GLOBOMYCIN, A NEW PEPTIDE ANTIBIOTIC WITH SPHEROPLAST-FORMING ACTIVITY

III. STRUCTURAL DETERMINATION OF GLOBOMYCIN

MUTSUO NAKAJIMA, MASATOSHI INUKAI, TATSUO HANEISHI,
AKIRA TERAHARA, MAMORU ARAI
Fermentation Research Laboratories

TAKESHI KINOSHITA and CHIHIRO TAMURA
Central Research Laboratories
Sankyo Co., Ltd.
2–58, 1-chome, Hiromachi, Shinagawa-ku, Tokyo 140, Japan

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The structure of globomycin has been determined by mass, PMR and CMR spectra and by chemical degradation. Globomycin is a new cyclic peptide antibiotic composed of L-serine, L-*allo*-threonine, glycine, N-methylleucine, L-*allo*-isoleucine and 3-hydroxy-2-methylnonaic acid. Among these components, L-*allo*-threonine, L-*allo*-isoleucine and 3-hydroxy-2-methylnonaic acid are novel components from natural products. A newly developed mass analysis has been introduced for determining the diastereoisomers of *allo*-threonine.

Globomycin (**I**) is a lipophilic cyclic peptide antibiotic produced by four different strains of the actinomycetes, *Streptomyces halstedii* No. 13912, *Streptoverticillium cinnamoneum* No. 15037, *Streptomyces neohygroscopicus* subsp. *globomyceticus* No. 15631 and *Streptomyces hagronensis* No. 17834. The antibiotic shows a specific activity against Gram-negative bacteria, inhibiting the cell wall synthesis to form spheroplasts in isotonic media. Taxonomy of the producing organisms and fermentation[1], isolation and properties of globomycin[2] have been reported in the preceding papers. This paper deals with the determination of the active structure of globomycin.

Globomycin (**I**) mp. 115°C, $[\alpha]_D^{25}$ 0 (*c* 1, $CHCl_3$), was obtained as neutral, colorless crystals soluble in organic solvents. The molecular formula was determined to be $C_{32}H_{57}N_5O_9$ (mol. wt. 655) by field desorption mass spectrometry and the result of elementary analysis fits this value. The IR spectrum indicates the presence of hydroxy, lactone or ester and amide groups (3300, 1740, 1660 cm^{-1}). The PMR and CMR spectra of globomycin suggested the presence of highly alkylated components, one N-methyl protons (3.2 ppm) and six carbonyl carbons (170.3, 171.8, 173.1, 174.7, 176.3, 178.9 ppm). The structural studies of globomycin have been achieved by the following chemical degradation studies (Scheme 1).

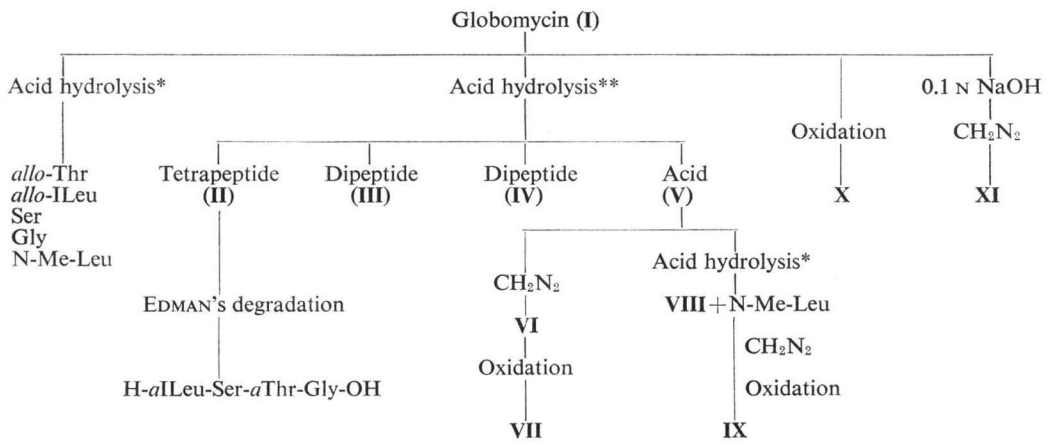
Acid hydrolysis (6 N HCl, 105°C, 16 hours) of globomycin (**I**) followed by amino acid analysis indicated presence of serine, glycine, threonine (or *allo*-threonine) and *allo*-isoleucine (Table 1).

