9$, 23.1 ($\delta\delta'$ Leu), 25.1 (γ Leu), 30.2 (C1 propargyl), 40.4 (β Leu), 43.6 (C1′ propargyl), 63.9 (α Leu), 82.4, 83.9 (C3,3′ propargyl), 91.0, 92.6 (C2,2′ propargyl), 124.8, 127.8 (C1,2 enediyne aromatic ring), 127.6, 127.7, 128.7, 131.1 (C3,4,5,6 enediyne aromatic ring), 176.5 (CO Leu). ^{1}H NMR ($CDCl_3$): $\delta = 0.94$, 0.96 (d, 6H, $\delta\delta'$ Leu, $^3J_{\gamma,\delta} = 6.6$ Hz), 1.37–1.48 (m, 2H, γ Leu), 1.57–1.77 (m, 2H, β Leu), 3.09–3.19 (m, 1H, α Leu), 3.61, 4.05 (d, 2H, H1 propargyl, $^2J_{H,H'} = 17.1$ Hz), 3.89, 4.51 (dd, 2H, H1′ propargyl, $^2J_{H,H'} = 17.6$ Hz, $^3J_{H,NH} = 3.5$ Hz, $^3J_{H',NH} = 9.4$ Hz), 7.17–7.36 (m, 4H, H3,4,5,6 enediyne aromatic ring), 7.54–7.63 (m, 1H, NHα).

(5S)-5-benzylbenz-4,7-diazacyclododeca-1,9-diyn-6-one ***(6d)*** Yield: 68 %. Colorless oil. R_f 0.44 (petrol ether–EtOAc 1:1). $[\alpha]_D$ +46.0° (*c* 0.5 MeOH). M_r 314.38. ^{13}C NMR ($CDCl_3$): $\delta = 30.4$ (C1 propargyl), 40.3, 40.8 (C1′ propargyl, β Phe), 66.8 (α Phe), 82.8, 84.5 (C2,2′ propargyl), 90.9, 92.9 (C3,3′ propargyl), 125.0, 128.0 (C1,2 enediyne aromatic ring), 127.3, 127.9, 127.9, 128.5, 128.9, 129.1, 129.2, 131.2 (δ,ε,ζ Phe, C3,4,5,6 enediyne aromatic ring), 137.5 (γ Phe), 175.4 (CO Phe). ^{1}H NMR ($CDCl_3$): $\delta = 2.65$, 3.32 (dd, 2H, β Phe, $^3J_{\alpha,\beta} = 4.1$ Hz, $^3J_{\alpha,\beta'} = 10.4$ Hz, $^2J_{\beta,\beta'} = 14.2$ Hz), 3.36, 3.88 (d, 2H, H1 propargyl, $^2J_{H,H'} = 17.4$ Hz), 3.39 (dd, 1H, α Phe, $^3J_{\alpha,\beta} = 4.1$ Hz, $^3J_{\alpha,\beta'} = 10.4$ Hz), 3.85, 4.62 (dd, 2H, H1′ propargyl, $^2J_{H,H'} = 17.6$ Hz, $^3J_{NH,H} = 3.1$ Hz, $^3J_{NH,H'} = 9.5$ Hz), 7.19–7.35 (m, 9H, H3,4,5,6 enediyne aromatic ring, δ,ε,ζ Phe), 7.65–7.70 (m, 1H, NHα).

*Synthesis of Boc-L-Lys(Boc)-δ-Gly-OH (**7**)*

Boc-L-Lys(Boc)-OH (200 mg, 0.057 mmol) and NMM (76 µL, 0.069 mmol) were dissolved in 2 mL dry DMF and cooled down to 0 °C. Isobutyl chloroformate (91 µL, 0.069 mmol) was added and the reaction was stirred for 10 min at 0 °C. 5-aminopentoic acid (81 mg, 0.069 mmol) was added and the solution was stirred for 30 min at 0 °C and then at room temperature overnight. The solvent was evaporated and the product extracted with EtOAc, washed with brine and water and purified by flash chromatography in petrol ether–EtOAc–AcOH 10:10:0.5.

Yield: 236 mg (92 %). Colorless oil. R_f 0.42 (petrol ether-EtOAc–AcOH 10:10:0,5). $[\alpha]_D = -7.5°$ (*c* 1.0 MeOH). $M_r = 445.55$. ^{13}C NMR (DMSO-d_6): $\delta = 21.9$ (β δ-Gly); 22.7 (γ Lys), 28.1, 28.2 (CH_3 Boc$^{\alpha}$, CH_3 Boc$^{\varepsilon}$), 28.5 (β Lys), 29.2 (γ δ-Gly), 31. 8 (δ Lys), 33.4 (α δ-Gly), 38.0 (δ δ-Gly), 39.1 (ε Lys), 54.3 (α Lys), 77.3, 77.9 (C Boc$^{\alpha}$, C Boc$^{\varepsilon}$), 155.2, 155.5 (CO Boc$^{\alpha}$, CO Boc$^{\varepsilon}$), 171.9 (CO Lys), 174.4 (CO δ-Gly). ^{1}H NMR (DMSO-d_6): $\delta = 1.36$, 1.37 (s, 18H, CH_3 Boc$^{\alpha}$, CH_3 Boc$^{\varepsilon}$), 1.29–1.50 (m, 10H, β,γ δ-Gly, β,γ,δ Lys), 2.18 (t, 2H, α δ-Gly, $^3J_{\alpha,\beta} = 7.2$ Hz), 2.83–2.89 (m, 2H, ε Lys), 2.97–3.09 (m, 2H, δ δ-Gly), 3.77, 3.85 (m, 1H, α Lys), 6.65 (d, 1H, $NH_{\alpha Lys}$, $^3J_{NH,\alpha} = 7.9$ Hz), 6.69 (br t, 1H, $NH_{\varepsilon Lys}$), 7.71 (t, 1H, $NH_{\delta\text{-Gly}}$, $^3J_{NH,\delta} = 6.0$ Hz).

