A Novel Glycopeptide Carrying a 3-Oxazolin-5-one Ring Obtained by Intra-molecular Cyclization

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The structure of a product, isolated during the synthesis of the semisynthetic glycopeptide MDL 63,246, was elucidated on the basis of spectroscopic methods and proved to be a novel glycopeptide containing a 3-oxazolin-5-one ring between positions 36 and 38. Subjected to acid hydrolysis this compound gave the corresponding pseudo aglycone and aglycone derivatives which maintained the original oxazolinone structure. Tested for antibacterial activity, these compounds showed a moderate activity against Gram-positive and inactive against Gram-negative bacteria.

MDL 63,246 (**II**) is a semisynthetic glycopeptide prepared by reacting the 6^B-decarboxy-6^B-hydroxymethyl derivative of the natural glycopeptide A-40926[1] (**I**) with 3,3-dimethylamino-1-propylamine (DMEPA) in the presence of PyBOP[††] as condensing agent (Fig. 2). In the course of some preliminary laboratory trials, carried out to determine the best conditions for this reaction, the formation of a less polar by-product was constantly observed in those runs performed with less than two equivalents of DMEPA. The same compound was subsequently obtained in a 70~75% yield by reacting **I** with PyBOP in the presence of two equivalents of triethylamine (TEA). After isolation, the structure of this compound was elucidated on the basis of a combination of ^{1}H-NMR, ^{13}C-NMR and fast atom bombardament spectroscopies. The results proved that this compound was the novel glycopeptide **III** (Fig. 1) which contains a 3-oxazolin-5-one ring between positions C-36 and C-38.

In this paper we report the synthesis and the structure elucidation of compound **III**, its physico-chemical properties and its two aglycones **IV** and **V** prepared by acidic hydrolysis of **III** in order to simplify the NMR spectra.

Tested for antibacterial activity, compounds **III**, **IV** and **V**, showed a moderate activity against Gram-positive bacteria some of which were clinically isolated resistant to teicoplanin.

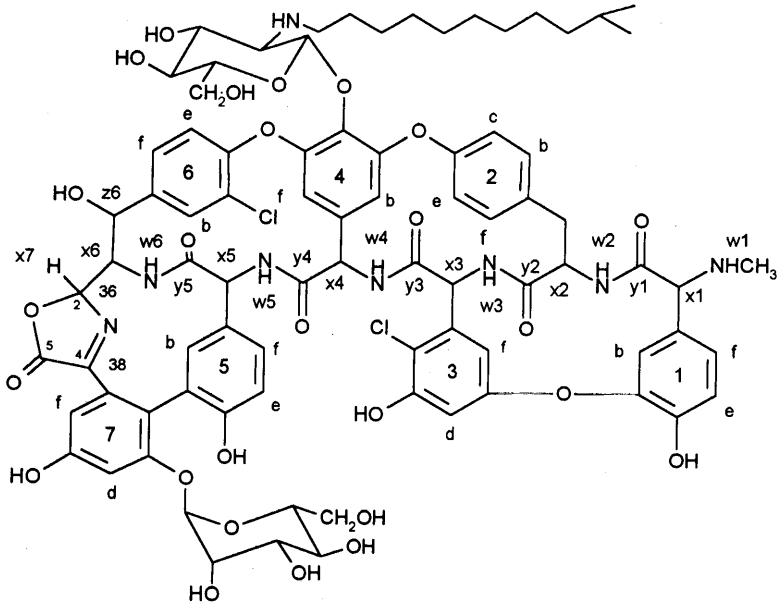
Chemistry

By reacting compound **I** with two equivalents of DMEPA in dimethyl sulfoxide (DMSO) at room temperature in the presence of PyBOP an high yield of compound **II** is usually obtained. In this case, PyBOP reacts with compound **I** to form the corresponding benzotriazolyl ester **Ia** (Fig. 3) which reacting with DMEPA produced the dimethylaminopropylamide **II** (ammonolysis reaction).[2] Gradually reducing the DMEPA/**I** molar ratio from 2 to 0.5, different mixtures of **II** and **III** were obtained in which the amount of compound **III** increased as the amount of DMEPA decreased. About 60% of **III** was obtained when 0.5 equivalents of DMEPA was used.