However, the conventional amino-acid analysis could not differentiate between threonine and *allo*-threonine by their retention time. Chromatographic separation of each amino acid obtained by acid hydrolysis was carried out on a column of Dowex 50W-X4 [H^+]. The isolated

Table 1. Amino acid analysis of globomycin.

Amino acid	Retention time (minutes)	μmole/μmole
*allo*Thr (II)	66	0.99
Ser	71	0.90
Gly	106	1.03
N-Me-Leu	114	1.02
*allo*Ileu	132	1.01

Scheme 1. Chemical degradation of globomycin



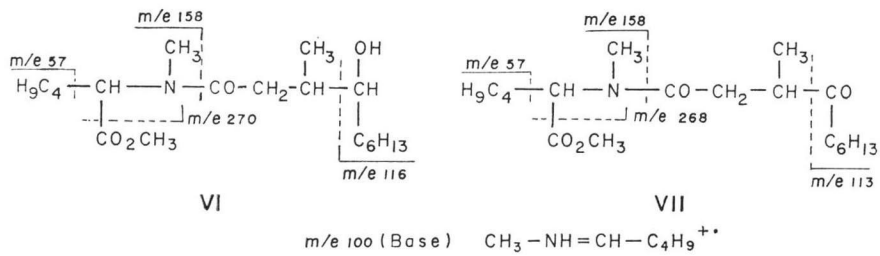
* 6 N HCl, 105°C, 16 hours,
** 6 N HCl, 37°C, 48 hours,

amino acids, serine, glycine and *allo*-isoleucine were identified by comparisons with authentic samples. For determination of the stereochemistry of threonine and *allo*-isoleucine, independent mass spectral analyses have been performed. The mass spectra of trimethylsilyl derivatives of authentic threonine and *allo*-threonine were recorded under the same conditions, and the relative ion intensities at *m/e* 218 and 219 were found to be significantly different. Thereafter, the direct comparison has been made between authentic DL-*allo*-threonine and the threonine species from globomycin and the mass spectra of the two compounds were exactly the same.

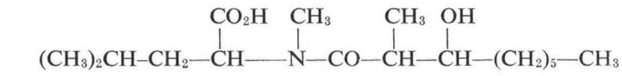
This seems to be the first example of differentiation between configurational isomers of an amino acid by mass analysis.

The structures of the components of globomycin (**I**) were elucidated by following procedures: Mild acid hydrolysis of globomycin (**I**) (6 N HCl, 37°C, 48 hours), afforded tetrapeptide (**II**), dipeptide (**III**), dipeptide (**IV**) and lipophilic acid component (**V**). Amino-acid analysis of tetrapeptide (**II**) indicated the presence of *allo*-isoleucine, serine, *allo*-threonine and glycine, in an approximate molar ratio of 1: 1: 1: 1. Dipeptide (**III**) and dipeptide (**IV**) were also confirmed to be an *allo*-isoleucine-serine unit and a glycine-*allo*-threonine unit by quantitative amino-acid analysis, respectively. EDMAN's degradation of tetrapeptide (**II**) indicated that the amino acid sequence of **II** from the N-terminal group was *allo*-isoleucine, serine, *allo*-threonine and glycine in this order of the linkage.[4]

Mass fragmentation analyses of **VI** and **VIII** established the structure **1** as the lipophilic acid moiety (**V**).



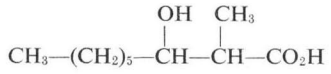
The structure **1** was further confirmed by the isolation of the amino acid from acid hydrolysate of **V**. The IR spectrum of **V** indicated the presence of the amide (1660 cm^{-1}) group. Further acid hydrolysis of **V** (6 N HCl, 105°C, 16 hours) yielded N-methylleucine and a hydroxy fatty acid (**VIII**).



1

The structure of N-methylleucine was confirmed by analyses of the mass (trimethylsilyl derivative) (Fig. 3), PMR spectrum [δ ppm; 0.9 (6 H, d), 1.75 (3H, m), 2.73 (3H, s), and 4.0 (H, t)] and by amino acid analysis in comparison with the authentic sample.