*Synthesis of amino acid derivatives **8***

Compound **7** (0.094 mmol) and NMM (0.094 mmol) were dissolved in 2 mL of dry DMF and cooled down to 0 °C. Isobutyl chloroformate (0.057 mmol) was added and the reaction mixture was stirred for 10 min at 0 °C. Compound **6** (0.047 mmol) was added and the solution was stirred for 30 min at 0 °C and then at room temperature overnight. The solvent was evaporated and the product purified by flash chromatography.

(5S)-5-methyl-4-[N-tert-butyloxycarbonyl-L-lysyl(N-tert-butyloxycarbonyl)-δ-glycyl]-benz-4,7-diazacyclododeca-1,9-diyn-6-one ***(8a)*** Yield: 32 %. Colorless oil. R_f 0.28 (EtOAc–petrol ether 3:1). M_r 665.82. ^{13}C NMR ($CDCl_3$): $\delta = 13.4$ (β Ala), 21.9 (β δ-Gly), 22.9 (γ Lys), (CH_3 Boc$^{\alpha}$,

CH_3 Boc$^{\varepsilon}$), 28.7 (β Lys), 29.8 (γ δ-Gly), 30.4 (C1 propargyl), 32.3 (δ Lys), 33.1 (C1′ propargyl), 33.4 (α δ-Gly), 39.1 (δ δ-Gly), 40.2 (ε Lys), 50.3 (α Ala), 54.7 (α Lys), 79.1, 80.0 (C Boc$^{\alpha}$, C Boc$^{\varepsilon}$), 82.0, 82.4 (C2,2′ propargyl), 88.4, 90.9 (C3,3′ propargyl), 125.9, 126.3 (C1,2 enediyne aromatic ring), 127.7, 128.3, 129.4, 130.8 (C3,4,5,6 enediyne aromatic ring), 155.8, 156.2 (CO Boc$^{\alpha}$, CO Boc$^{\varepsilon}$), 170.6, 172.15, 174.9 (CO Ala, CO Lys, CO δ-Gly). ^{1}H NMR ($CDCl_3$): $\delta = 1.35$ (d, 3H, β Ala, $^3J_{\alpha,\beta} = 7.1$ Hz), 1.42, 1.43 (s, 18H, CH_3 Boc$^{\alpha}$, CH_3 Boc$^{\varepsilon}$), 1.48–1.85 (m, 8H, β,γ δ-Gly, β,γ Lys), 2.43–2.73 (m, 4H, α δ-Gly, δ Lys), 3.04–3.14 (m, 2H, δ δ-Gly), 3.16–3.24 (m, 2H, ε Lys), 3.69, 4.59 (dd, 2H, H1 propargyl, $^3J_{\mathrm{H,NH}} = 4.5$ Hz, $^3J_{\mathrm{H',NH}} = 8.1$ Hz, $^2J_{\mathrm{H,H'}} = 17.7$ Hz), 3.94–4.06 (m, 1H, α Lys), 4.27, 4.35 (d, 2H, H1′ propargyl, $^2J_{\mathrm{H,H'}} = 18{,}9$ Hz), 4.66–4.75 (m, 1H, $NH_{\delta\mathrm{Gly}}$), 5.21 (d, 1H, $NH_{\alpha\mathrm{Lys}}$, $^3J_{\alpha,\mathrm{NH}} = 8{,}1$ Hz), 5.33 (q, 1H, α Ala, $^3J_{\alpha,\beta} = 7.1$ Hz), 6.44 (br s, 1H, $NH_{\varepsilon\mathrm{Lys}}$), 7.0 (br s, 1H, NH_{amide}), 7.18–7.39 (m, 4H, H3,4,5,6 enediyne aromatic rings).

(5S)-5-isopropyl-4-[N-tert-butyloxycarbonyl-L-lysyl(N-tert-butyloxycarbonyl)-δ-glycyl]-benz-4,7-diazacyclododeca-1,9-diyn-6-one ***(8b)*** Yield: 29 %. Colorless oil. R_f 0.37 (EtOAc–petrol ether 3:1). M_r 693.87. ^{13}C NMR ($CDCl_3$): $\delta = 18.2$, 19.7 ($\gamma\gamma'$ Val), 21.7 (β δ-Gly), 22.7 (γ Lys), 28.3, 28.4 (CH_3 Boc$^{\alpha}$, CH_3 Boc$^{\varepsilon}$), 28.8 (β Lys), 29.4 (γ δ-Gly), 29.7 (C1 propargyl), 30.2 (β Val), 31.9 (δ Lys), 32.7 (C1′ propargyl), 33.9 (α δ-Gly), 37.1 (δ δ-Gly), 38.8 (ε Lys), 54.5 (α Lys), 61.3 (α Val), 78.5, 79.1 (C Boc$^{\alpha}$, C Boc$^{\varepsilon}$), 81.7, 82.5 (C2,2′ propargyl), 88.7, 90.8 (C3,3′ propargyl), 125.6, 126.2 (C1,2 enediyne aromatic ring), 127.6, 128.2, 129.5, 130.7 (C3,4,5,6 enediyne aromatic ring), 171.9, 172.4, 173.2 (CO Val, CO δ-Gly, CO Lys). ^{1}H NMR ($CDCl_3$): $\delta = 0.81$, 1.01 (d, 6H, $\gamma\gamma'$ Val, $^3J_{\beta,\gamma} = 7{,}1$ Hz, $^3J_{\beta,\gamma} = 6.4$ Hz), 1.45, 1.46 (s, 18H, CH_3 Boc$^{\alpha}$, CH_3 Boc$^{\varepsilon}$), 1.49–1.85 (m, 8H, β,γ δ-Gly, β,γ Lys), 2.29–2.79 (m, 6H, α δ-Gly, δ Lys, β Val), 3.07–3.26 (m, 4H, δ δ-Gly, ε Lys), 3.63–3.76, 4.55–4.81 (m, 2H, H1 propargyl, α Val), 3.94–4.07 (m, 1H, α Lys), 4.27–4.35 (br s, 2H, H1′ propargyl), 5.14 (br s, 1H, $NH_{\alpha\mathrm{Lys}}$), 6.34 (br s, 1H, $NH_{\varepsilon\mathrm{Lys}}$), 6.81 (br s, 1H, 1H, NH_{amide}), 7.22–7.75 (m, 4H, H3,4,5,6 enediyne aromatic ring).

