AN UNEXPECTED INTRAMOLECULAR CYCLIZATION OF ISOTHIOURONIUM TEICOPLANINS

II. REACTION MECHANISM AND BIOLOGICAL ACTIVITY

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N^{15}-Isothiouronium derivatives of teicoplanin and its aglycone submitted to alkaline condition give rise to an intramolecular cyclization. The structures of the new γ-lactam derivatives were determined by using ^{1}H NMR, IR and fast atom bombardment mass spectra. The cyclization mechanism was interpreted on the basis of the identification of the intermediate structure.

The poor *in vitro* antibacterial activity of the new cyclic compounds and the negligible affinity for the synthetic peptidoglycan model Ac_2-L-Lys-D-Ala-D-Ala is probably due to the lack of the N-17 amidic proton and to the lack of the basic character of the nitrogen in position 15.

Teicoplanin (Targocid) is a new glycopeptide antibiotic[1] recently introduced in some countries for the treatment of severe Gram-positive bacterial infections.[2]

Teicoplanin (CTA) consists (Fig. 1) of five major closely related factors (A2/1~5) differing in the *N*-acyl chain linked with β-D-glucosamine at position C-56. CTA also contains one α-D-mannose and one *N*-acetyl-α-D-glucosamine at C-42 and C-34 positions, respectively.[1] All three sugars can be removed by acidic hydrolysis under selected conditions obtaining the related aglycone (TD) (Fig. 1).[3]

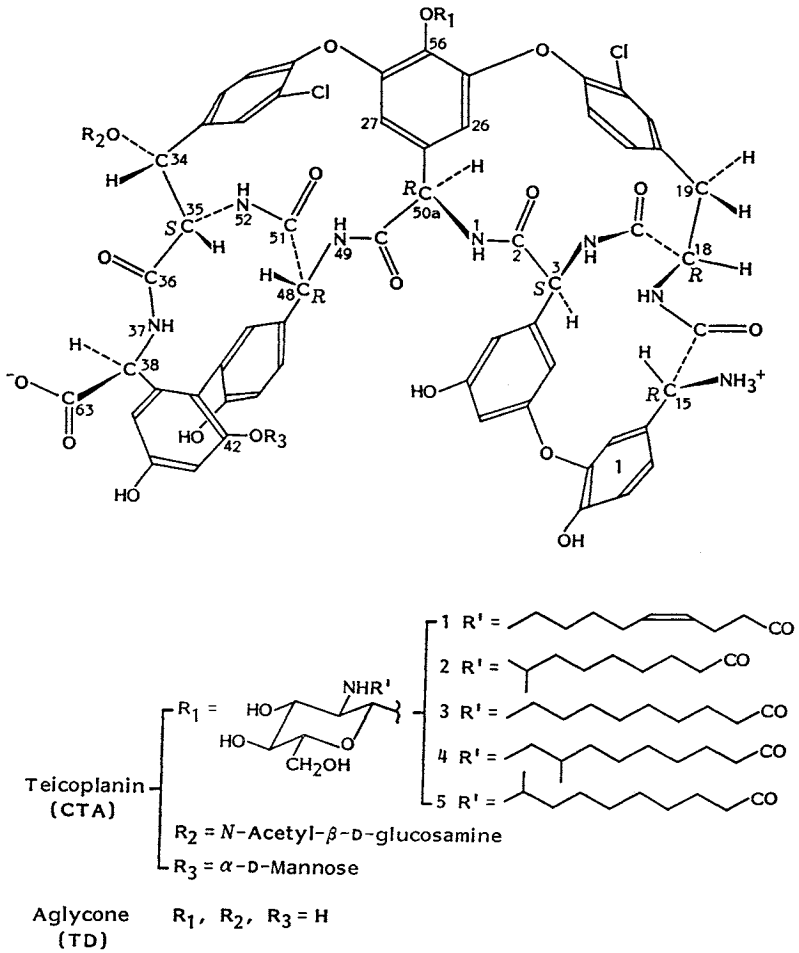
In a program of synthetic modification directed to extend the antibacterial activity of CTA and TD against Gram-negative bacteria, it was considered of interest to introduce the guanidine group into the structure of the above compounds to verify if the positively charged guanidine derivatives can penetrate the cell-wall of Gram-negative microorganisms better than the parent compound. In the literature are reported several natural antibiotics containing a guanidine unit (pK=13.6)[4] showing some activity against Gram-negative bacteria. Streptomycin[5] is the best example of a guanidine carrying antibiotic active against Gram-negative bacteria.

