Novel Teicoplanins by Directed Biosynthesis

AMERIGA LAZZARINI, ANGELO BORGHI, LUIGI FRANCO ZERILLI, PIETRO FERRARI, LUIGI COLOMBO and GIAN CARLO LANCINI

Lepetit Research Center,
Via R. Lepetit 34, I-21040 Gerenzano (Varese), Italy

(Received for publication August 2, 1996)

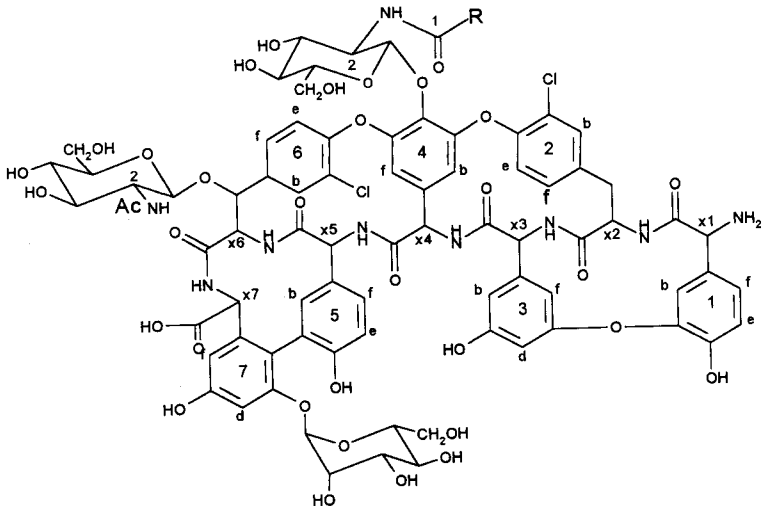
Teicoplanin[1], a clinically useful glycopeptide antibiotic[2], is produced by *Actinoplanes teichomyceticus* as a complex of five main components, denoted factors A2-1 to A2-5, differing in the structure of their acyl side chain, which is linear in A2-1 and A2-3 and branched in the other components[3]. In a previous paper[4] it was shown that the acyl moieties of the teicoplanin complex originate from stepwise shortening, through the usual β-oxidation mechanism, of long chain fatty acids constituting the membrane of *A. teichomyceticus* cells[4]. In fact there is a clear correspondence between the relative amount of a given fatty acid in the cell membrane and the amount of the teicoplanin component having as side chain the structurally related branched or linear acid. For instance, the amount of component A2-3, characterized by a linear C10:0 acyl chain, depends on the amount of oleic acid, whereas the production of A2-1, characterized by a linear C10:1 acyl moiety, is entirely dependent on the addition of linoleate to the medium. This suggested the possibility of obtaining new members of the teicoplanin family by feeding *A. teichomyceticus* cultures with different eighteen carbons fatty acids having specific structural features. Our assumption was that these acids would be shortened (starting from the carboxyl group) to the proper length, *i.e.* ten carbons, and condensed to the core molecule.

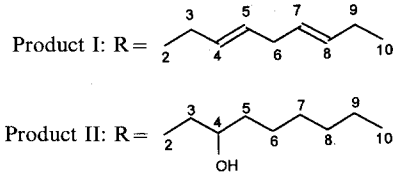
Therefore, it could be predicted that addition of linolenic acid (which is a linear eighteen carbon chain bearing double bonds in the 9th, 12th and 15th positions) would give a teicoplanin having an acyl moiety characterized by two double bonds in the 4th and 7th positions, and that ricinoleic acid (which is a linear eighteen carbon chain bearing a double bond in the 9th position and a hydroxy group in the 12th position) would give a teicoplanin having an acyl moiety characterized by a hydroxyl in the 4th position. The preparation and physico-chemical characterization of these two novel teicoplanins bearing a 4,7-decadienoic acyl moiety (I) and a 4-hydroxy-decanoic acyl moiety (II) are reported hereby.