(5S)-5-isobutyl-4-[N-tert-butyloxycarbonyl-L-lysyl(N-tert-butyloxycarbonyl)-δ-glycyl]-benz-4,7-diazacyclododeca-1,9-diyn-6-one ***(8c)*** Yield: 31 %. Colorless oil. R_f 0.47 (EtOAc–petrol ether 3:1). M_r 707.90. ^{13}C NMR ($CDCl_3$): $\delta = 21.7$ (β δ-Gly), 22.2, 22.9 ($\delta\delta'$ Leu), 22.7 (γ Lys), 24.8 (γ Leu), 28.3, 28.4 (CH_3 Boc$^{\alpha}$, CH_3 Boc$^{\varepsilon}$), 28.8 (β Lys), 29.4 (γ δ-Gly), 30.2 (C1 propargyl), 31.9 (δ Lys), 32.8 (C1′ propargyl), 33.5 (α δ-Gly), 37.1 (δ δ-Gly), 38.8 (ε Lys), 39.9 (β Leu), 52.9, 54.6 (α Lys, α Leu), 79.2, 79.3 (C Boc$^{\alpha}$, C Boc$^{\varepsilon}$), 81.9, 82.5 (C2,2′ propargyl), 88.4, 90.9 (C3,3′ propargyl), 126.1, 126.3 (C1,2 enediyne aromatic ring), 127.6, 128.2, 129.4, 130.7 (C3,4,5,6 enediyne aromatic ring), 171.9, 175.2, 176.7 (CO Leu, CO δ-Gly, CO Lys). ^{1}H NMR ($CDCl_3$): 0.86, 0.95 (d, 6H, $\delta\delta'$ Leu, $^3J_{\gamma,\delta} = 6.7$ Hz), 1.43, 1.44 (s, 18H, CH_3 Boc$^{\alpha}$, CH_3 Boc$^{\varepsilon}$), 1.46–1.82 (m, 11H, β,γ Leu, β,γ Lys, β,γ δ-Gly), 2.26–2.57, 2.64–2.74 (m, 4H, α δ-Gly, δ Lys), 3.05–3.24 (m, 4H, δ δ-Gly, ε Lys), 3.68, 4.59 (d, 2H, H1 propargyl, $^3J_{\mathrm{H,NH}} = 4.4$ Hz, $^3J_{\mathrm{H',NH}} = 8.4$ Hz, $^2J_{\mathrm{H,H'}} = 17.6$ Hz), 3.98 (br s, 1H, α Lys), 4.23–4.32 (m, 2H, H1′ propargyl), 5.12 (br s, 1H, $NH_{\alpha\mathrm{Lys}}$), 5.24 (t, 1H, α Leu, $^3J_{\alpha,\beta} = 7.3$ Hz), 6.31 (br s, 1H, $NH_{\varepsilon\mathrm{Lys}}$), 6.87 (br s, 1H, NH_{amide}), 7.20–7.36 (m, 4H, H3,4,5,6 enediyne aromatic ring).

Synthesis of amino acid derivatives ***9***

Compound **8** was dissolved in 1 mL TFA–H_2O 9:1 and stirred for 1 h at room temperature. Solvent was evaporated and the product was purified on HPLC. Residual TFA was removed by flushed through a silicagel filled column (20 % NH_3 in EtOAc was used as an eleunt).

(5S)-5-methyl-4-(L-lysyl-δ-glycyl)-benz-4,7-diazacyclododeca-1,9-diyn-6-one ***(9a)*** Yield: 43 %. R_T 10.7 min (40 % MeOH in 0.1 % TFA). Purity 98.7 %. HRMS (MALDI): *m/z* $[M+H]^+$ calcd for $C_{26}H_{35}N_5O_3$, 466.2813; found: 466.2816. M_r 465.59. ^{1}H NMR (DMSO): $\delta = 1.27$–1.68 (m, 11H, β Ala, β,γ δ-Gly, β,γ Lys), 2.71–2.78 (m, 4H, δ Lys, α δ-Gly), 3.30 (α Lys, δ δ-Gly—under the HOD signal from DMSO), 3.61–3.68 (m, 2H, ε Lys), 3.94 (s, 2H, H1 propargyl), 4.26, 4.52 (d, 2H, H1′ propargyl, $^2J_{\mathrm{H,H'}} = 17.6$ Hz), 4.36–4.45 (m, 1H, α Lys), 4.80 (q, 1H, α Ala, $^3J_{\alpha,\beta} = 7.4$ Hz), 7.36–7.51 (m, 4H, H3,4,5,6 enediyne aromatic ring).