An explanation of these results can be given assuming that an intramolecular cyclization yielding the oxazolinone ring, gradually starts when less than two equivalents of DMEPA are available for the ammonolysis reaction. In this case, the anion of the enolic form of the 36-amide group (**1b**) cyclized intramolecularly on the C=O of the benzotriazolyl ester producing the 2-oxazolin-5-one ring **IIIa**. This mechanism is analogous to that suggested for

[††] PyBOP is 1-H-benzotriazol-1-yl-oxy tripyrrolidinophosphonium-hexafluorophosphate.

Fig. 1. Structure of compound **III** with proton nomenclature.



The nomenclature is that proposed by D. H. WILLIAMS group[12].

the base-catalyzed racemization process in many peptide activate species[3]. However, in our case the racemization process is incomplete because the intermediate **IIIa** rapidly isomerized into the more stable 3-oxazolin-5-one derivative **III**.

The hydrolysis of **III** in trifluoroacetic acid at room temperature or in diluted mineral acid at 50~60°C gave the pseudo aglycone **IV** and the aglycone **V** maintaining the 3-oxazolin-5-one ring (Fig. 2). The cleavage of the 36~38 amide bond of the oxazolinone ring was possible only in concentrated mineral acids at 50°C where the aglycone **VI** is formed in few hours.

The structure of **VI** was elucidated by DQF-COSY experiments and their attributions resulted in complete agreement with those of a teicoplanin derivative in which the peptide chain was opened between amino acids 6 and 7[4].

Structure Elucidation

The potentiometric analysis of compound **III** performed in methyl cellosolve-water (4:1) and its insolubility in aqueous $NaHCO_3$ solution indicated the absence of any carboxy group. The molecular weight was 1700.5 Daltons, by 18 Da less than the starting product, and the presence of a 1784 cm^{-1} band in the infrared spectrum characteristic of a 3-oxazolin-5-one system[5] (**III**), suggested the presence of an oxazolinone ring.

This value is significantly different from that expected for a 2-oxazolin-5-one system **IIIa** which is around 1835 cm^{-1} [6].

^{1}H-NMR Assignments

Proton resonances have been assigned by homonuclear proton resonance correlation spectroscopy using DQF-COSY[7], TOCSY[8] and NOESY[9] experiments and HMQC[10] spectra for proton carbon correlation *via* 1-J coupling.

The preliminary ^{1}H-NMR analyses of compound **III** clearly indicated the presence of the long fatty acid chain and the sugar moieties.

In Table 2 the proton chemical shifts of compound **V** are compared with those of the A-40926 aglycone portion **VIII**. The proton chemical shifts, similar for the right part of the molecules, are significantly different in the left part where the oxazolinone ring is present. Analysing the DQF-COSY spectrum of aglycone **V**, it appears that one of the seven cross peaks, usually present in the amide proton/alpha proton region, is missing. The six cross peaks observed can be attributed to the residues 1~6 while the signal for the last amino acid (w7/x7) is missing. An additional singlet, not present in the starting compound, that appears in the aromatic region is attributed to the oxazolone proton x7. The attribution of this proton was obtained by a NOESY experiment in which this proton experiences cross relaxation to x6 and z6.

It is noteworthy that the signals corresponding to 5b

Fig. 2. Synthesis of compound **III**, its aglycones **IV**, **V** and compound **VI**.