Esterification of **VIII** with diazomethane followed by oxidation with pyridinium chlorochromate[5] in dichloromethane gave the keto-ester (**IX**) whose molecular formula was established as $C_{11}H_{22}O_3$ by high resolution mass spectrometry. The IR spectrum showed the presence of two carbonyl groups (1740, 1720 cm^{-1}). Mass fragmentation analysis of **IX** also indicated the presence of hexylketone moiety *via m/e* 85 (C_6H_{13}) and *m/e* 113 (COC_6H_{13}). The 100 MHz PMR spectrum of **IX** (Fig. 4) supported that this hexylketone moiety should be in a straight chain, *via* broad methyl triplet (0.9 ppm), methylene triplet (2.5 ppm) and the other peaks of methylenes (1.3 ~ 1.8 ppm). A methyl doublet observed at 1.3 ppm suggested that a secondary methyl group should be present. Furthermore, spin decoupling experiments revealed that the methyl doublet was collapsed by irradiation at 3.55 ppm which may be due to the adjacent proton deshielded by the two carbonyl groups. From these data, the structure of the hydroxy fatty acid (**VIII**) should be 3-hydroxy-2-methylnonaic acid, **2**.



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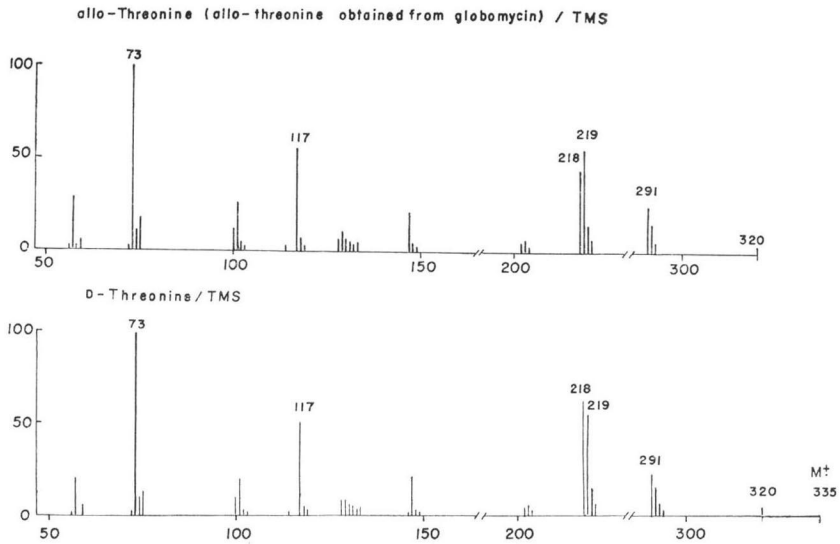
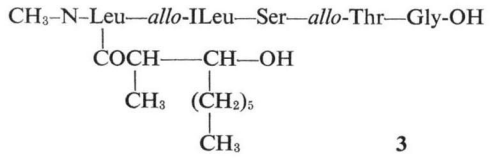
Esterification of lipophilic acid component **V** with diazomethane afforded monomethyl ester derivative (**VI**), $C_{18}H_{35}NO_4$. Oxidation of **VI** with pyridinium chlorochromate in dichloromethane

Fig. 1. Mass spectra of trimethylsilyl derivatives of threonine and *allo*-threonine.



afforded the ketone (**VII**). Accordingly, the structure of lipophilic acid component **V** should be completed as **1.**

The gross structure of globomycin (**I**) was determined as follows: Oxidation of globomycin with pyridinium chlorochromate in dichloromethane gave the product **X** whose molecular formula, $C_{32}H_{53}N_5O_9$, was determined by high resolution mass spectrometry. The amino acid analysis of **X** revealed that both serine and *allo*-threonine of globomycin were possessed of a free hydroxyl group. Mild alkaline hydrolysis (0.1 N NaOH, room temperature, one hour) of globomycin followed by esterification with diazomethane afforded the derivative **XI**. The PMR spectrum of **XI** showed the ester methyl signal at 3.7 ppm and N-methyl signal at 3.0 ppm. The IR spectrum of **XI** also indicated the presence of ester carbonyl (1745 cm^{-1}) along with amide carbonyl (1660 cm^{-1}). The presence of three hydroxyl groups in **XI** was confirmed by mass analysis of the trimethylsilyl derivative of **XI** (M^+ 903). Based on these facts the linear peptide structure for the product obtained by alkaline hydrolysis of globomycin was given as **3.**



