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Peptide Derivatives of Tylosin-Related Macrolides

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Abstract—Approaches to the synthesis of model compounds based on the tylosin-related macrolides desmycosin and *O*-mycaminosyltylonolide were developed to study the conformation and topography of the nascent peptide chain in the ribosome tunnel using specially designed peptide derivatives of macrolide antibiotics. A method for selective bromoacetylation of desmycosin at the hydroxyl group of mycinose was developed, which involves preliminary acetylation of mycaminose. The reaction of the 4"-bromoacetyl derivative of the antibiotic with cesium salts of the dipeptide Boc-Ala-Ala-OH and the hexapeptide MeOTr-Gly-Pro-Gly-Pro-Gly-Pro-OH led to the corresponding peptide derivatives of desmycosin. The protected peptides Boc-Ala-Ala-OH, Boc-Ala-Ala-Phe-OH, and Boc-Gly-Pro-Gly-Pro-Gly-Pro-OH were condensed with the C23-hydroxyl group of *O*-mycaminosyltylonolide.

Key words: tylosin, desmycosin, O-mycaminosyltylonolide, peptide derivatives of macrolides

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INTRODUCTION

The antibiotic tylosin belongs to the class of macrolides, which can bind to the large subunit of bacterial ribosome and inhibit protein biosynthesis [1].[2] Tyl (**I**) is a derivative of 16-membered lactone that has a mycarosylmycaminose disaccharide residue attached at C5 position and a mycinose monosaccharide residue, at C23 position.

The Tyl-related antibiotics contain the same lactone ring with a smaller number of carbohydrate residues: Des (**II**) is devoid of a mycarose residue, and OMT (**III**) lacks the residues of mycarose and mycinose.

Macrolide antibiotics are of interest not only as therapeuticals but also as convenient tools for studying the functional aspects of protein biosynthesis. A structural analysis of the complexes of ribosomal subunits with antibiotics is also important for understanding the molecular mechanisms of the binding of antibiotics and the reasons for antibiotic resistance, as well as for the efficient design of drugs. Crystalline complexes of Tyl and other antibiotics with the 50S subunit of the bacterial ribosome have recently been obtained [2, 3]. The X-ray diffraction analysis of these complexes with a resolution of about 3Å showed that antibiotics are bound in the ribosomal tunnel near PTC [4, 5]. In this case, the disaccharide substituent at C5 of Tyl extends along the tunnel toward PTC, and the mycinose residue at C23 of the lactone ring is directed toward the tunnel exit.

The known spatial orientation of Tyl in the ribosome tunnel allows one to construct peptide derivatives of the antibiotic at positions C20 and C23 whose peptide fragments extend, correspondingly, toward the PTC (arbitrarily designated as "upstream the tunnel") and in the opposite direction ("downstream the tunnel"). These peptide fragments would modulate to some extent the nascent peptide chain in the ribosomal tunnel both near the PTC and nearby the tunnel exit.

These peptide derivatives of macrolides may serve as useful tools for studying the conformation and topography of a polypeptide being synthesized in the ribosomal tunnel. Previously we have synthesized peptide derivatives of Tyl and Des using their aldehyde groups in C19 position [6].

Here we describe the synthesis of a number of peptides and approaches to their conjugation with Des (**II**) and OMT (**III**) at the hydroxy groups at C4" and C23, respectively.

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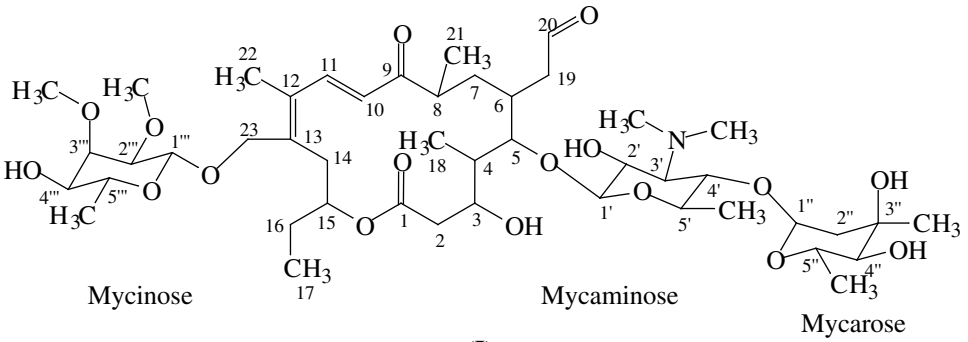
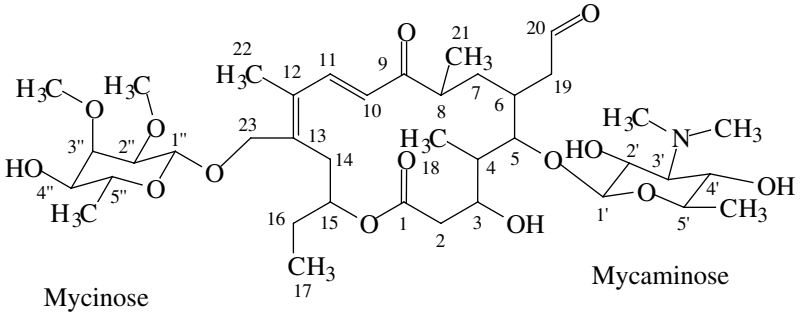
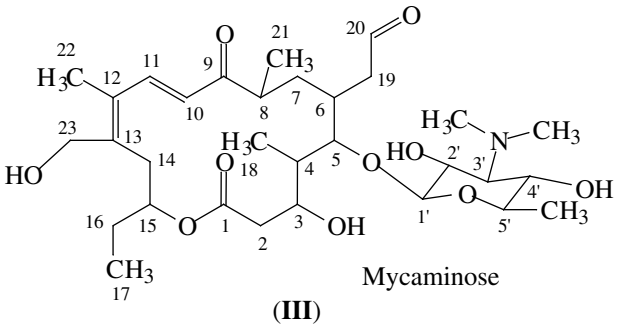
[2] Abbreviations: βAla, β-alanine; DCC, *N*,*N*'-dicyclohexylcarbodiimide; Des, desmycosin; DIEA, diisopropylethylamine; DMAP, 4-dimethylaminopyridine; DNPH, 2,4-dinitrophenylhydrazine; Glyc, glycolyl; HBTU, *N*-hydroxybenzotriazolyluronium hexafluorophosphate; MeOTr, 4-monomethoxytrityl; OMT, *O*-mycaminosyltylonolide; PTC, the peptidyl transferase center; Tyl, tylosin. All amino acids of the *L* series.