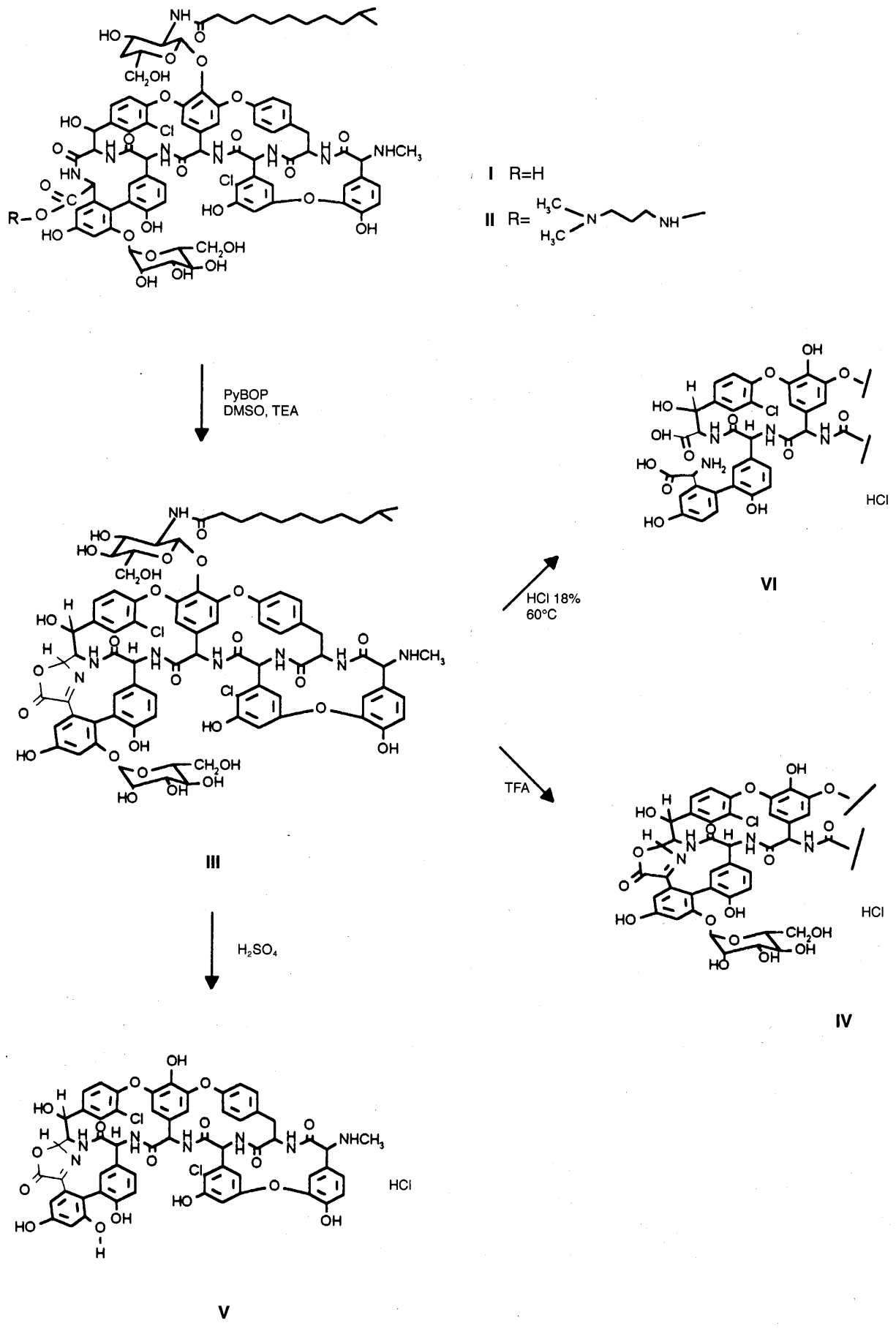


Fig. 3. Mechanism of formation of compound **III**.