Chemistry

The displacement of methylmercaptane from an isothiouronium salt is a well-known method for preparing guanidine.[6] Therefore, some previously described[7] isothiouronium derivatives of CTA and TD (**I**, Scheme 1) were selected for their transformation into differently substituted guanidine derivatives (**II**). Surprisingly, by utilizing different primary amines, instead of the expected compounds **II**, compounds **IV**, carrying a γ-lactam ring between C-15 and C-16, were obtained. The new structures were determined by ^{1}H NMR, IR and fast atom bombardment mass spectra (FAB-MS) spectroscopies.

By reaction of isothiouronium derivatives of TD, for example **Ic**, with primary amines (methylamine and ethylenediamine) in MeOH the formation of two peaks, **IIIc** and **IVc**, were detected by

Fig. 1. Structures of the components of teicoplanin (CTA) and the aglycone (TD).



HPLC (Fig. 2) and by heating up to 40°C a rapid transformation of **IIIc** into **IVc** was obtained. The formation of the two peaks was independent of the amine used. The same result was obtained using triethylamine (TEA) in MeOH. When the reaction was carried out in CH_3CN - H_2O - TEA only peak **IIIc** formed. This compound was isolated and quantitatively transformed into peak **IVc** by dissolution in MeOH at 40°C.

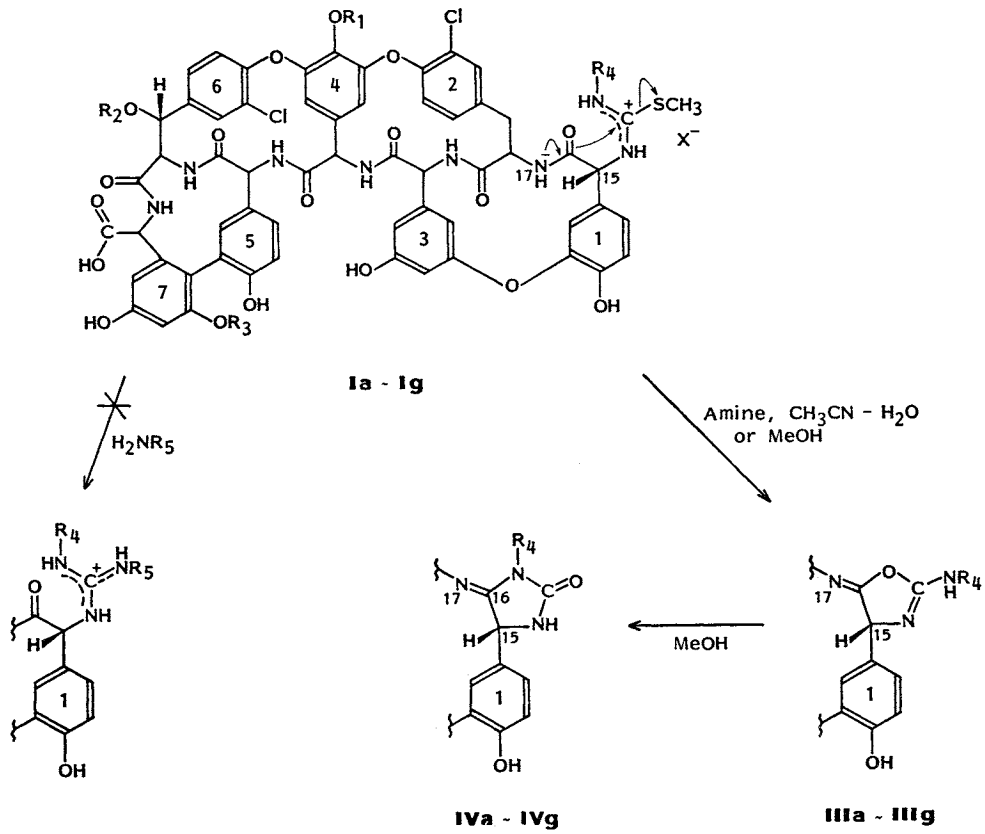
Starting from **Id** the corresponding intermediate compound **IIId** was also isolated. Compounds **IV**, obtained from the corresponding CTA or TD derivatives (**Ia**~**Ig**), showed a decreased solubility in acidic water as compared with that of the starting antibiotics, as expected due to the reduced basicity of C(15)-N.