(**I**)



(**II**)



(**III**)

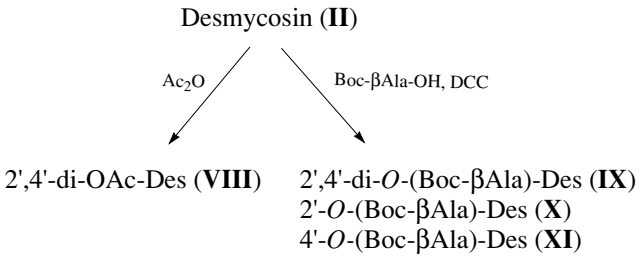
RESULTS AND DISCUSSION

The initial stage of the study involved the synthesis of the starting antibiotics Des (**II**) and OMT (**III**), which can arise during an acidic hydrolysis of Tyl [7, 8].

The maintaining of Tyl for 72 h in a hydrochloric acid solution under mild conditions (pH 2.8, 20°C) gave chromatographically homogeneous Des (yield 92%), which was identical in characteristics to that described in literature [7].

The preparation of pure OMT (**III**) meets some difficulties. Acidic hydrolysis of Tyl under rigorous conditions (pH 1.7, 100°C, 50 h) [8] was accompanied by the formation of large amounts of by-products, which required a thorough separation of the mixture on a silica gel column under the conditions of gradient elution. The homogeneous substance isolated had a higher melting temperature (132–135°C) compared to that reported for OMT in literature (115–118°C). Therefore, the structure of the antibiotic obtained was additionally confirmed by mass spectrometry and ^{1}H and ^{13}C NMR spectra.

We chose the strategy of the synthesis of Des and OMT peptide derivatives on the basis of the known structures of the antibiotics and the available literature data on the reactivity of their functional groups. The Des molecule has three reactive hydroxy groups: 2'- and 4'-OH groups in the mycaminose residue and 4''-hydroxy group in the mycinose residue. In the context of the problem under study, the 4''-hydroxy group is well suited for the introduction of peptide fragments that would occupy the expected downstream-the-tunnel position. A great number of biologically active Des derivatives at different functional groups, including the 4''-hydroxy group of mycinose, have been reported; however, no peptide derivatives at this position are known. The 2'- and 4'-hydroxy groups of mycaminose are more readily acylated than the 4''-hydroxy group of mycinose [9]. We used this fact when developing the approaches to the synthesis of 4''-*O*-peptidyldesmycosins: the introduction of 2',4'-diprotected Des into the



Scheme 1.

reaction with bromoacetyl bromide was considered with the aim of subsequent acylation of the resulting Des bromoderivative by cesium salts of carboxylic acids [10].

OMT (**III**) has a primary hydroxy group at C23, which can be selectively acylated by highly reactive reagents both with the preliminary protection of the mycaminose hydroxy groups and without it [11]. Dipeptide derivatives of the antibiotic in position 23 obtained by the acylation of the amino group preliminarily introduced into this position have been reported [12]. We considered this pathway of synthesis to be rather complicated and, therefore, used another approach, which involved the direct acylation of 23-hydroxyl with the preliminary blocking of the 2',4'-hydroxy functions of mycaminose.

We considered two possible ways when developing the approaches to the synthesis of peptidyl macrolides. One approach consists in the attachment of the first from the *C*-end amino acid to the antibiotic followed by a stepwise elongation of the chain by the methods of classical peptide chemistry. Another way involves the acylation of the antibiotic by the preliminarily synthesized peptide. We thought the first way to be more promising, since the activation of amino acids used in this method, unlike that of peptides, would be accompanied by a lesser racemization. In addition, this method enabled one to use a combinatorial approach to obtain a great number of analogues.

Our preliminary studies showed that it is possible to obtain peptidyl macrolides in this way using Boc-amino acids; however, under standard conditions, with the removal of the protection group, antibiotics underwent a severe degradation and resinification, which made us to abandon this approach and seek for other suitable protecting groups.

The second approach, namely, the acylation of antibiotics by the ready model peptides, proved to be more successful. To this end, we used short fragments (**IV**)–(**VII**), which were obtained in the synthesis of more long target peptide sequences:

Boc-Ala-Ala-OH (**IV**),

Boc-Ala-Ala-Phe-OH (**V**),

Boc-Gly-Pro-Gly-Pro-Gly-Pro-OH (**VI**), and

MeOTr-Gly-Pro-Gly-Pro-Gly-Pro-OH (**VII**).

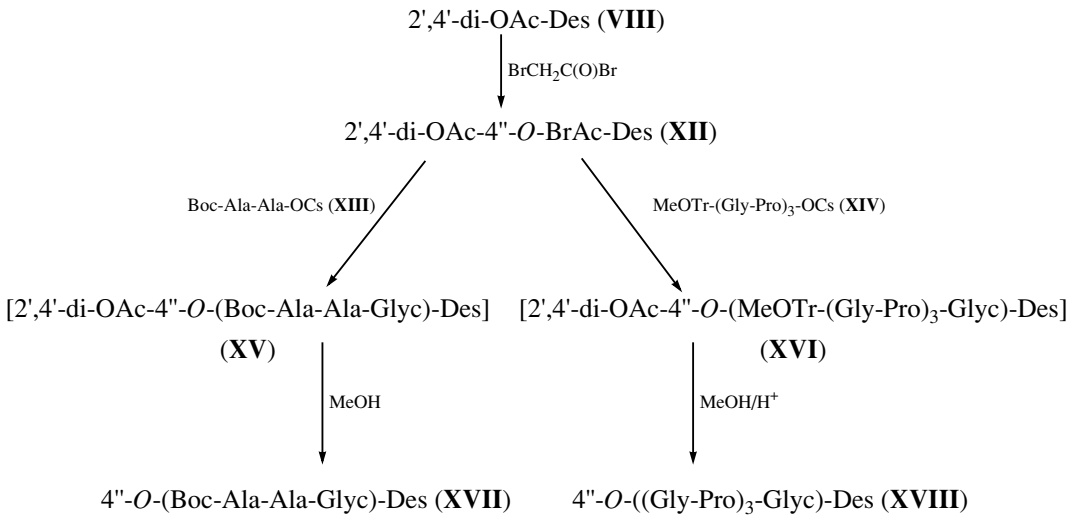
As mentioned above, peptide derivatives of macrolides can serve as tools for studying the conformation and functioning of the nascent peptide chain in the ribosomal tunnel. Therefore, peptides of different structures and spatial organization, in particular, those tending to form α helices, β structures, and unordered structures, are of interest. In the choice of these peptides, we were followed the general theoretical concepts on amino acid residues that prefer a particular secondary structure [13].