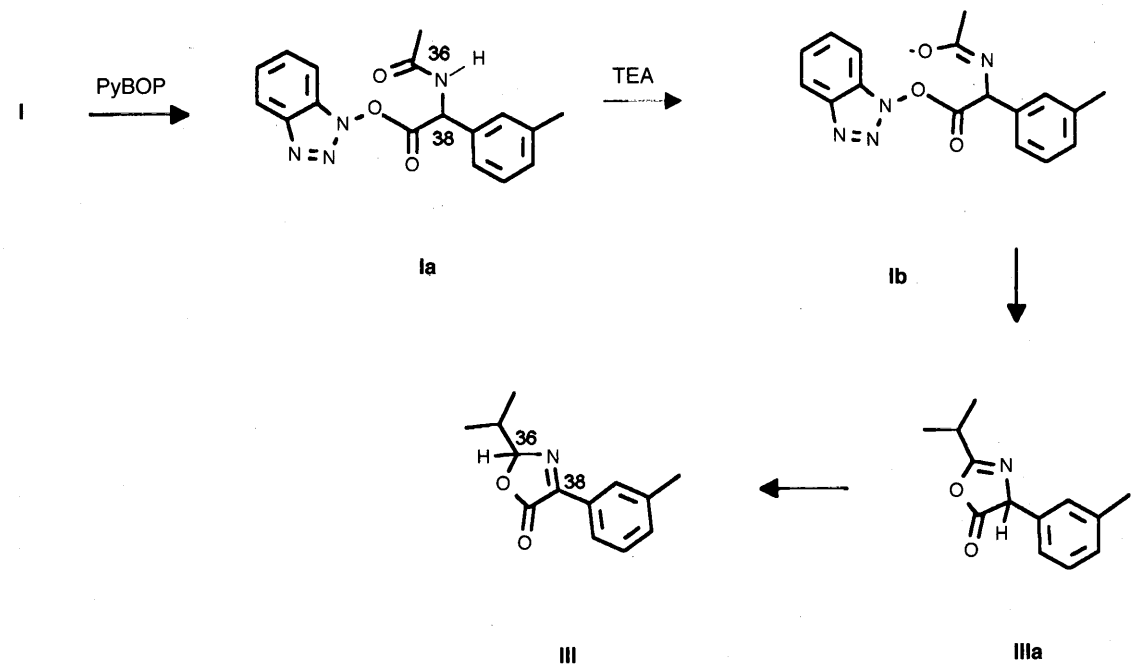


Table 1. Physico-chemical properties of **I**, **II**, **III**, **IV**, and **V**.

Compound		MASS	HPLC	IR absorptions	UV
No.	Formula	*m/z*	Rt (minutes)	(Nujol mull)	max ($E^{1\%}_{cm}$)
I	$C_{83}H_{90}Cl_2N_8O_{28}$	1718.5	14.7	— 1653, 1587	281 (58.75) MeOH
II	$C_{88}H_{102}Cl_2N_{10}O_{27}$	1802.7	19.4	— 1653, 1585, 1065	281 (60.2) Acetic acid 0.1 N
III	$C_{83}H_{88}Cl_2N_8O_{27}$	1700.5	28.9	1784, 1664, 1587, 1040	280 (64.89) MeOH, 280 (56.38) HCl 0.1 N, 305 (101.1) KOH 0.1 N
IV	$C_{65}H_{55}Cl_2N_8O_{22}$	1357.0	21.1	1782, 1672, 1587, 1055	Not determined
V	$C_{59}H_{45}Cl_2N_8O_{17}$	1193.5	22.5	1782, 1663, 1585, 1022	Not determined

and 5f in the molecule containing the oxazolone ring experience a strong upfield shift with respect to the corresponding protons in **VIII**. This shift is interpretable as due to an anisotropic effect of the oxazolinone carbonyl moiety.

These results and the data obtained with the IR and mass spectroscopy analysis confirmed the presence of an oxazolinone ring. This hypothesis is also supported by the presence of an additional signal at 9.40 ppm in the ^{1}H-NMR spectrum of component **V** isolated as hydrochloride which can be attributed to the protonated nitrogen (NH+) of the oxazolone ring.

^{13}C-NMR Assignments

A complete assignment of all carbon resonances of **V** has been achieved by HMQC and HMBC[11] spectra. Table 2 compares the carbon chemical shifts of this compound with those of the A-40926 aglycone portion **VIII**. These data reveal some differences for the carbon resonances of the first residue which can be explained by a different ionization status of the terminal amino function since **VIII** has been investigated in the non-protonated state.

In the carbonyl region two out of the seven expected signals are shifted towards higher field. The five signals between 170.9 and 167.0 ppm can be assigned to y1 ~ y5. The resonance at 164.1 ppm shows heteronuclear long

Table 2. NMR data for compound **V** as hydrochloride (DMSO-d_6 at 259°K) in comparison with those of A-40926 aglycone VIII (DMSO-d_6 at 303°K).