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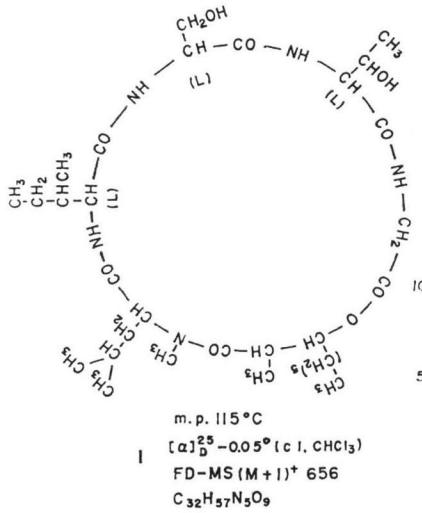
Consequently, all evidences described above have unequivocally elucidated the cyclic peptide structure of globomycin as **I.**



I

Fig. 2. Mass spectra of DMF-DM derivatives of isoleucine and *allo*-isoleucine.

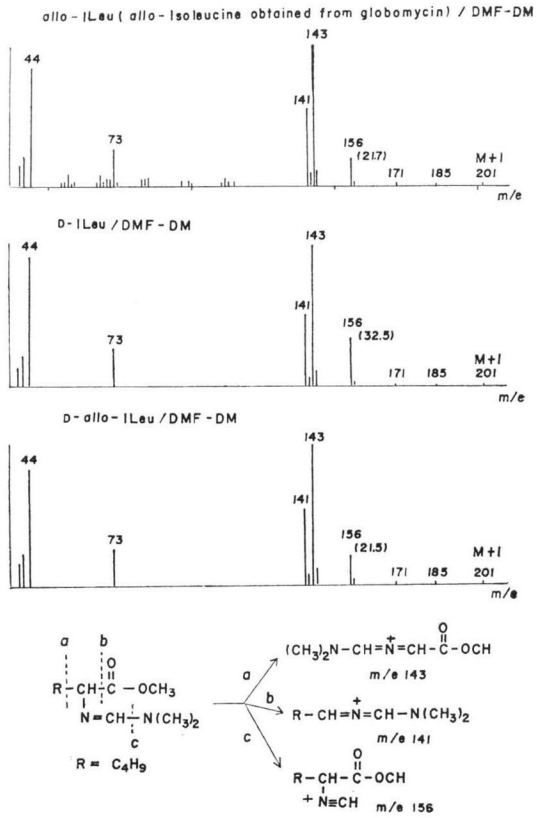


Fig. 3. Mass spectrum of trimethylsilyl derivative of N-methylleucine.

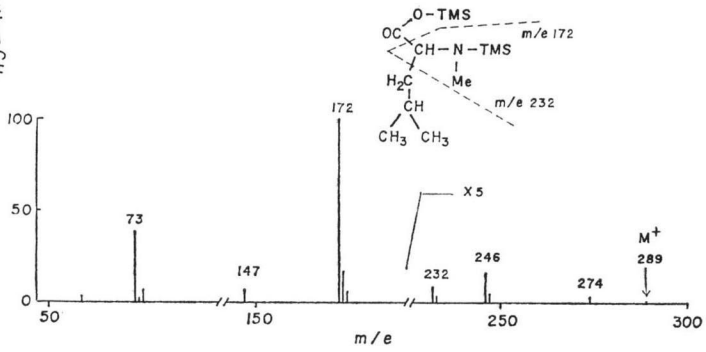
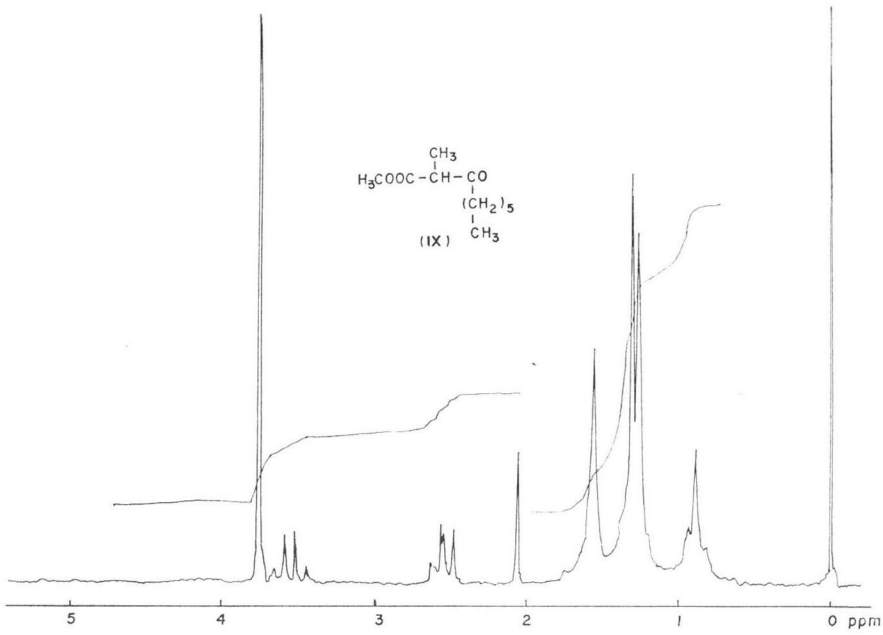