The peptides (**IV**) and (**V**) were synthesized by the classical method using DCC in the presence of HOBt. The reactions were carried out in DMF under cooling (0°C), which ensures only a negligible racemization degree [14]. Carboxyl groups were protected by esterification. The synthesis of peptide (**V**) was carried out from the *C*-terminus to obtain first Boc-Ala-Phe-OEt (**Va**), which was then coupled with Boc-Ala-OH after the removal of its Boc-group by TFA. The peptides esters were saponified with alcoholic alkali. As a result, peptidyl acids (**IV**) and (**V**) necessary for the conjugation with OMT were obtained. For the modification of Des, dipeptide (**IV**) was converted into the corresponding cesium salt [10, 15].

Peptide (**VI**) was obtained by the solid-phase synthesis using Merrifield resin [16], Boc-amino acids, and DCC as an activating agent. Boc groups were eliminated by a solution of hydrogen chloride in dioxane, and the peptide was cleaved from the polymer by transesterification with methanol in the presence of DIEA. Purification by column chromatography on silica gel gave peptide (**VI**) as a homogeneous substance (by TLC and HPLC). The structure of the peptide was confirmed by amino acid analysis and mass spectrometry.

As mentioned above, the use of the Boc-group for the synthesis of peptidyl macrolides is not optimal, and, therefore, a 4-monomethoxytrityl group was in some cases substituted for Boc group, which can be removed under milder conditions [17]. For example, the treatment of the deblocked Boc-hexapeptide (**VI**) with 4-methoxytriphenylchloromethane in DMF for 16 h at 40°C led to the peptide (**VII**). It was purified on a silica gel column; however, the mass spectrum of peptide (**VII**) showed only the peak corresponding to the detritylated derivative.

Peptide derivatives of Des were synthesized as follows. At the first step, the reaction of the antibiotic with acetic anhydride resulted in a 2',4'-diacetyl derivative of Des (**VIII**), whose molecular mass was confirmed by mass spectrometry (Scheme 1). It has earlier been shown that the 4"-hydroxyl group of mycinose is not affected under the acetylation of diethyl acetal of the antibiotic by acetic anhydride [9]. This fact was confirmed by the results of the Des acylation by anhydride $(Boc\text{-}\beta Ala)_2O$. It was shown by NMR spectroscopy that the acylation proceeds strictly at 2'- and 4'-hydroxyl groups of mycaminose without affecting the mycinose 4"-hydroxyl. Even with a twofold excess of the acylat-



Scheme 2.

ing agent, a mixture of 2'- and 4'-isomers (**X**) and (**XI**) instead of the disubstituted derivative (**IX**) is formed.

The positions of acyl groups in (**IX**) were confirmed by the methods of two-dimensional correlation spectroscopy COSY and HMBC. The COSY spectra allowed us to unambiguously assign the signals of mycinose and two nonequivalently substituted mycaminose fragments. In this case, the chemical shifts of the mycinose ring protons corresponded to the signals of the starting compound. Two nonequivalent amino sugar residues were characterized by a considerable low-field shift of the signal of protons at C2' (δ 4.98 ppm, m) or at C4' (δ 4.80 ppm, t) relative to the signals of the corresponding protons of the starting compound, indicating that the hydroxyl at C2' or C4' underwent acylation. This conclusion is confirmed by the presence of cross-peaks between the signals of carbonyl atoms of acetyl groups and the protons at C2' or C4'.

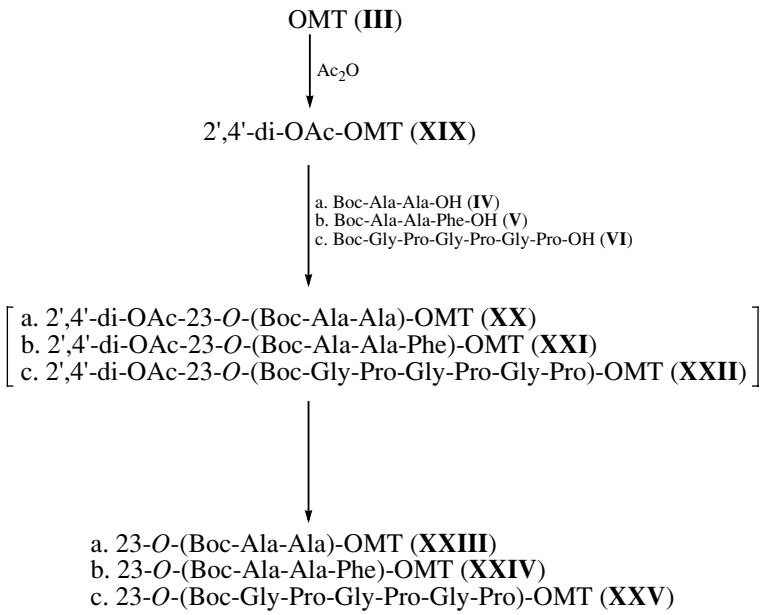
The diacetyl derivative of Des (**VIII**) was then put in reaction with bromoacetyl bromide at –15°C in the presence of pyridine (Scheme 2). The mass spectrum of the bromoacetyl derivative of Des (**XII**) showed a clear peak of the target compound, which proves with a high degree of probability that bromoacetylation occurs at position 4'' of the mycinose residue of Des.