(5S)-5-isopropyl-4-(L-lysyl-δ-glycyl)-benz-4,7-diazacyclododeca-1,9-diyn-6-one ***(9b)*** Yield: 30 %. R_T 14.3 min (40 % MeOH in 0.1 % TFA). Purity 96.9 %. HRMS (MALDI): *m/z* $[M+H]^+$ calcd for $C_{28}H_{39}N_5O_3$, 494.3125; found: 494.3128. M_r 493.64. ^{1}H NMR (CD_3OD): $\delta = 0.86$–0.93 (m, 6H, $\gamma\gamma'$ Val), 1.41–1.65 (m, 8H, β,γ δ-Gly, β,γ Lys), 2.24–2.36 (m, 6H, α δ-Gly, δ Lys, β Val), 3.17–3.19 (m, 1H, α Lys), 3.28–3.35, 3.40–3.43 (m, 4H, δ δ-Gly, ε Lys), 3.62–3.66, 4.53–4.55 (m, 2H, H1 propargyl), 4.04–4.08 (m, 1H, α Val), 4.19–4.22 (m, 2H, H1′ propargyl), 7.60–7.63 (m, 2H, H4,5 enediyne aromatic ring), 7.70–7.73 (m, 2H, H3,6 enediyne aromatic ring).

(5S)-5-isobutyl-4-(L-lysyl-δ-glycyl)-benz-4,7-diazacyclododeca-1,9-diyn-6-one ***(9c)*** Yield: 33 %. R_T 13.3 min (43.5 % MeOH in 0.1 % TFA). Purity 97 %. HRMS (MALDI): *m/z* $[M+H]^+$ calcd for $C_{29}H_{41}N_5O_3$, 508.3282; found: 508.3288. M_r 507.67. ^{1}H NMR (CD_3OD): $\delta = 0.92$,

0.96 (d, 6H, δδ′ Leu, $^{3}J_{\gamma,\delta} = 6.7$ Hz), 1.35–1.90 (m, 11H, β,γ Leu, β,γ Lys, β,γ δ-Gly), 2.25–2.34 (m, 4H, α δ-Gly, δ Lys), 2.91–2.97 (m, 1H, α Lys), 3.13–3.26 (m, 4H, ε Lys, δ δ-Gly), 3.78, 4.46 (d, 2H, H1 propargyl, $^{2}J_{H,H'} = 17.6$ Hz), 4.19–4.23 (m, 1H, α Leu), 4.42 (s, 2H, H1′ propargyl), 7.24–7.40 (m, 4H, H3,4,5,6 enediyne aromatic ring).

*Synthesis of H–L-Lys-δ-Gly-OH (**10**)*

Compound **7** (20 mg, 0.045 mmol) was dissolved in 1 mL TFA–H_2O 9:1 and stirred for 1h at room temperature. Solvent was evaporated and the product is purified by HPLC. Residual TFA was removed on a Dowex 1 × 2 200 (Ac) column (water was used as an eluent).

Yield: 36 %. R_T 8.2 min (35 % MeOH in 0.1 % TFA). Purity 98.2 %. HRMS (MALDI): *m/z* $[M+H]^+$ calcd for $C_{11}H_{23}N_3O_3$, 246.3259; found: 246.3257. M_r 245.32. ^{13}C NMR (CD_3OD): δ = 23.1 (β δ-Gly), 24.4 (γ Lys), 28.3 (δ Lys), 30.0 (γ δ-Gly), 32.6 (α δ-Gly), 36.9 (β Lys), 40.4, 40.5 (δ δ-Gly, ε Lys), 54.6 (α Lys). ^{1}H NMR (CD_3OD): δ = 1.43–1.51, 1.53–1.66, 1.67–1.74, 1.80–1.92 (m, 10H, β,γ δ-Gly, β,γ,δ Lys), 2.24 (t, 2H, α δ-Gly, $^{3}J_{\alpha,\beta} = 7.4$ Hz), 2.94 (t, 2H, δ Lys, $^{3}J_{\gamma,\delta} = 7.5$ Hz), 3.29–3.32 (m, 2H, δ δ-Gly), 3.80 (t, 1H, α Lys, $^{3}J_{\alpha,\beta} = 6.4$ Hz).

*Synthesis of (5S)-5-methyl-4-[N-tert-butyloxycarbonyl-L-lysyl(N-tert-butyloxycarbonyl)]-benz-4,7-diazacyclododeca-1,9-diyn-6-one (**11**)*

Boc-L-Lys(Boc)-OH (23 mg, 0.066 mmol) and NMM (16 μL, 0.132 mmol) were dissolved in dry DMF and cooled down to 0 °C. Isobutyl chloroformate (10 μL, 0.073) was added and the reaction mixture was stirred for 10 min at 0 °C. Compound **6a** (10 mg, 0.055 mmol) was added and the solution was stirred for 30 min at 0 °C and then at room temperature overnight. The solvent was evaporated and the product purified by flash chromatography in EtOAc–petrol ether 2:1.