Fig. 4. 100 MHz PMR spectrum of IX in $CDCl_3$.



Although, the determination of stereochemistry of globomycin has not been completed yet, the isolated amino acids, serine, *allo*-threonine and *allo*-isoleucine were confirmed to be L isomers by direct comparison of their specific rotations with those of the authentic samples. Measurement of specific rotation of N-methylleucine afforded the value of $[\alpha]_D^{25}+3.2°$ (*c* 0.44, 2 N HCl), indicating the mixture of L and D forms, although this might be resulted from racemization in hydrolysis process. So far as we know, it would be the first evidence that L-*allo*-isoleucine and L-*allo*-threonine exist as natural amino acids.

Experimental

PMR spectra were obtained on Hitachi R 24 and 100 MHz PMR was determined by Varian 100 HR spectrometer, CMR spectra were recorded on Varian XL-100 (25.2 MHz). Electron impact mass spectra were measured on JEOL-O1SG mass spectrometer using the direct-inlet probe and field desorption mass spectra on a JEOL-O1SG-FI/FD mass spectrometer with a double focusing JEOL JMS-O1SG instrument equipped with a combined f.d.f.i.e.i. ion source. GC/MS spectra were obtained by JEOL JMS D-100. Amino acid analysis was carried on a Hitachi KLA-5 automatic amino-acid analyzer.

Acid hydrolysis of globomycin

(a) A 300-mg sample of globomycin was hydrolyzed in 1 ml of 6 N HCl in a sealed tube for 16 hours at 105°C. The hydrolysate was concentrated under vacuum for several times from water to remove excess HCl. The residue was subjected to Dowex 50W-X4 [H^+] and the crude amino acid mixture was eluted with 0.5 N NH_4OH solution. Cellulose column chromatography (Avicel, Funakoshi Yakuhin Co., Ltd., Japan) of amino acid mixture with *iso*-PrOH-H_2O (80~90 v/v %) as eluent afforded *allo*-isoleucine (27 mg): $[\alpha]_D^{20}$ +9.02° (*c* 0.4, H_2O): *allo*-threonine (10 mg): $[\alpha]_D^{20}$ +29.5° (*c* 0.2, 2 N HCl): serine (15 mg): $[\alpha]_D^{22}+11.5°$ (*c* 1.5, 2 N HCl), glycine (33 mg) and N-methylleucine (15 mg).

(b) A 300-mg sample of globomycin was hydrolyzed in 50 ml of 6 N HCl for 48 hours at 37°C.

The hydrolysate was extracted with ethyl acetate. After washing with water and drying (Na_2SO_4), the solvent was evaporated *in vacuo* to yield lipophilic acid component **V** (130 mg). FD Mass spec: $(M+1)^+$ 316 ($C_{17}H_{33}NO_4$); IR ($CHCl_3$): 3300, 1720, 1620 cm^{-1}. The aqueous hydrolysate was concentrated under vaccum for several times from water to remove excess HCl. The concentrated hydrolysate was lyophilized to afford the peptide mixture (150 mg). Separation of the peptide mixture by high voltage paper electrophoresis at pH 1.8, CH_3COOH - HCOOH - H_2O (75: 25: 900), afforded tetrapeptide **II**, dipeptide **III** and dipeptide **IV**.