4''-Bromoacetyl derivative of Des (**XII**) was a convenient starting compound for the attachment of peptide fragments. Dipeptide Boc-Ala-Ala-OH and hexapeptide MeOTr-Gly-Pro-Gly-Pro-Gly-Pro-OH, which were introduced into the reaction with the bromoacetyl derivative (**XII**) as Cs salts (**XIII**) and (**XIV**) according to Scheme 2, were used as protected peptides.

The reaction was carried out in anhydrous DMF at 50–60°C. The proposed reaction products (**XV**) and (**XVI**) were purified by column chromatography on silica gel in systems containing chloroform and methanol. TLC showed that these derivatives are homogeneous; they absorb in UV-light and are detected by DNPH and ninhydrin after exposure in TFA vapors. HPLC indicated that the compounds contained impurities. The mass spectra of peptidyl desmycosins showed peaks corresponding to structures (**XVII**) and (**XVIII**); i.e., deacetylated products and, in the case of (**XVII**), also a detritylated derivative. The absence of peaks of compounds (**XV**) and (**XVI**) in the mass spectra suggested that, most probably, the *O*-acetyl groups in the mycaminose residue and the trityl function are very unstable under the conditions of chromatography on silica gel in methanol-containing systems. These facts are consistent with the literature data on the lability of 2'- and 4'-acetyl groups of mycaminose in the presence of methanol [9, 18].

Peptide derivatives of OMT were synthesized by Scheme 3. OMT was treated with acetic anhydride in dichloromethane at room temperature as described in [19] to give a 2',4'-diacetyl derivative of OMT (**XIX**). In this case, the primary hydroxyl at C23 does not enter the reaction [18, 19]. Compound (**XIX**) was obtained in a good yield and was homogeneous by TLC and HPLC. No constants of this compound, except for the chromatographic mobility in one system, were given in the patent [19], and we measured its mass spectrum, which confirmed the structure (**XIX**). This derivative was then coupled with protected peptides by the peptide chemistry methods.

We first used DCC as the coupling reagent in the presence of DMAP as a catalyst for dipeptide Boc-Ala-Ala-OH (**IV**). It is known that the condensation by the DCC method [20] proceeds in high yields but can be accompanied by a substantial racemization, especially in the presence of DMAP [21]. To suppress the racemization, the reaction was carried out in dichloromethane at a low temperature (0°C) in the presence of a minimum amount of base (0.1–0.2 equiv). The isolation and



Scheme 3.

purification of the proposed reaction product (**XX**) on silica gel gave a chromatographically homogeneous substance, which corresponded to the deacetylated derivative 23-*O*-(Boc-Ala-Ala)-OMT (**XXIII**) as confirmed by its mass spectrum. We were unable to determine the optical purity of the compound (as well as of other peptidyl macrolides) because of a low quantity of the material. In addition, without depreciating the importance of this aspect, we should emphasize that most attention in this study was paid to the search for approaches to the synthesis of peptidyl macrolides.

Like the dipeptide (**IV**), diacetate (**XIX**) was condensed with hexapeptide (**VI**) for which the conditions of the reaction (DCC, DMAP, –5°C, the solution in dichloromethane and DMF) were more appropriate since the *C*-terminal proline usually shows no tendency to racemize [22]. Tripeptide (**V**) was coupled with diacetate (**XIX**) using the uronium reagent HBTU [23], which reduces racemization to a minimum but requires a particular precaution because of its high reactivity.

The resulting OMT peptide derivatives (**XX**), (**XXI**), and (**XXII**) were purified on a silica gel column. The chromatographically homogeneous compounds (by TLC) were analyzed by mass spectrometry. In all cases, we registered the peaks corresponding to target compounds (**XXIII**)–(**XXV**), which contain no acetyl groups. Like Des, this can be explained by an increased lability of the acetyl groups of 3-deoxy-3-dimethylaminoglucose during the purification on silica gel columns in methanol-containing systems [9, 18].

Thus, using model compounds, we elaborated approaches to the synthesis of specially designed peptide derivatives of macrolides, which can be used as tools in the study of the conformation and topography of the nascent polypeptide chain in the ribosome tunnel.

EXPERIMENTAL

The following reagents were used: derivatives of amino acids (Fluka and Bachem); DCC, bromoacetyl bromide, and DMAP (Merck, Germany); and *N*-hydroxybenzotriazolyluronium hexafluorophosphate (Fluka, Switzerland). Other reagents, including Tyl (OOO Mosagrogen), were of domestic manufacture.

TLC of peptide derivatives was carried out on silica gel 60 F254 plates (Merck, Germany), column chromatography, on silica gel 60 (0.063–0.2 mm) (Fluka, Switzerland).

The following solvents systems were used: (*1*) 14 : 1 : 5 isopropanol–25% ammonia–water; (*2*) 7 : 3 chloroform–methanol; (*3*) 9 : 1 chloroform–methanol; (*4*) 4 : 1 chloroform–methanol; (*5*) 50 : 25 : 2 benzene–acetone–acetic acid; (*6*) 65 : 4 : 25 chloroform–25% ammonia–methanol; (*7*) 8 : 1 : 0.1 dichloromethane–methanol–25% ammonia; (*8*) 4 : 1 : 0.06 dichloromethane–methanol–25% ammonia; (*9*) 4 : 1 benzene–acetone; and (*10*) 60 : 45 : 20 chloroform–methanol–32% acetic acid.

Compounds absorbing the UV-light were monitored using a Brumberg chemiscope. Compounds with a free α-amino group were detected by ninhydrin reagent [ninhydrin (0.2 g) in a mixture of acetone (100 ml), water (5 ml), and acetic acid (5 ml)], and compounds containing a Boc group were detected by the same method after heating the plate at 90°C. Tyl, Des, and OMT as well as their derivatives were detected using 0.4% DNPH solution in 2 M HCl.

Acidic hydrolysis of peptides was executed under standard conditions (6 M HCl, 105°C, 24 h) in sealed ampoules. The content of amino acids in hydrolyzates was determined using an amino acid analyzer, model 835 (Hitachi, Japan).