Structure Determination

The IR spectra of compounds **IVa**~**IVg** showed an absorption in the range of 1730~1740 cm^{-1}, attributable to the C=O stretching of a γ-lactam ring. The frequency is higher by 10~20 cm^{-1} than a five member cyclic urea and is due to the electron withdrawing character of the exo-cyclic double bond C(16)=N(17).

FAB-MS spectrometry shows the MW's of the synthesized compounds (Table 1) to be in accor-

Scheme 1.



II $R_5 = CH_3$, $CH_2CH_2NH_2$

	R_4	Starting compound
a	CH_3	CTA
b	C_2H_5	CTA
c	H	TD
d	CH_3	TD
e	C_2H_5	TD
f	$CH_2C_6H_5$	TD
g	C_6H_{11}	TD

dance with the proposed structure.

The selected ^1H NMR spectral attributions, reported in Table 2, were based on the comparison with data for teicoplanin and other glycopeptides,[3] on selective decoupling and 2D correlation spectroscopy (COSY) experiments. The structure of compounds **IV** is well characterized by their ^1H NMR spectra in comparison with parent compounds. In particular, the cyclic ureide linked to C-15 and C-16 is evidenced by the disappearance of 17-H signal, which was involved in the isoamidic equilibrium N(17)H-C(16)O↔N(17)=C(16)OH necessary for the ring closure, and the significant change in chemical shifts of the protons

Fig. 2. Reversed-phase HPLC profile of the reaction.

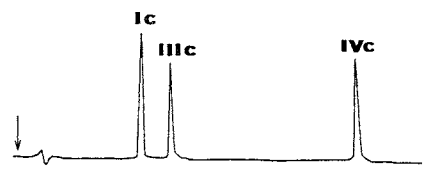
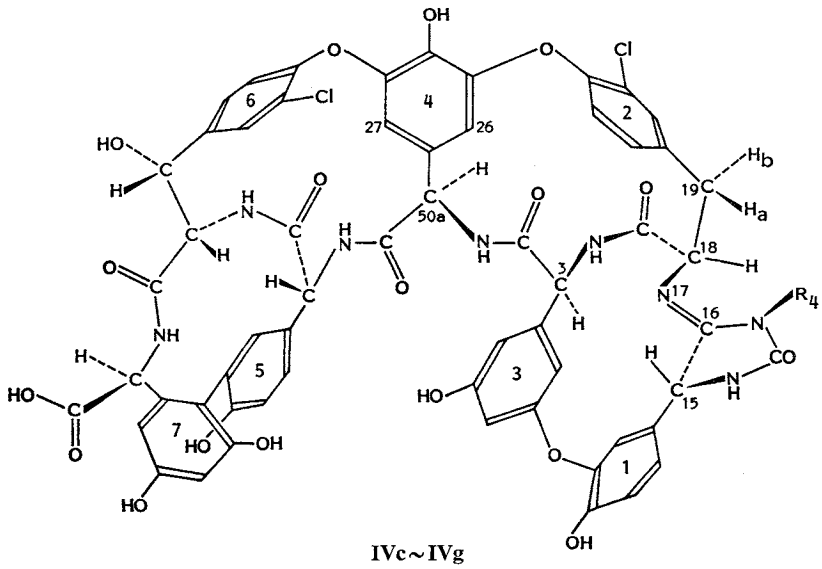


Table 1. Analytical data.