Position	V		VIII		Position	V		VIII	
	$^{13}C^a$	$^1H^a$	$^{13}C^a$	$^1H^a$		$^{13}C^a$	$^1H^a$	$^{13}C^a$	$^1H^a$
w1	—	9.67/9.41	—	—	4f	103.70	5.25	103.70	5.8
X1	62.2	5.54	65.90	5.40	w5	—	8.90	—	8.42
Y1	168.20	—	171.90	—	x5	57.30	5.10	53.60	4.36
1-CH_3	30.80	2.50	34.10	2.36	y5	170.90	—	169.00	—
1a	120.40	—	128.80	—	5a	125.90	—	126.20	—
1b	122.40	7.25	117.20	6.65	5b	132.30	6.33	135.50	7.12
1c	141.70	—	141.50	—	5c	123.80	—	121.10	—
1d	150.10	—	146.90	—	5d	154.60	—	155.50	—
1e	118.20	7.06	118.00	6.92	5e	115.20	6.73	116.50	6.7
1f	127.80	7.25	125.70	7.09	5f	123.20	6.28	125.70	6.7
w2	—	8.71	—	7.49	w6	—	6.61	—	6.68
x2	57.20	4.74	54.40	4.99	x6	52.50	5.42	61.70	4.13
y2	169.00	—	169.20	—	y6/Oxa-C2	99.70	—	167.50	—
z2	36.30	2.99	37.40	2.82	z6	70.50	5.49	71.50	5.44
2a	132.50	—	133.30	—	6a	140.70	—	142.00	—
2b	131.70	6.99	130.70	7.11	6b	127.60	7.42	127.20	7.78
2c	123.00	6.81	124.10	7.21	6c	125.50	—	126.20	—
2d	154.60	—	154.70	—	6d	149.10	—	148.70	—
2e	122.00	7.17	122.60	7.05	6e	124.60	7.27	123.20	7.24
2f	131.10	7.93	131.0	7.66	6f	126.90	7.60	127.20	7.44
w3	—	8.03	—	7.67	w7	—	9.41	—	8.36
x3	55.10	5.81	54.0	6.08	x7/Oxa-C4	164.10	6.41	56.70	4.42
y3	167.00	—	167.10	—	y7/Oxa-C5	163.40	—	172.50	—
3a	137.20	—	138.90	—	7a	132.30	—	136.10	—
3b	112.50	—	113.70	—	7b	117.30	—	117.70	—
3c	154.60	—	154.40	—	7c	155.70	—	156.40	—
3d	105.50	6.71	106.20	6.58	7d	104.90	6.57	102.40	6.4
3e	155.50	—	154.40	—	7e	157.80	—	157.10	—
3f	104.10	6.76	105.50	6.52	7f	107.90	6.44	105.80	6.27
w4	—	6.57		7.43	OH-6	—	6.11	—	—
x4	55.90	4.73	55.0	5.58	OH-1	—	10.00	—	—
y4	168.30	—	170.10	—	OH-3	—	10.63	—	—
4a	124.90	—	127.40	—	OH-4	—	9.40	—	—
4b	106.40	5.8	108.0	5.63	OH-5	—	9.17	—	—
4c	149.30	—	149.30	—	OH-7c	—	9.71	—	—
4d	133.80	—	134.20	—	OH-7e	—	9.87	—	—
4e	149.30	—	147.20	—					

[a] Chemical shifts in ppm.

range correlations in the HMBC spectrum to the protons 7f and x7. Therefore it can be attributed to carbon C4 of the oxazolinone ring. The second carbon at 163.4 does not show any correlation and is assigned to be C5 (small 3J coupling constant to x7). It must be mentioned that also the carbon resonance of x7 does not experience any long range coupling to x6 (2J), z6 or w6 (3J). However, the 3J-coupling between the z6 carbon and the x7 proton is clearly observed and confirms the oxazolinone structure of **III**.

Antibacterial Activity

The biological activity data of compounds **III**, **IV** and **V**, tested against a panel of microorganisms in comparison with teicoplanin, are summarized in Table 3.