The optical rotation of peptides was measured on a Perkin-Elmer polarimeter (model 341) at a wavelength of 589 nm. Melting points were measured on a PHMK instrument (VEB Wagetechnic Rapido).

Reversed-phase HPLC was carried out on a Milichrom A-02 chromatograph (Econova) on a ProntoSIL-120-5-C18 AQ column (2.0 × 75 mm, 5 μm) in a 15 to 70% gradient of B in A for 20 min: (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile; elution rate 100 μl/min; detection at 214 and 300 nm.

Molecular masses were determined by MALDI TOF mass spectrometry on an Ultraflex instrument (Bruker Daltonics, Germany) equipped with a laser (λ 337 nm).

Proton and two-dimensional spectra for solutions of substances in $CDCl_3$ were recorded at 303 K on a Bruker DRX-500 spectrometer with an operating frequency of 500.13 MHz for protons; and ^{13}C spectra (with uncoupling from protons), on a Bruker AM-300 spectrometer with an operating frequency of 75.43 MHz. The multiplicities of signals in ^{13}C spectra were determined by the INEPT procedure. The residual signals of chloroform (δ_H 7.27 ppm, δ_C 77.0 ppm) were used as internal standards. Two-dimensional spectra were recorded using standard methods of the Bruker company (COSY, in the magnitude presentation; for HSQC and HMBC, $^1J_{CH}$ 135 Hz and $^3J_{CH}$ 8 Hz, respectively, were used). Chemical shifts are given in ppm, and spin–spin coupling constants, in Hz.

Des (II). A solution of Tyl (5.0 g, 5.47 mmol) in water (100 ml) and 1 N HCl (10 ml) (pH 3) was kept for 4 days; pH was adjusted to 8.5 by adding dry sodium bicarbonate; and extracted with chloroform (4 × 20ml). Combined extracts were dried by magnesium sulfate, filtered, and evaporated to dryness to give 3.85 g (91.6%) of (**II**); mp. 95–120°C (literature data [7]: mp 95–115° C; R_f 0.45 (2) and R_f 0.5 (7). MC: $[M + H]^+$ 772.8 (100%) (calc. for $C_{39}H_{65}NO_{14}$: 771.8).

***O*-Mycaminosyltylonolide (III).** Concentrated sulfuric acid (600 μl) was added to a solution of Tyl (10 g, 10.92 mmol) in water (500 ml), pH was adjusted to 1.68; the solution was refluxed for 50 h and extracted with chloroform (3 × 130 ml). The aqueous phase was adjusted to pH 9.5 with 25% ammonia and again extracted with chloroform (4 × 200 ml). The combined organic extracts were dried with anhydrous $MgSO_4$ and concentrated to one fifth of its volume. Diethyl ether was added to achieve complete precipitation. After the precipitate was filtered, washed with ether, and dried, 6.62 g of (**III**) was obtained. One gram of the product was purified on a silica gel column (25 × 3 cm) in a gradient of system № 2 to № 8; yield of (**III**) 380 mg (38%); mp 132–135°C (literature [8]: mp 115–118°C; R_f 0.26 (2) and 0.3 (7); MS: $[M + H]^+$ 598.7 (100%) (calc. for $C_{31}H_{51}NO_{10}$: *M* 597.8); ^1H NMR, *COSY*, *HSQC* ($CDCl_3$, 500 MHz): 9.64 (1 H, s, H20), 7.28 (1 H, d, *J* 15.0, H11), 6.29 (1 H, d, *J* 15.0, H10), 5.86 (1 H, d, *J* 10.7, H13), 4.93 (1 H, ddd, *J* 9.4, 9.4, and 1.7, H15), 4.22 (1 H, d, *J* 7.3, H1'), 3.80 (1 H, d, *J* 10.5, H3), 3.70 (3 H, m, H5, H23), 3.44 (1 H, dd, *J* 10.0 and 7.7, H2'), 3.23 (1 H, m, H5'), 3.03 (1 H, t, *J* 9.0, H4'), 2.90 (1 H, dd, *J* 17.7 and 9.6, H19), 2.84 (1 H, m, H14), 2.50 (1 H, br s, H8), 2.47 [3 H and 1 H, s and m, $N(CH_3)_2$, H2], 2.34 (2 H, m, H3', H19), 2.10 (1 H, br s, H6), 1.91 (1 H, d, *J* 16.5, H2), 1.81 (1 H, m, H16), 1.58 (4 H, m, H4, H7, H16), 1.40 (1 H, m, H7), 1.78 (3 H, s, H22), 1.22 (3 H, d, *J* 6.4, H6'), 1.17 (3 H, d, *J* 6.6, H22), 0.97 (3 H, d, *J* 6.6, H21), 0.90 (3 H, t, *J* 7.3, H14); ^{13}C NMR, *INEPT*, *HSQC* ($CDCl_3$, 500 MHz): 203.5 (C9), 203.0 (C20), 173.9 (C1), 148.0 (C11), 141.8 (C13), 135.8 (C12), 118.9 (C10), 104.0 (C1'), 81.2 (C5), 75.1 (C15), 73.3 (C5'), 70.9 (C2'), 70.8 (C4'), 70.2 (C3'), 67.0 (C3), 62.4 (C23), 47.2 (C14), 44.5 (C8), 43.7 (C19), 41.7 ($N(CH_3)_2$), 40.3 (C4), 39.5 (C2), 32.8 (C7), 32.6 (C6), 25.6 (C16), 17.8 (C6'), 17.3 (C21), 13.1 (C22), 9.7 (C17), 9.0 (C18).