Compound	IR[a] (C=O, cm^{-1})	Formula	MW	FAB-MS $(M+H)^+$	Method	HPLC retention time (minutes)	
						Isothiouronium salt[c]	Reaction product
IIIc	—	$C_{59}H_{44}N_8O_{18}Cl_2$	1,223.95	nd	B	4.0	4.4
IIId	—	$C_{60}H_{46}N_8O_{18}Cl_2$	1,237.97	1,237	B	5.2	6.5
IVa	1735	$C_{90}H_{98}N_{10}O_{33}Cl_2$[b]	1,918.71	1,940.5[d]	A	12.5	19.0
IVb	1735	$C_{91}H_{100}N_{10}O_{33}Cl_2$[b]	1,932.74	1,953.4[d]	A	14.3	20.6
IVc	1730	$C_{59}H_{43}N_7O_{19}Cl_2$	1,223.95	1,223.95	A	4.0	4.6
IVd	1740	$C_{60}H_{46}N_8O_{18}Cl_2$	1,237.97	1,238	A	5.2	14.5
IVe	1738	$C_{61}H_{48}N_8O_{18}Cl_2$	1,252.0	1,251.3	A	7.2	17.0
IVf	1735	$C_{66}H_{50}N_8O_{18}Cl_2$	1,314.07	1,313.3	A	14.6	21.5
IVg	1730	$C_{65}H_{54}N_8O_{18}Cl_2$	1,306.09	1,305	A	17.6	24.5

[a] Determined in Nujol mull. [b] Formula of factor 2 of CTA. [c] Starting material I. [d] M+Na.
nd: Not determined.



IVc~IVg

Table 2. Assignments of the signals of the ^1H NMR spectra[a] of compounds **IV** in comparison with teicoplanin aglycone (TD) hydrochloride.

	TD·HCl	IVc	IVd	IVe	IVf	IVg
R_4		nd	2.91 (CH_3)	1.10 (CH_3) 3.20 (CH_2)	4.63, 4.78 (CH_2) 7.24 (C_6H_5)	1.35, 1.68 ($(CH_2)_5$) 3.99 (CH)
15-H	5.47	5.09	4.85	4.84	4.98	4.72
18-H	4.92	4.66	4.34	4.42	4.45	4.36
19-H_a	2.87	3.09	2.70	2.75	2.60	2.78
19-H_b	3.35	3.47	3.15	3.19	3.15	3.16
3-H	5.35	5.56	5.42	5.38	5.34	5.10
50a-H	5.60	5.74	5.54	5.54	5.54	5.42
26-H	5.50	5.45	5.30	5.39	5.40	5.41
27-H	5.08	5.32	5.28	5.30	5.31	5.20

[a] Recorded in DMSO-d_6 solution with TMS as internal reference (δ 0.00) with a 250 MHz Bruker spectrometer equipped with an Aspect 3000 computer.
nd: Not determined.

Table 3. *In vitro* activity.[a]

	MIC (μg/ml)									
	CTA	IVa	IVb	TD	IVc	IVd	IVe	IVf	IVg	IIId
Staphylococcus aureus TOUR	0.125	4	4	0.06	0.5	8	2	1	4	1
S. aureus TOUR +30% bovine serum	0.5	32	32	0.25	8	128	32	32	128	32
S. epidermidis ATCC 12228	0.12	2	2	0.01	0.12	2	1	0.5	2	0.5
Streptococcus pyogenes C 203 SKF 13400	0.06	0.25	1	0.06	2	32	2	2	8	2
S. pneumoniae UC 41	0.06	2	1	0.06	1	16	4	1	4	2
S. faecalis ATCC 7080	0.12	2	4	0.12	2	64	8	4	16	4

[a] See the Experimental section.

from 15-H to 27-H. This variation is interpreted as being due to the insertion of a C=N and a new ring into the core of the molecule, a fact which causes a steric rearrangement involving various anisotropic effects.