Yield: 26 %. Colorless oil. R_f 0.43 (EtOAc–petrol ether 2:1). $[\alpha]_D$ +54.0° (*c* 1.0 MeOH). M_r 566.69. ^{13}C NMR ($CDCl_3$): δ = 15.5 (β Ala), 23.1 (γ Lys), 29.6 (δ Lys), 28.3, 28.5 (CH_3 Boc^{α}, CH_3 Boc^{ε}), 30.2 (C1 propargyl), 33.2 (C1′ propargyl), 32.4 (β Lys), 40.1 (ε Lys), 50.4, 54.4 (α Lys, α Ala), 81.1, 81.2 (C Boc^{α}, C Boc^{ε}), 82.1, 83.1 (C2,2′ propargyl), 89.3, 90.8 (C3,3′ propargyl), 125.8, 126.2 (C1,2 enediyne aromatic ring), 127.9, 128.4, 129.6, 130.7 (C3,4,5,6 enediyne aromatic ring), 155.6, 156.9 (CO Boc^{α}, CO Boc^{ε}), 168.8, 170.3 (CO Lys, CO Ala). ^{1}H NMR ($CDCl_3$): δ = 1.39–1.47 (m, 21H, β Ala, CH_3 Boc^{α}, CH_3 Boc^{ε}), 1.54–1.89 (m, 6H, β,γ,δ Lys), 3.01–3.18 (m, 2H, ε Lys), 3.57–5.38 (m, 6H, α Lys, α Ala, H1,1′ propargyl), 7.13–7.39 (m, 4H, H3,4,5,6 enediyne aromatic ring).

*Synthesis of (5S)-5-methyl-4-(L-lysyl)-benz-4,7-diazacyclododeca-1,9-diyn-6-one (**12**)*

Compound **11** (16 mg, 0.028 mmol) was dissolved in 1 mL TFA–H_2O 9:1 and stirred for 1h at room temperature. Solvent was evaporated and the product was purified on HPLC in 25 % MeOH. Isolated product was dissolved in 20 % NH_3 in EtOAc and flushed through silicagel filled column.

Yield: 87 %. Colorless oil. R_T 33.7 min (25 % MeOH in 0.1 % TFA). Purity 96.6 %. HRMS (MALDI): *m/z* $[M+H]^+$ calcd for $C_{21}H_{27}N_4O_2$, 367.4645; found: 367.4640. M_r 366.46. ^{13}C NMR (CD_3OD): δ = 11.9 (β Ala), 21.7 (γ Lys), 26.4 (δ Lys), 29.9, 30.6, 33.2 (C1,1′ propargyl, β Lys), 38.6 (ε Lys), 50.9, 51.4 (α Ala, α Lys), 81.9, 83.2 (C2,2′ propargyl), 88.2, 90.4 (C3,3′ propargyl), 124.8, 125.4 (C1,2 enediyne aromatic ring), 128.6, 129.2, 129.7, 131.3 (C3,4,5,6 enediyne aromatic ring), 170.8, 171.4 (CO Ala, CO Lys). ^{1}H NMR (CD_3OD): δ = 1.29 (d, 3H, β Ala, $^{3}J_{\alpha,\beta} = 6.7$ Hz), 1.58–1.72, 2.37–2.62 (m, 6H, β,γ,δ Lys), 2.94 (t, 1H, α Lys, $^{3}J_{\alpha,\beta} = 7.1$ Hz), 3.84, 4.29 (d, 2H, H1 propargyl, $^{2}J_{H,H'} = 18.1$ Hz), 4.41, 4.58 (d, 2H, H1′ propargyl, $^{2}J_{H,H'} = 19.6$ Hz), 5.29 (q, 1H, α Ala, $^{3}J_{\alpha,\beta} = 6.7$ Hz), 7.28–7.50 (m, 4H, H3,4,5,6 enediyne aromatic ring).

Results and discussion

Design of enediyne-based APN inhibitors

Crystal structure of the APN-bestatin complex revealed extended conformation of the inhibitor with pharmacophores occupying S1 and S1′ pocket (phenyl and isobutyl unit, respectively) placed away from each other (Ito et al. 2006). Having in mind this conformation, we designed enediyne–peptide chimeras **9** with general structure presented in Fig. 1A. Enediyne moiety is embedded within the 12-membered ring, and can be considered as amino acid N→C bridging unit. Having in mind affinity of APN (Ala>Phe>Leu>Arg), hydrophobic amino acids alanine, valine, leucine and phenylalanine were used as carriers of the enediyne moiety. Secondary amino group is bound through a flexible linker to the lysine residue. Binding to the S1 pocket is targeted with the aromatic ring of the enediyne moiety, while hydrophobic amino acid side chain aims the S1′ pocket. Rough MM2 calculations show that the distance between the enediyne aromatic ring and the amino acid side chains is around 8 Å (derivative **9c**, Fig. 1B), while the distance between two bestatin chromophores in the active site of APN is 8.4 Å (Fig. 1C). These calculations also pointed two carbonyl groups towards the same direction aiming to coordinate Zn^{2+} ion.