The fermentation strain was *Actinoplanes teichomyceticus* ATCC31121. A frozen stock culture of *A. teichomyceticus* was added to 500-ml Erlenmeyer flasks containing 100 ml of seed medium composed of 1% glucose, 0.4% Bacto-peptone (DIFCO), 0.4% Bacto yeast extract (DIFCO), 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% KH_2PO_4 and 0.4% K_2HPO_4 in distilled water. After incubation at 28°C for 48 hours on a rotary shaker at 200 rpm (5 cm throw) 200 ml of the seed culture was used to inoculate 4 liters of production medium C in a 5-liter jar. Medium C composition: 2% glucose (sterilized separately), 0.5% yeast extract, 0.15% asparagine, 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% $CaCO_3$, 0.01% NaCl, 0.01% $CaCl_2 \cdot 2H_2O$, 1 ml/liter standard mineral solution. Standard mineral solution (g/liter): boric acid (0.5), $CuSO_4 \cdot 5H_2O$ (0.04), KI (0.1), $FeCl_3 \cdot 6H_2O$ (0.2), $MnSO_4 \cdot H_2O$ (0.4), $FeSO_4 \cdot 7H_2O$ (0.4) and ammonium molybdate (0.2).

The jar was maintained at 28°C with stirring and aeration at 1/2 vol/vol/minute 24 hours after inoculation either of 4 g/liter Linseed oil (a triglyceride containing 50% linolenic acid (*cis*-9, *cis*-12, *cis*-15-octadecatrienoic acid), 30% oleic acid (*cis*-9-octadecenoic acid), and 20% linoleic acid (*cis*-9, *cis*-12-octadecadienoic acid) in the case of I, or of 4 g/liter Castor oil (a triglyceride

Fig. 1. Structures of products I and II.





containing 87% ricinoleic acid (*d*-12-hydroxy-*cis*-9-octadecenoic acid and 13% of other fatty acids) in the case of II, was added, and fermentation was continued for a further 72 hours. The fermentation broths were filtered on buchner.

The filtrate from each jar was assayed by HPLC for the presence of new peaks in the teicoplanin complex. Analytical HPLC was performed under the following conditions:

Column: Erbasil C18, 5 μm. 150 × 4.6 mm i.d.
Mobile phase: A, 0.2% HCO_2NH_4/CH_3CN (9/1)
B, 0.2% HCO_2NH_4/CH_3CN (3/7)
Gradient: %B: 0.5 ~ 55 linear in 32 minutes
Flow: 1.5 ml/minute
Detector: UV, 254 nm.

In addition to the peaks of teicoplanin components, peaks were observed having relative retention times of about 0.80 (21.4 minutes) in the case of I, or of about 0.54 (12.0 minutes) in the case of II, with respect to that of the main component of teicoplanin, T-A2-2 (26 minutes). On the basis of peak areas, the filtrates contained about 1 g of the new product in the case of I or 7 g in the case of II.

The filtered broths were loaded on an affinity chromatography column prepared with 1.5 liters of D-Ala-D-Ala affinity resin, prewashed with 1% NH_4OH (1 vol.) and H_2O (2 vol.); the elution was carried out with 1% NH_4OH. The column was washed with 1% ammonium formate (pH 6), followed by 0.6% Tris base solution (1 vol.) and eluted with 1% NH_4OH. Various fractions were obtained at different pH cuts, containing different percentages of the new peaks relative to the entire lipoglycopeptide content. The appropriate portions were pooled and loaded onto columns of 60 g of silanized silica gel 60 (Merck) prewashed with CH_3OH and H_2O. A gradient elution was performed by gradually mixing 1 liter of a solution of 5% CH_3CN-0.2% ammonium formate with 1 liter of a 40% CH_3CN-0.2% ammonium formate solution. The columns were washed with 1 liter of 0.2% ammonium formate and eluted under the previously defined gradient conditions. The first portion of the eluent (0.3 liter for I and 1.5 liters for II) was discarded and the fractions containing the product (10 fractions of 10 ml for I and 8 fractions of 15 ml for II) were separately collected.

The two fractions of each product with the highest content of the new peak were pooled and ultrafiltered on a 1000 MW globular protein exclusion limit membrane and the retentate was lyophilized. The obtained products (20 mg of I and 90 mg of II), in which the new substances amounted to about 85% of the total lipoglycopeptide content, were considered sufficiently pure for structure determination.