*tert-Butyloxycarbonyl-alanyl-alanine (**IV**)*

Methyl ester of *tert*-butyloxycarbonyl-alanyl-alanine (IVa). A solution of DCC (3.0 g, 14.5mmol) in DMF (10 ml) was added to a mixture of Ain-alanine (1.89 g, 10 mmol) and IIAt (2.0 g, 14.8 mmol) in DMF (15 ml) under cooling (0°C) and stirring, and stirring was continued for 2 h at 0°C. Then a solution of alanine methyl ester hydrochloride (1.5 g, 10.7 mmol) and DIEA (1.82 ml, 10.7 mmol) in DMF (15 ml) was added to the reaction mixture, and the mixture was kept for 12 h at room temperature. After the completion of reaction, the reaction mixture was filtered, and the filtrate was diluted with water tenfold and extracted with ethyl acetate (3 × 100 ml). The ethyl acetate extract was washed with 0.05 M H_2SO_4 (3 × 60 ml), water (60 ml), 5% $NaHCO_3$ (3 × 60 ml), and saturated NaCl solution (50 ml); dried with anhydrous $MgSO_4$; filtered; and evaporated on a rotor evaporator. The residue was crystallized with petroleum ether; yield of (**IVa**) 2.25 g (82%); R_f (5) 0.80.

A solution of (**IVa**) (274 mg, 1.0 mmol) in methanol (5 ml) was mixed with 2 N NaOH (0.6 ml), and the mixture was stirred for 3 h. Methanol was evaporated, the pH of the solution was adjusted to 3 by 0.05 M H_2SO_4, extracted with ethyl acetate (3 × 20 ml); the organic phase was washed with water and a saturated NaCl solution; dried by anhydrous $MgSO_4$; filtered; the filtrate was evaporated to a small volume; and (**IV**) was precipitated with petroleum ether; yield 180 mg (70%); R_f (*5*) 0.50; mp 95–97°C; $[\alpha]_D^{20}$ –31° (*c* 1; methanol) (literature mp 97–98°C; $[\alpha]_D^{20}$ –33° (*c* 1; methanol) [24]).

*tert-Butyloxycarbonyl-alanyl-alanyl-phenylalanine (**V**)*

Ethyl ester of *tert*-butyloxycarbonyl-alanyl-phenylalanine (Va). A solution of DCC (7.17 g, 34.8 mmol) in DMF (15 ml) was added to a solution of Boc-alanine (5.10 g, 27 mmol) and HOBt (4.72 g, 34.8 mmol) in DMF (35 ml) at 0°C under stirring. The stirring was continued for 2 h under cooling. Then a solution of phenylalanine ethyl ester hydrochloride (6.5 g, 28.4 mmol) and triethylamine (6.77 ml, 48.3 mmol) in DMF (35 ml) was added to the reaction mixture, and the mixture was stirred for 12 h at room temperature. The reaction mixture was treated as in the case of (**IVa**). Yield of (**Va**) 8.43 g (90%); mp 105–106°C (from diethyl ether); R_f 0.91 (*4*), 0.82 (*5*). Amino acid analysis: Ala 1 (1), Phe 1.15 (1).

Ethyl ester of alanyl-phenylalanine trifluoroacetate (Vb). A solution of (**Va**) (8.1 g, 22.3 mmol) in TFA (15 ml) was kept for 1 h at room temperature, evaporated, and the residue was three times coevaporated with methanol and dissolved in methanol (3 ml). The peptide was precipitated with diethyl ether to give 7.50 g of a white crystalline (**Vb**); yield 90%; mp 157–159°C. R_f (1) 0.80 and (6) 0.79.

Ethyl ester of *tert*-butyloxycarbonyl-alanyl-alanyl-phenylalanine (Vc). A solution of DCC (3.83 g, 18.6 mmol) in DMF (8 ml) was added to a solution of Boc-Ala (2.34 g, 12.4 mmol) and HOBt (2.53 g, 18.6 mmol) in DMF (16 ml) at 0°C. The stirring was continued for several hours under cooling. Then a solution of (**Vb**) trifluoroacetate (4.95 g, 13.1 mmol) and triethylamine (3.32 ml, 23.6 mmol) in DMF (16 ml) was added to the reaction mixture, and stirring was continued for 12 h at room temperature. The reaction mixture was treated as described for (**IVa**) to give 5.09 g (90%) of (**Vc**); mp 139–140°C (ethyl acetate–petroleum ether); R_f (4) 0.85 and (5) 0.50.

(**V**) was synthesized as described for (**IV**) in methanol starting from (**Vc**) (500 mg, 1.15 mmol) and 2 M NaOH (690 μl, 1.38 mmol); yield 448 mg (95%); mp 113–115°C. R_f (4) 0.73 and (5) 0.21; HPLC: RT 15.2 min (gradient 0 to 60% of B in A for 20 min); $[\alpha]_D^{20}$ −34° (*c* 1; DMF); MS: 408.6 $[M + H]^+$ (calc. for $C_{20}H_{29}N_3O_6$: *M* 407.5. Amino acid analysis: Ala 2.14 (2), Phe 1 (1).

*tert-Butyloxycarbonylglycyl-prolyl-glycyl-prolyl-glycyl-proline (**VI**)*

Methyl ester of *tert*-butyloxycarbonylglycyl-prolyl-glycyl-prolyl-glycyl-proline (VIa). Ain-Pro-polymer (3.5 g; 0.75 mmol/g of resin; the total amount of amino acid 2.625 mmol) was placed in a reaction flask, and dioxane (30 ml) was added. Threefold excesses of Boc-Gly-OH and Boc-Pro-OH derivatives, and DCC were applied using a 10% solution of DCC in dichloromethane. The following operations were successively carried out (volume of washing solution was 30 ml and that at condensation, 20 ml): deblocking with a 4 N HCl solution in dioxane (30 min), washing with dioxane, washing with dichloromethane, neutralization with 10% solution of Et_3N in dichloromethane (10 min), washing with dichloromethane, addition of a solution of Boc-amino acid in dichloromethane (5 min), addition of a solution of DCC in dichloromethane (2 h), and washing with dichloromethane. At each step of the synthesis, the completeness of the coupling reaction was monitored using the Kaiser test [25]. After the completion of the synthesis, the peptidyl polymer was transferred from the Merrifield flask to a round bottom flask, and the solution (152 ml, 0.46 mM) was treated at room temperature with DIEA in methanol under stirring. The polymeric support was then filtered, and the filtrate was evaporated on a rotor evaporator. The product was obtained as a yellow oil, which was purified by column chromatography on silica gel (50 g) in system 3 to give 660 mg (55%) of (**VIa**) as a white hard oil; mp 150°C; R_f (3) 0.58 and (4) 0.85; HPLC: RT 18.2 min (gradient 0 to 50% of B for 25 min); $[\alpha]_D^{20}$ −48° (*c* 1; DMF); MS: 595.2 $[M + H]^+$ (calc. for $C_{27}H_{42}N_6O_9$: 594.7). Amino acid analysis: Gly 1.31 (1), Pro 1 (1).