Esterification of lipophilic acid component **V**

Esterification of **V** (130 mg) with diazomethane in ethyl acetate gave the crude methyl ester **VI**. The crude methyl ester was chromatographed on a column of silica gel prepared and eluted with $CHCl_3$ - MeOH (10: 1). Initial fractions gave methyl ester **VI** (80 mg) as oily compound. Mass spec: M^+ 329 ($C_{18}H_{35}NO_4$); IR ($CHCl_3$): 3300, 1745, 1630 cm^{-1}.

Oxidation of methyl ester **VI**

To a solution of **VI** (37 mg) in dry dichloromethane (5 ml), pyridinium chlorochromate (43 mg) was added. The reaction mixture was kept for 24 hours at room temperature with magnetic stirring. The reaction mixture was extracted with ethyl acetate. After washing with water and drying (Na_2SO_4), the solvent was evaporated and the residue was purified by preparative thin-layer chromatography on silica gel to yield oxidation product **VII** (13 mg): $[\alpha]_D^{20}$ $-31.37°$ (*c* 0.73, $CHCl_3$); Mass spec: M^+ 327 ($C_{18}H_{33}NO_4$); IR ($CHCl_3$): 1745, 1720, 1620 cm^{-1}; PMR ($CDCl_3$); 0.9~1.1 (9 H), 1.1~1.9 (14 H), 2.5 (2H, t), 3.0 (3H, s), 3.7 (H, q), 5. 3 (H, t).

Acid hydrolysis of **VII**

A 20-mg sample of **VII** was hydrolyzed in 6 N HCl at 105°C for 16 hours. The hydrolysate was extracted with ethyl acetate and the aqueous layer was concentrated. This concentrate was applied onto cellulose (Avicel) column. The column was eluted with 80% aqueous CH_3CN, and fractions of 10 ml each were collected. Fractions No. 9 and 10 were combined and concentrated to yield N-methylleucine (4 mg). The ethyl acetate extract from the hydrolysate was condensed and the residue was treated with diazomethane followed by silica gel chromatography for purification to yield methyl 3-hydroxy-2-methylnonate **VIII** (6 mg). Mass spec: M^+ 202 ($C_{11}H_{22}O_3$); IR ($CHCl_3$): 3300, 1730 cm^{-1}.

Oxidation of methyl 3-hydroxy-2-methylnonate **VIII**

The solution of **VIII** (6 mg) in dry dichloromethane was oxidized with pyridinium chlorochromate. The reaction mixture diluted with dichloromethane was washed with water to remove excess reagents. After drying (Na_2SO_4), the solvent was evaporated *in vacuo* to yield the crude oxidation product, which was further purified by a preparative thin-layer chromatography on silica gel to give 2 mg of methyl 3-keto-2-methylnonate (**IX**) as colorless oil. Mass spec: M^+ 200 *m/e* 113, *m/e* 85; IR ($CHCl_3$): 1745, 1720 cm^{-1}.

Oxidation of globomycin

A 20-mg sample of globomycin (0.033 m mole) in dry dichloromethane (3 ml) was oxidized by pyridinium chlorochromate (40 mg) for 24 hours at room temperature with magnetic stirring. To the reaction mixture, 10 ml of water was added, and then the whole mixture was extracted with ethyl acetate. After washing with NaCl-saturated water and drying (Na_2SO_4), the solvent was evaporated. The residue was subjected to silica gel preparative thin-layer chromatography ($CHCl_3$ - MeOH, 10: 1) to yield 7 mg of oxidation product **X**. Mass spec: M^+ 651 ($C_{32}H_{53}N_5O_9$); IR ($CHCl_3$): 3400, 1740, 1680 cm^{-1}. Amino acid analysis (μmole/μmole): *allo*-isoleucine (1.03), serine (0.05), *allo*-threonine (0), glycine (0.98).

Alkaline hydrolysis of globomycin

The solution of globomycin (5 mg) dissolved in methanolic 0.1 N NaOH (2 ml) was kept for 30 hours at room temperature, and then the solution was neutralized with 0.1 N HCl. After the solvent was evaporated *in vacuo*, the residue (16 mg) was treated with trifluoroacetic acid followed by esterification with diazomethane to yield the crude methyl ester derivative **XI**. The purification was carried out

on preparative thin-layer chromatography of silica gel, AcOEt - CH_3CN (1: 1) to afford **XI** (4.8 mg). Mass spec: M^+ 687 ($C_{33}H_{61}N_5O_{10}$); IR ($CHCl_3$): 3300, 1750, 1660 cm^{-1}.

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