All three compounds showed moderate *in vitro* activity against Gram-positive bacteria and resulted inactive against Gram-negative bacteria. In particular, compound **III** showed an appreciable activity against *Streptococcus pneumoniae* UC41 and *S. piogenes* C203 (MIC 0.13 and 0.25 respectively) while compound **V** showed a good activity against isolates of *S. aureus*, *Staphylococcus*

Table 3. Antimicrobial activity of compounds **III**, **IV** and **V** in comparison with teicoplanin[a].

Organism	MIC (μg/ml)			
	III	**IV**	**V**	Teicoplanin
Staphylococcus aureus Tour	0.5	2	0.5	0.125
S. aureus Smith	1	4	n.d.	0.5
S. aureus clin. isolate	16	16	0.5	8
S. epidermidis ATCC 12228	8	8	0.5	0.25
S. epidermidis clin. isolate	8	4	0.5	8
S. haemolyticus clin. isolate	32	32	2	32
Streptococcus pyogenes C203	0.25	4	2	0.125
S. pneumoniae UC41	0.13	4	1	0.063
Enterococcus faecalis ATCC7080	1	4	0.5	0.125
Neisseria gonorrhaeae	>128	>128	>128	>128
Escherichia coli SKF12140	>128	>128	>128	>128
Pseudomonas aeruginosa ATCC105	>128	>128	>128	>128

[a] Minimum inhibitory concentrations (MICs) were determined by broth microdilution using inocula 5×10^5 CFU/ml and incubation at 37°C. Staphilococci and enterococci were grown in Iso-Sensitest broth (Oxoid), streptococci in Todd-Hewitt broth (Difco) and *Neisseria gonorrhoeae* in GC Base broth (Difco) +1% Iso VitaleX (BBL). *N. gonorrhoeae* was incubated for 48 hours in 5% CO_2; all other organisms were incubated for 20~24 hours in air.

epidermidis, and *S. haemoliticus* (MICs 0.5, 0.5 and 2 respectively) which are resistant to teicoplanin.

Experimental

Reactions, intermediates and final products were checked by HPLC analysis.Chromatograms were obtained with a Beckman C8, 5 μ (250 × 5 mm) column using a HP 1090 L liquid chromatograph equipped with a 10 μl injector and a UV detector at 254 nm. The mobile phases were: (A) 0.025 M aq. NaH_2PO_4/CH_3CN in a ratio 95 : 5 (v/v) and (B) CH_3CN. Elution was obtained with a linear gradient of phase B in phase A from 10% to 45% in 25 minutes at the flow rate of 0.9 ml/minute.

IR

The spectra were obtained using a IFS Fourier Transformation Bruker spectrophotometer.

NMR

All the spectra have been recorded on Bruker DRX 600 and AM 500 at 296 K in DMSO-d_6. The data were processed on a Aspect station with the UXNMR software from Bruker. Homonuclear experiments COSY, TOCSY and NOESY were performed with a spectral width of 12 ppm. In all of the experiments, spectra were recorded with 512 increments in t_1 and 4096 complex data points in t_2. For the NOESY 32 transient were averaged for each t_1 value, for COSY and TOCSI 16 transients. Mixing times of 70 or 150 ms were used for TOCSY and NOESY spectra, respectively. For HMQC spectra 512 increments (16 scans) with 4096 complex data points in t_2 were collected using a sweep width of 12 ppm in the proton and 165 ppm in the carbon dimension. The HMBC spectra were acquired with a sweep of 12 in the proton and 200 ppm in the carbon dimension. A total of 48 transients were averaged for each of 512 increments in t_1, and 4096 complex points in t_2 were recorded. A delay of 70 ms was taken for the development of long range correlations.

Mass

MALDI-TOF spectra were acquired on a Kompact MALDI III by KRATOS (Manchester UK) operating in positive linear mode. Ions formed by a pulsed ultraviolet (UV) laser beam (nitrogen laser = 337 nm) were 20 KeV of kinetic energy.