A solution of (**VIa**) (260 mg, 0.438 mmol) in methanol (3.5 ml) was treated with 2 M NaOH (0.265 ml, 0.53 mmol). The reaction mixture was stirred for 3 h at room temperature and then treated as described for (**IV**); yield of (**VI**) 180 mg (precipitation from ethyl acetate with hexane) (71%); R_f (6) 0.41 and (10) 0.79; HPLC: RT 13.3 min (gradient 0 to 60% of B for 20 min); MS: 581.8 $[M + H]^+$ (calc. for $C_{26}H_{40}N_6O_9$: 580.6).

4-Monomethoxytritylglycyl-prolyl-glycyl-prolyl-glycyl-proline (VII). TFA (0.5 ml) was added to hexapeptide (**VI**) (50 mg, 0.086 mmol), the mixture was kept for 1 h at room temperature, and excess TFA was removed on a rotor evaporator. The residue was several times coevaporated with absolute methanol, dissolved in DMF (0.5 ml), after which DIEA (31 μl, 0.18 mmol) and MeOTrCl (50 mg, 0.162 mmol) were added. The mixture was kept for 16 h at 40°C, evaporated in a vacuum, and the residue was purified by column chromatography on silica gel in 7 : 3 chloroform–methanol system containing 0.5% pyridine. Yield of (**VII**) 47 mg (73%); R_f (2) 0.43 and (4) 0.35; MS:480.4 $[M + H - MeOTr]^+$; calc. for $C_{21}H_{31}N_6O_7$: M 479.4.

2',4'-Di-*O*-acetylDes (VIII). Ac_2O (75 μl, 0.8 mmol) was added to a solution of Des (183 mg, 0.25 mmol) in dichloromethane (7 ml) under cooling with ice and stirring. The stirring was continued for 1 h at room temperature; the reaction mixture was poured into a diluted ammonia solution (0°C) and extracted twice with dichloromethane. The organic extracts were dried with anhydrous $MgSO_4$ and evaporated, to give (**VIII**); yield of 183 mg (85.5%); R_f 0.91 (2), R_f 0.65 (3), and 0.11 (9); MS: $[M + H]^+$ 856.9 (100%), $[M + Na]^+$ 878.8 (36%); calc. for $C_{43}H_{69}NO_{16}$: *M* 855.9.

2'- and 4'-*O*-[*N*-(*tert*-butyloxycarbonyl)-β-alanyl]-Dess (X) and (XI). Boc-βAla-OH (282 mg, 1.5 mmol) was dissolved in a mixture of DMF (50 μl) and dichloromethane (0.5 ml), the solution was cooled to 0°C, and a cooled (0°C) solution of DCC (154.5 mg, 0.75 mmol) in dichloromethane (0.5 ml) was added. The mixture was stirred for 1 h under cooling, and a solution in anhydrous dichloromethane (0.4 ml) of Des (270 mg, 0.35 mmol) dried over P_2O_5 in a vacuum at 63°C was added. After 10 min, DMAP (17 mg, 0.14 mmol) was added, and the mixture was stirred at room temperature for 12 h. The precipitate was filtered off and washed with dichloromethane. The filtrate was twice washed with saturated sodium bicarbonate solution and water and dried with anhydrous $MgSO_4$. After evaporation, a chromatographically homogeneous mixture (230 mg) of (**X**) and (**XI**) was obtained: TLC: R_f 0.95 (2); HPLC: RT 18.1 min (RT of the starting Des 11.7 min); MS: [*M*] 943.4 (calc. for $C_{47}H_{78}N_2O_{17}$: *M* 943.02; ^{1}H NMR ($CDCl_3$, 500 MHz), δ, ppm: (**X**): 4.98 (1 H, m, H2'), 4.56 (1 H, d, *J* 8.0, H1'), 4.31 (1 H, d, *J* 8.0, H1'), 3.75 (1 H, s, H3''), 3.55 (1 H, m, H5''), 3.29 (1 H, m, *J* 6.5, H5'), 3.22 (1 H, d, *J* 7.0, H4''), 3.10 (1 H, t, *J* 8.5, C4'), 3.05 (1 H, dd, *J* 8.0, <2.0, H2''), 2.55 (1 H, t, *J* 8.0, C3'); (**XI**): 4.80 (1 H, t, *J* 8.5, H4'), 4.56 (1 H, d, *J* 8.0, H1'), 4.25 (1 H, d, *J* 8.0, H1'), 3.75 (1 H, s, H3''), 3.55 (1 H, m, H5''), 3.35 (1 H, m, H2'), 3.29 (1 H, m, *J* 6.5, H5'), 3.22 (1 H, d, *J* 7.0, H4''), 3.05 (1 H, dd, *J* 8.0, <2.0, H2''), 2.56 (1 H, t, *J* 8.5, H3').

2',4'-Di-*O*-acetyl-4''-*O*-bromoacetyldesmycosin (XII). 2',4'-Di-*O*-acetylDes (171 mg, 0.2 mmol) was dried by twofold coevaporation with anhydrous pyridine, dissolved in dry dichloromethane (3 ml), mixed with anhydrous pyridine (50 μl), and cooled to –15°C. Bromoacetyl bromide (50 μl, 0.95 mmol) was added to the reaction mixture under stirring. A white solid was precipitated, which dissolved after addition of dichloromethane (3 ml). After 10 min of stirring at –10 to –15°C, the reaction mixture was transferred to a mixture of ice and dichloromethane. The organic layer was separated, washed with 5% sodium bicarbonate and saturated sodium chloride solution, dried with $MgSO_4$, and evaporated on a rotary evaporator. Dry ether was added to the resulting oily residue, and the mixture was evaporated several times to give (**XII**) as a light yellow precipitate; yield: 110 mg (56.3%); R_f 0.31 (9); MS: $[M]^+$ 975.6 (100%), unidentified peaks *m/z* 1007.7 (15%) and 1027.7 (37%); calculated for $C_{45}H_{70}BrNO_{17}$: *M* 976.8.