Structure Determination of 4,7-Decadienoyl-teicoplanin (I)

All the ESI-MS spectra were recorded on an API III+ triple quadrupole mass spectrometer (PE-Sciex, Thornhill, ON, Canada) equipped with an articulated ion spray interface. Tuning and calibration were performed on both the first (Q1) and the third quadrupole using a solution of polypropylene glycols (PPG's) in 3 mM ammonium acetate. Samples were infused *via* a 75 μm i.d. fused silica capillary tubing to the ion spray tip which was held at a potential of +5.5 kV. A syringe pump (Model 22, Harvard Apparatus, MA, U.S.A.) controlled the delivery of the sample at a rate of 5.0 μl/minute. Zero grade compressed air was used as nebulizer gas (pressure set at 55 psi). A curtain gas (99.999% UHP nitrogen) flow of 0.8 liter/minute was employed. The interface heater was set at 60°C. Mass spectra were obtained at a dwell time of 1.00 msec (Q1 scan range 400 ÷ 2000 u, 10 scans averaged) and a step size of 0.10 u. The orifice voltage was maintained at 70 V. In the ESI-MS spectrum of (I) together with the ion corresponding to the protonated molecule ($[M+H]^+$, *m/z* 1874.6 and $[M+2H]^{++}$, *m/z* 937.8) some other fragment ions, corresponding to the losses of the acylglucosamine and acetylglucosamine moieties, are present.

FAB-MS spectrum was recorded on a Finnigan MAT 8430 double focusing mass spectrometer. Experimental conditions: 3 kV Xe atoms at 1 mA current; matrix: thioglycerol/glycerol (1/1, v/v); sample dissolved in methanol and added to the matrix on the target. In the FAB-MS spectrum the isotopic clusters of the quasi-molecular ions $(M+Na)^+$ and $(M+H)^+$ indicate 1896.7 and 1874.7 as the masses of the lowest mass isotopes. These values are 4 mass units smaller than those of teicoplanin A2-2[5].

^1H NMR spectra were recorded in DMSO-d_6 solution at 500 MHz with a Bruker AM-500 spectrometer equipped with an Aspect 3000 computer. COSY spectrum was obtained in the phase sensitive mode, double quantum filter.TOCSY experiment was based on Homonuclear Hartmann-Hann transfer with mixing by composite pulse cycle using inverse and phase sensitive mode (TPPI) optimized for long range couplings. ^1H NMR spectral assignments are reported in Table 1. The attributions of the majority of the H-atoms were done on the basis of the 2-D experiments and by comparison with the known data for teicoplanin[5,6]. Whereas the aglycone appears unchanged, differences are observed in the acyl moiety. The terminal group of the aliphatic chain is a CH_3, as shown by a triplet at 0.90 ppm. This CH_3 is attached to a CH_2 shown by a multiplet at 1.99 ppm which, as deduced from the COSY spectrum, is near an ethylenic double bond on the basis of its chemical shift and its coupling to a proton, whose signal is at about 5.2 ppm. Furthermore a CH_2 indicated by a multiplet at 2.65 ppm must be placed between this double bond and a second double bond. Again, on the basis of coupling and chemical shift criteria, a CH_2 at 2.08 ppm is placed between the second double bond and the CH_2 at 2.19 ppm, which is in the α position with respect to the amidic C=O. The ^1H NMR and COSY results are thus in accordance with a teicoplanin-like molecule, with the

Table 1. Assignments of the main signals of the ^1H NMR spectrum of product I.
(Numbering system of Fig. 1)

Proton	Multiplicity	δ (ppm)
10 CH_3	t	0.90
9 CH_2	m	1.99
8 =CH	m	5.33
7 =CH	m	5.22
6 CH_2	m	2.67
5 CH	m	5.32
4 CH	m	5.28
3 CH_2	m	2.19
2 CH_2	m	2.12
Acetyl group of glucosamine	s	1.86
C2-H of acetyl glucosamine	m	3.37
Anomeric H of acetylglucosamine	d	4.37
NH of acetylglucosamine	d	7.90
C2-H of acylglucosamine	m	3.76
Anomeric H of acylglucosamine	d	5.30
Anomeric H of mannose	s	5.24
z_2, z'_2	m	2.83, 3.27
x_6	m	4.10
x_5, x_7	d	4.42~4.32
x_2	m	4.81
z_6	s	5.35
4b	s	5.58
4f	s	5.12
x_3	d	5.28
x_4	d	5.70
7f	s	6.32
7d	s	6.46
6b	s	7.77

s: Single, d: doublet, m: multiplet.