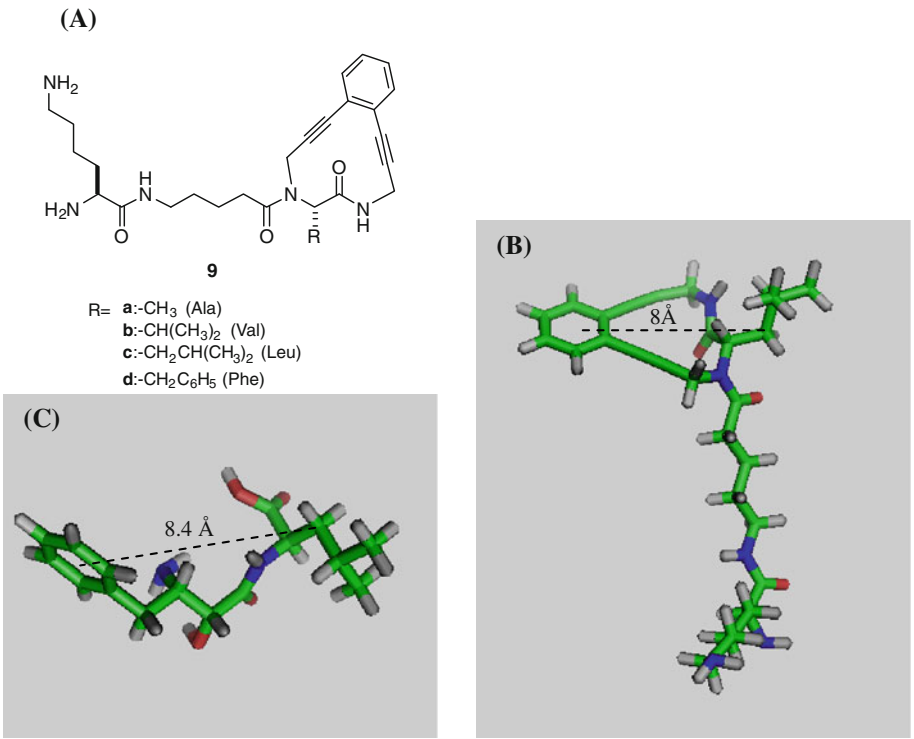


Fig. 1 **A** Chemical structures of designed peptide-based enediyne derivatives; **B** optimized structure of **9c** obtained by the MM2 calculation; **C** bestatin conformation in the APN active site (Ito et al. 2006)

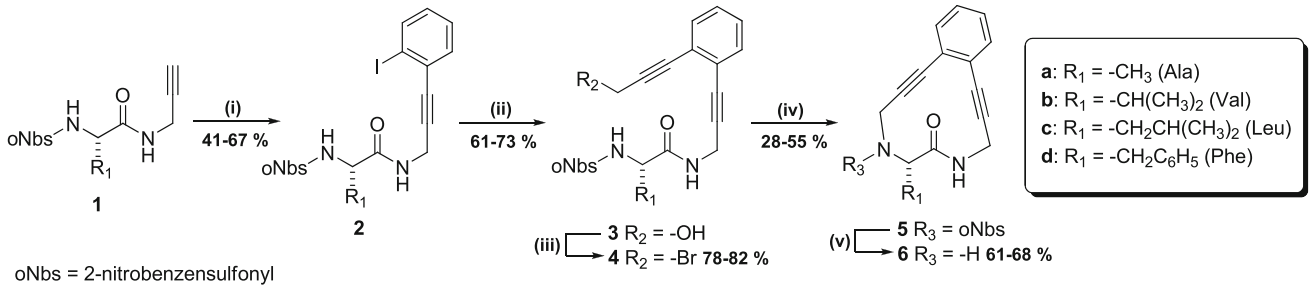
The rigidity of the enediyne-containing ring ensures extended-like conformation, but requires introduction of free NH_2 group for the interaction with the glutamic residue in the vicinity of S1 pocket; we opted for the L-lysine as an amino group donor. It has already been shown that presence of carbonyl group capable of Zn^{2+} coordination, as well as the free amino group in L-lysine-derived compounds, contributes to the APN inhibition (Wang et al. 2008). Additional flexible linker was placed between enediyne "head" and the lysine residue thus to enable closer interaction of the lysine amino group with acidic binding site of the APN.

Known feature of enediyne-related compounds is thermally or photochemically triggered Bergman cyclization producing diradical species and leading to the DNA cleavage (Smith and Nicolaou 1996) or protein degradation (Fouad et al. 2005). The 12-membered ring comprising enediyne moiety is expected to be stable toward the Bergman cyclization owing to the large distance between terminal acetylene carbon atoms (*cd* distance). It was shown that cyclic enediynes $cd = 3.2–3.3$ Å undergo cyclization at room temperature (10-membered and some 11-membered rings), while enediynes with $cd > 3.7$ Å require elevated temperatures for the cyclization (Nicolaou et al. 1992). MM2 calculations showed that the *cd* distances in compounds **9** are around 4.2 Å; therefore, the Bergman cyclization is not expected under the conditions applied in this work.

Synthesis

The key step in the synthesis of 12-membered enediyne rings **6** is intramolecular N-alkylation of C-terminal modified amino acids Ala, Val, Leu and Phe (Scheme 1). Amino acid derivatives **1** (Gredičak et al. 2008) were submitted to two consecutive Sonogashira couplings (Sonogashira et al. 1975) that result in moderate yields of **2** and **3**. When aliphatic reactant(s) are involved, the Sonogashira coupling must be performed under rigorous reaction conditions and generally results in lower yields (Gredičak and Jerić 2009). The typical procedure includes halogen arene and terminal alkyne mixed with piperidine, $Pd(PPh_3)_4$ and CuI in THF under the argon atmosphere. Compound **4** was obtained in satisfying yields as a result of the S_N2 substitution, utilizing PBr_3 in THF. The closing of the 12-membered ring **5** was performed by intramolecular N-alkylation reaction in DMF with K_2CO_3. It is worth noting that we tried to obtain **5** directly from **3** using Mitsunobu reaction conditions (Mitsunobu and Yamada 1967), but only traces of **5** were obtained. The last step was the removal of the oNbs protection group with PhSH and K_2CO_3.

Stability of prepared macrocycles **6** toward the Bergman cyclization was tested at this stage by the temperature-dependent FT-IR spectroscopy, an established method for simple and rapid monitoring of thermally induced physical and chemical rearrangements (Gredičak et al. 2010).