4''-*O*-(*tert*-Butyloxycarbonyl-alanyl-analyl-glycolyl)-Des (XVII). Derivative (**XII**) (10 mg, 0.010 mmol) and the preliminarily prepared cesium salt of Boc-alanyl-alanine (a methanol solution of the peptide was mixed with a 2 M aqueous cesium carbonate solution in the equivalent ratio, evaporated to dryness, and thoroughly dried) (3.74 mg, 0.010 mmol) were dissolved in DMF (100 μl). The solution was kept in a thermostat for 68 h at 45°C. The reaction mixture was then diluted with water and twice extracted with chloroform. The organic layer was washed with water and dried over anhydrous $MgSO_4$. After the evaporation of chloroform, the residue (2.2 mg) was examined by chromatography: R_f 0.59 (2); the substance absorbs in UV-light and is developed by ninhydrin and DNPH. HPLC: RT 22.3 min; MS: $[M + H]^+$ 1073.3; calc. for $C_{52}H_{85}N_3O_{20}$: *M* 1072.13.

4''-*O*-(Glycyl-prolyl-glycyl-prolyl-glycyl-prolyl-glycolyl)-Des (XVIII). Peptide (**VII**) (12 mg, 0.016 mmol) was mixed with cesium carbonate (5.2 mg, 0.016 mmol) and dissolved in ethanol (1 ml). The solution was evaporated to dryness and then twice evaporated with dry pyridine. 2',4'-Di-*O*-acetyl-4''-*O*-bromoacetyl-Des (**XII**) (14 mg, 0.014 mmol) and anhydrous DMF (0.5 ml) were added to the residue and kept for 24 h at 50°C under stirring. The reaction mixture was evaporated, equal volumes of water and dichloromethane were added, and the mixture was intensively shaken. The organic layer was separated, washed with water, and dried with molecular sieves 4Å. Purification of the product on a silica gel column in system 2 yielded 3 mg (14.5%) of **(XVIII)**; MS: $[M + H]^+$ 1293.3; calc. for $C_{62}H_{97}N_7O_{22}$: *M* 1292.36.

2',4'-Di-*O*-acetyl-IIO (XIX) [19]. A solution of OMT (0.5 g, 0.84 mmol) and acetic anhydride (0.63 ml, 6.68 mmol) in acetone (4.5 ml) was stirred for 2 h at room temperature, evaporated, and then coevaporated with toluene (2 × 5 ml). The residue was dried in a vacuum desiccator to get (**XIX**); yield 0.56 g (98%); R_f 0.9 (2) and 0.3 (9); mp 127–133°C, HPLC: RT 10.4 min (gradient 20–80% B for 20 min); MS: $[M + H]^+$ 682.7 (100%), and *m/z* 640.5 [AcOMT] (54%), 598.5 [OMT] (23%); calc. for $C_{35}H_{55}NO_{12}$: *M* 681.8.

23-*O*-(*tert*-Butyloxycarbonyl-alanyl-alanyl)-OMT (XXIII). A solution of DCC (3.1 mg, 0.015 mmol) in dichloromethane (50 μl) was added to a solution of 2',4'-di-*O*-acetyl-OMT (**XIX**) (7.8 mg, 0.01mmol), DMAP (0.2 mg), and peptide (**IV**) (4 mg, 0.015 mmol) in dichloromethane (100 μl) under stirring and cooling (0°C). The reaction mixture was stirred for additional 36 h at room temperature, washed with a diluted ammonia, and evaporated. The residue was purified on a silica gel column (3 × 0.8 cm) in system 2; R_f 0.95 (2). The substance was detected with DNPH and ninhydrin; MS, *m/z*: 840.4 $[M + H]^+$ (100%), 598.4 [OMT] (68%); calc. for $C_{42}H_{69}N_3O_{14}$: *M* 839.5.

23-*O*-(*tert*-Butyloxycarbonyl-alanyl-alanyl-phenylalanyl)-OMT (XXIV). A solution of 2',4'-di-*O*-acetyl-OMT (**XIX**) (5 mg, 0.07 mmol), Boc-Ala-Ala-Phe-OH (**V**) (7.4 mg, 0.019 mmol), and HBTU (5 mg, 0.013 mmol) in a mixture of dichloromethane and DMF (100 μl) was stirred for 12 h at room temperature and evaporated. Dichloromethane (1 ml) was added, and the mixture was washed with a diluted ammonia and dried using molecular sieves 4 Å. After evaporation, the residue was fractionated on a silica gel column (20 × 5 mm) in system 2, collecting the substance with R_f 0.91. The substance was homogeneous by TLC; it

absorbs in UV-light and is detected by DNPH and ninhydrin; MS, *m/z*: 988.0 $[M + H]^+$ 988.0; $[M + H - Boc]^+$ 887.9; calc. for $C_{51}H_{78}N_7O_{15}$: *M* 987.1.

23-*O*-(*tert*-Butyloxycarbonyl-glycyl-prolyl-glycyl-prolyl-glycyl-prolyl)-OMT (XXV). A solution of (**XIX**) (11.2 mg, 0.015 mmol), DMAP (0.3 mg, 0.0025 mmol), and hexapeptide (**VI**) (11.6 mg, 0.02 mmol) in a mixture of dichloromethane (100 μl) and DMF (50 μl) was cooled to –5°C, and a solution of DCC (4.1 mg, 0.02 mmol) in dichloromethane (100 μl) was added under stirring. The reaction mixture was stirred for additional 12 h at room temperature, washed with a diluted ammonia over anhydrous $MgSO_4$, and evaporated. The residue was purified on a silica gel column (30 × 10 mm) in system 2. A substance with R_f 0.91 was isolated. It was homogeneous by TLC, absorbed UV-light, and was detected by DNPH and ninhydrin upon heating; MS, *m/z*: 1161.2 $[M + H]^+$ (100%), 1183.2 $[M + H]^+$ (54%), 682.7 [Ac_2-OMT], 640.9 [OMT]; calc. for $C_{57}H_{89}N_7O_{15}$: *M* 1160.3.

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