Table 2. Assignments of the main signals of the ^1H NMR spectrum of product II.
(Numbering system of Fig. 1)

Proton	Multiplicity	δ (ppm)
10 CH_3	t	0.85
9 CH_2/5 CH_2	m	1.25/1.13
4 CH(OH)	m	3.25
3 CH_2	m	1.46
2 CH_2	m	2.01, 2.19
Acetyl group of glucosamine	s	1.89
C2-H of acetylglucosamine	m	3.37
Anomeric H of acetylglucosamine	d	4.36
NH of acetylglucosamine	d	8.04
C2-H of acylglucosamine	m	3.76
Anomeric H of acylglucosamine	d	5.25
Anomeric H of mannose	s	5.25
z_2, z'_2	m	2.85, 3.27
x_6	m	4.10
x_5, x_7	d	4.37
x_2	m	4.85
z_6	s	5.33
4b	s	5.58
4f	s	5.12
x_3	d	5.27
x_4	d	5.67
7f	s	6.28
7d	s	6.47
6b	s	7.80

s: Single, d: doublet, m: multiplet.

normal acyl chain substituted by a 4,7-decadienoic group.

Structure Determination of 4-Hydroxydecanoyl-teicoplanin (II)

In the ESI-MS spectrum of (II) together with the ion corresponding to the protonated molecule ($[M+H]^+$, m/z 1894.4 and $[M+2H]^{++}$, m/z 947.7) some others fragment ions corresponding to the losses of the acylglucosamine and acetylglucosamine moieties are present.

FAB-MS spectrum was recorded on Kratos MS50 double focusing mass spectrometer. Experimental conditions: 6 kV Xe atoms at 1 mA current; thioglycerol matrix acidified with 0.1 N CH_3COOH; sample dissolved in methanol and added to the matrix on the target. In the FAB-MS spectrum the isotopic clusters of the quasi-molecular ions $(M+Na)^+$ and $(M+H)^+$ indicate 1916.7 and 1894.7 as the masses of the lowest mass isotopes. These values are 16 mass units larger than those of teicoplanin A2-2[5].

^1H NMR and ^1H-^1H COSY spectra were obtained under the same experimental conditions as above. Selected spectral attributions of the H-atoms done on the basis of ^1H-^1H COSY results and by comparison with the known data for teicoplanin[5,6] are reported in Table 2. Whereas the aglycone portion of the molecule appears unchanged, differences are observed in the acyl moiety. The aliphatic chain is linear as the terminal group is a CH_3 which gives rise to a triplet at 0.85 ppm in the ^1H NMR spectrum. The integral values for the ^1H NMR signals of the acyl chain fit a decanoyl chain. In the ^1H-^1H COSY spectrum a signal at 1.46 ppm due to the CH_2 which is in beta position to the carbonyl of the acyl chain shows a coupling with the adjacent CH_2 in alpha to the acyl carbonyl and also with a proton attributable to a CH–OH group, on the basis of the chemical shift value of 3.25 ppm. The CH–OH in turn appears to be coupled with another CH_2 whose signal is located at 1.25 ppm, in the region of the remaining CH_2 of the chain. The ^1H NMR and COSY results are thus in accordance with a teicoplanin-like molecule, where the normal acyl chain is constituted by a 4-OH decanoic group.

It was observed that the complex obtained after the addition of Linseed oil to the fermentation medium is modified in composition with respect to the standard teicoplanin complex. In particular, by HPLC analysis, in addition to the formation of I, an increase has been noticed in the amounts of components TA-2-1 and TA-2-3, relatively to component TA-2-2. This is explained by the presence in Linseed oil triglyceride of 20% linoleic acid and 25% oleic acid, which are already known[4] to give origin to TA-2-1 and TA-2-3, re-

spectively. On the contrary, the composition of teicoplanin complex did not change after addition of Castor oil but, in addition to the new peak corresponing to II, some small peaks have been noticed in the HPLC profile due to the presence of other fatty acids in Castor oil. Both in the cases (addition of Linseed oil or Castor oil), the formation of the new products I or II did not depress the amount of teicoplanin complex produced.

The new teicoplanin-like complexes obtained by adding either linseed oil or Castor oil to the fermentation broths, further confirm that the acyl moiety of teicoplanins derives from the shortening of fatty acids having 16~18 carbon chains. It also suggests that other teicoplanins modified in the acyl chain might be obtained by addition of suitable long chain fatty acids.

The antibacterial activity of the two new complexes obtained resembles that of the teicoplanin complex.The new teicoplanin-like compounds I and II are somewhat less active than the teicoplanin complex against all Gram-positive strains tested and, like teicoplanin, are inactive against *E. coli* and *P. aeruginosa*.

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