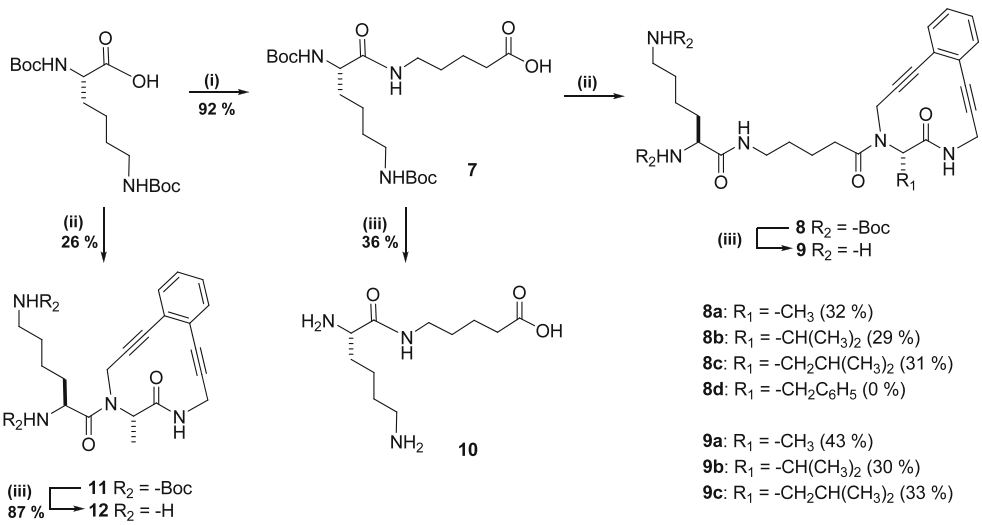
Scheme 1 *(i)* 1,2-diiodobenzene (1.2 eqv.), piperidine (2 eqv.), $Pd(PPh_3)_4$ (0.02 eqv.), CuI (0.1 eqv.) THF; *(ii)* propargyl alcohol (3 eqv.), piperidine (2 eqv.), $Pd(PPh_3)_4$ (0.02 eqv.), CuI (0.1 eqv.) THF; *(iii)* PBr_3 (2 eqv.), THF; *(iv)* K_2CO_3 (2 eqv.), DMF; *(v)* PhSH (2 eqv.), K_2CO_3 (2 eqv.), DMF

Compounds were heated up to 240 °C without any changes observed in FT-IR spectra, proving stability of the 12-membered enediyne rings.

With enediyne macrocycles **6** in hands, final derivatives **9** with incorporated L-lysine have been prepared (Scheme 2). Boc-protected L-lysine was coupled with 5-aminopentoic acid by a mixed anhydride method to provide **7**. The same method was applied for the coupling of **7** with **6** to give **8** in moderate yields, while **8d** could not be obtained. Although the obtained yields are considered satisfactory with regard to secondary amine as a participant, several other coupling methods were tried, focusing on **6d**. The BOP/HOBt, HATU and DCC/HOSu methods provided lower yields of the compounds **8**, while **8d** could not be obtained by any method used, most likely due to the sterical hindrances of the phenylalanine aromatic ring over nitrogen. Final compounds **9** were obtained by Boc deprotection in acidic conditions. Following the same procedure, compound **10** was prepared from **7**. The reason for the synthesis of **10** is to exclude that eventually observed inhibition stems only from the "dipeptide" part (L-lysine + flexible linker) of the molecule.

APN inhibition test

Enediyne macrocycles **6a–d** and dipeptide mimic **10** were submitted to the APN inhibition experiments to see whether two structural elements alone can inhibit the enzyme. The inhibition experiments with derivatives **6** revealed that only valine-based macrocycle **6b** caused 30 % inhibition at 150 μM concentration. Dipeptide **10** was not inhibitory at 200 μM concentration and at 1 mM it lowered the enzyme activity only moderately (25.3 ± 7.5 %). The inhibition experiments conducted with enediyne conjugates **9** showed diverging results. The most prominent inhibitor was the alanine-based derivative **9a**, manifesting an IC_{50} value at 33.7 ± 11.4 μM concentration. Valine-based derivative **9b**, in spite of causing some inhibition as a tailless macrocycle **6b**, did not show any inhibition whatsoever, even at 400 μM concentration, and similar result was obtained with the leucine-based derivative **9c**. Clearly, conjugation of the alanine-related enediyne "head" with L-lysine through a flexible linker fulfilled basic structural and conformational requirements for the APN inhibition. In addition, we examined the role of flexible linker in positioning of **9a** into the APN active site, and prepared compound **12**



Scheme 2 *(i)* ClOCOiBu (1.2 eqv.), NMM (2 eqv.), 5-aminopentoic acid (1.2 eqv.); *(ii)* **6** (1.2 eqv.), ClOCOiBu (1.2 eqv.), NMM (2 eqv.); *(iii)* TFA–H_2O 9:1 (v/v)

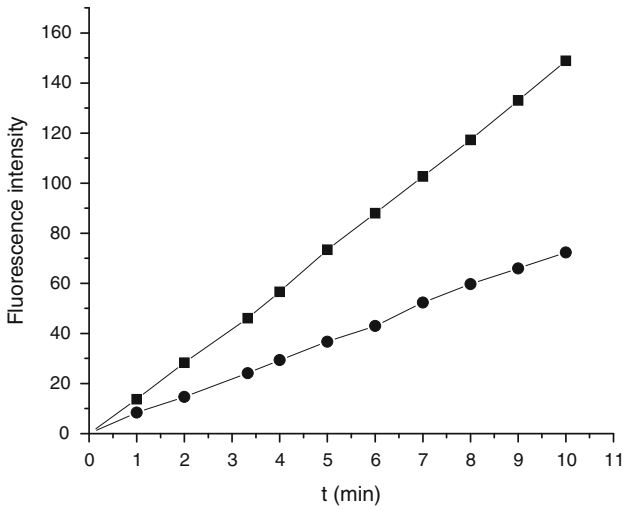


Fig. 2 Hydrolysis rate plots of Leu-2NA cleavage by APN in the absence (*filled square*) or in the presence (*filled circle*) of compound **9a**

(Scheme 2) with L-lysine directly bound to the secondary amine of the alanine-related enediyne "head". Linkerless derivative **12** was found to be far less active towards APN than the corresponding **9a**, with IC_{50} value above 1 mM.