The resolution in the linear mode for this kind of compounds is R = 270 M/dM, with a mass accuracy of 0.04%. This accuracy is based on the calibration of the instrument at the start of each measurements with the singly charged protonated molecular ions produced from a standard mixture of dihydroxybenzoic acid (DHB), the undecapeptide substance P (MW 1347.6) and MDL 63,246 used as reference substance.

General Procedure for the Synthesis of Mixtures of MDL 63,246 (II) and III

To a solution containing 1 mmole of compound **I** in 20 ml of DMSO, 0.5~2 mmoles of DMEPA were slowly added with stirring at room temperature. After 30 minutes a solution of 1.4 mmoles of Py-BOP in 5 ml of DMSO, was dripped into the mixture. Stirring was continued for an additional hour then the mixture was poured on a ten fold volume of stirred ethyl acetate and the solid obtained was filtered and dried under vacuum at 45°C. Yields from 1.5 to 50% of **III** were obtained varying the DMEPA/I molar ratio from 2 to 0.6 respectively.

Preparation of Compound III with TEA and PyBOP

A 250 ml round bottomed flask was charged with 80 ml of DMSO, 8 g of compound **I** (assay 83%; 3.9 mmoles) and 1.15 ml of triethylamine (8.2 mmoles). The mixture was stirred at room temperature for 10 minutes then a solution of 2.7 g of PyBOP (5.1 mmoles) in 10 ml of DMSO was slowly added. Stirring was continued for additional 1.5 hours then the mixture was poured in 600 ml of ethyl acetate. The suspension obtained was stirred for 30 minutes then the white solid was filtered and dried at room temperature yielding 9.2 g of crude compound **III**.

Purification of Compound III

A portion of 1 g of crude **III** was purified by preparative HPLC using a Lichrosphere RP 8, 5 μ, 250 × 25 mm Merck column. Elution was obtained with a linear gradient from 10% to 60% of phase B (0.02 M aq. ammonium formate - acetonitrile 90 - 10) in phase A (0.02 M aq. ammonium formate - acetonitrile 90 - 10) at a flow rate of 3 ml/minute.

About 40 ml solution, containing the purified compound **III**, was collected after 19 injections. The solvent was evaporated and the white solid obtained was washed with 10 ml of distilled water and lyophilized yielding 90~100 mg of pure title compound.

Preparation of the Pseudo Aglycone IV

A solution of 2 g of crude **III** in 20 ml of trifluoroacetic acid (TFA) was stirred overnight at room temperature and then was poured in 200 ml of water. The white solid obtained was filtered off and dried yielding 1.3 g of crude **IV**. This material was chromatographated on silanized silicagel (200 g) using a linear gradient from 10% to 30% of acetonitrile in water previously adjusted at pH 2.5 with 10% hydrochloric acid. The fractions containing the title compound were pooled and the solvent was removed under vacuum at 40°C. The residual aqueous solution was lyophilized yielding 560 mg of white solid **IV** as hydrochloride.

Preparation of the Aglycone V

A solution of 1 g of crude **III** in 20 ml of methanol and 0.3 ml of H_2SO_4 was refluxed at 60°C for 3 hours. After cooling the mixture was diluted with water (20 ml) and THF (10 ml) and chromatographated on silanized silicagel (200 g) eluting the column with a linear gradient from 10% to 30% of methanol in water previously adjusted at pH 2.5 with 10% hydrochloric acid. The fractions containing the title compound were collected and evaporated under vacuum at 45°C obtaining a white solid residue that was crystallized from MeOH/ethyl ether affording 300 mg of pure **V** as hydrochloride.

Opening of the Oxazolinone Ring—Preparation of Compound VI

A solution of 1 g of crude **III** in 20 ml of 18% hydrochloric acid was heated at 60°C for 22 hours. After cooling the mixture was diluted with 50 ml of water and chromatographated on silanized silicagel (150 g) eluting the column with a 1:9 MeOH - H_2O mixture. The fractions containing the title compound were collected, evaporated under vacuum obtaining a white solid residue that was dissolved in MeOH (10 ml) and treated with an excess of ethyl ether to give 25 mg of pure **VI** as hydrochloride.

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