To verify if the inhibition by **9a** is maintained over the APN assay period (10 min), the enzyme activity was followed continuously (Fig. 2). The activity decline was observed in the presence of **9a** from the beginning till the end of measurement of enzyme reaction.

Since lysine residue could be recognized at the P1 position of the APN substrates, susceptibility of the most active enediyne macrocycle **9a** and the dipeptide mimic **10** to the hydrolysis by the APN was examined. Results obtained by the TLC and the HPLC–MS analysis revealed that both compounds are stable toward the enzymatic hydrolysis. Moreover, presence of only **9a** in the reaction mixture incubated with enzyme confirms that structural properties of the enediyne conjugate and not chemical reaction of the enediyne moiety are responsible for the observed activity. These findings are in accordance with previously published data on the recognition role of enediyne-related compounds as enzyme inhibitors (Lin et al. 2001; Dutta et al. 2009; Chandra et al. 2011).

Addlagatta et al. (2008) investigated the structural basis for the unusual specificity of *E. coli* APN. This enzyme cleaves adjacent to the nonpolar amino acids Phe and Tyr but also next to the polar residues Lys and Arg. They determined the structure of the APN in complex with different amino acids (Addlagatta et al. 2008). The authors found that hydrophobic walls of the S1 pocket accommodate nonpolar or largely nonpolar side chains of Phe and Tyr, but also hydrocarbon part of the polar Lys and Arg side chains. The bottom and top of the S1 pocket are polar and provide interactions with α-amino group and charged groups of the Lys and Arg side chains. This combination of hydrophobic and hydrophilic binding surfaces accounts for the ability of enzyme to cleave Lys, Arg, Phe, and Tyr. Drag et al. have shown, by examining a library of 61 natural and unnatural amino acids substrates, that the most favored amino acids in mammalian APN synthetic (fluorogenic) substrates have rather large hydrophobic side chains, indicating an open S1 pocket (Drag et al. 2010). These authors revealed a large and hydrophobic character for the S1 pocket of mammalian (human, pig and rat) APN (Drag et al. 2010).

Considering structure of enediyne–peptide conjugates presented in this work, S1 pocket can accommodate either hydrophobic aromatic part of enediyne "heads" or hydrocarbon part of the lysine side chain. Obviously, the hydrocarbon linker contributes to the binding into the APN active site, since the compound **12** was much less potent, compared to the **9a**. It can be speculated that presence of linker allowed interaction of free NH_2 group with glutamic acid side chain of the APN active site. Alternatively, the ε-amino group of the lysine side chain could contact with polar top of the S1 subsite (Addlagatta et al. 2008). Since we did not observe the hydrolytic cleavage of Lys residue, upon APN incubation with **9a**, we speculate that the enzyme interaction with the ε-amino group of the lysine side chain, and accommodation of enediyne "head" in S1 subsite, is more likely.

Finally, it was important to compare our results with other known APN inhibitors. Different classes of compounds have been prepared and tested as potential inhibitors of the APN, but also of some other zinc-dependent metallopeptidases, like leucine aminopeptidase (Drag et al. 2005) and matrix metalloproteinases-2 (MMP-2) (Wang et al. 2008). Peptidomimetics containing 3-galloylamido-*N*′-substituted-2,6-piperidinedione-*N*-acetamide, showed high inhibitory activity against MMP-2 and low activity against APN, except two derivatives with IC_{50} values of 3.1 and 5.2 μM (Li et al. 2007). A series of peptidomimetic L-iso-glutamine derivatives exhibited selective inhibition against APN with IC_{50} values in μM range (Li et al. 2009). A broad comparison study using phosphinate inhibitors yielded a potent APN inhibitor with an IC_{50} about 60 nM (Grzywa et al. 2010). Amino-benzosuberone derivatives, a low molecular weight, nonpeptidic compounds are among the most active APN inhibitors found so far, with inhibitory constant in picomolar range (Maiereanu et al. 2011). Regarding derivatives of L-lysine, synthesized compounds were inferior APN inhibitors compared with MMP-2 and only 1 compound out of 20 showed inhibition activity comparable with bestatin (Wang et al. 2008). Other compounds were less active (IC_{50} values higher than 100 μM) and fall into the same range as compounds considered here.

Preliminary results on small number of enediyne–peptide conjugates presented in this work, point toward enediyne–amino acid chimeras as new pharmacophore for the APN S1/S1′ pockets and identified enediyne macrocycle **9a** as the most promising candidate for further studies (IC_{50} value 33.7 ± 11.4 μM). To approach efficiency of the most active APN inhibitors, in the second generation of enediyne–peptide conjugates, emphasis will be given to the modification of linker length and introduction of better zinc-binding moieties.

Conclusions

New class of enediyne–peptide conjugates has been prepared with the aim to inhibit aminopeptidase N. The key step in the synthesis was C-terminal modification of hydrophobic amino acids Ala, Val, Leu and Phe and subsequent intramolecular N-alkylation. The fused enediyne–amino acid "heads" were attached to the L-lysine through a flexible 5-aminopentoic acid-derived linker. Alanine-related derivative **9a** showed the highest APN inhibition potential ($IC_{50} = 33.7 \pm 11.4$ μM), considerably higher than derivative **12** missing the linker part. These results will be used as a starting point for further structural tunings comprising linkers of different length and possibly substituents.

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