The formation of **IV** can be explained with the formation under alkaline conditions of the base of isothiourea (**I**), less stable than the corresponding salt, followed by the nucleophilic displacement of CH_3SH by participation of the enol-amide (N(17)=C(16)OH) through an intramolecular reaction favored with respect to the intermolecular reaction with an amine (Scheme 1). The oxazolinic structure **III** is stable in CH_3CN or CH_3CN - H_2O while it rearranges in the presence of MeOH to give structure **IV**. The only structural requirement investigated for obtaining this cyclization is the nature of substituent R_4. In fact, when R_4 is phenyl or *p*-F-phenyl, cyclization does not occur.

Antibacterial Activity

Compounds **IV** which lack the two requisites believed essential for the activity of glycopeptide antibiotics, (*i.e.*, the basic character of C(15)-N and the amidic proton N(17)-H, important for the formation of the complex with the carboxyl group of the dipeptide D-Ala-D-Ala terminal of the muramylpentapeptide)[8~11] show a poor *in vitro* antibacterial activity.

Compounds **IIId** and **IVa**~**IVg** were assayed for antibacterial activity against selected microorganisms. All the compounds tested show a negligible activity on Gram-positive with MICs ten times or higher than the reference compounds CTA and TD (Table 3), while they result to be inactive against the Gram-negative bacteria. Compound **IIIc** was tested because of the general poor activity of these cyclic derivatives.

Binding Studies

The binding strengths (K_A) to the synthetic peptidoglycan analogue Ac_2-L-Lys-D-Ala-D-Ala of compounds **IIId** and **IVa**~**IVg** were measured by UV differential spectroscopy.[12] The experiments were run in phosphate buffer at pH 9 due to the insolubility of the compounds in the generally used citric buffer at pH 5. All the measurements indicated a weak binding strength ($K_A < 10^3$).

Conclusion

The isothiouronium salts of CTA and TD (**I**) undergo an intramolecular cyclization in the presence of amines instead of giving the expected guanidine derivatives **II** (Scheme 1). The pathway of this reaction, producing a γ-lactam ring between position 15 and 16 (**IV**) of parent antibiotics, has also been verified with the isolation and identification of the oxazoline intermediate (**IIIc** and **IIId**). The new structures **IIId** and **IVa**~**IVg** show a low affinity constant for Ac_2-L-Lys-D-Ala-D-Ala and a

negligible activity against Gram-positive bacteria, as a predictable consequence of the modifications, *i.e.*, the absence of the protonable C(15)-N amino group and the loss of N-17 proton. This latter seems to be essential in participating in the complex formation with the terminal carboxyl group of the muramylpentapeptide.[10]

Experimental

Evaporation of solvents was carried out, after adding BuOH to prevent foaming, with a rotary evaporator at 45°C under vacuum. If not otherwise stated, the intermediates and the final products were washed with Et_2O and dried at 50°C under vacuum.

Products were purified by reversed-phase column chromatography on silanized silica gel (0.063 ~ 0.2 mm, Merck) by eluting with a linear gradient of CH_3CN in water.

Monitoring of the reactions and the chromatographic fractions and control of the purity of the compounds was done by HPLC with a chromatograph Hewlett-Packard 1090L equipped with a UV detector at 254 nm and a column ODS-Hypersil (C-18) 5 μm, 100×4.6 mm.

Injection volume 10 μl. Flow rate 1 ml/minute.

Mobile phases: (A) 0.02 aq NaH_2PO_4 (pH 4.7); (B) CH_3CN; linear gradient as follows:

Minutes	0	1	24	29	34
% B	20	20	35	47	20

IR spectra (Nujol) were recorded on a Perkin-Elmer 580 spectrometer. ^1H NMR spectra were recorded with a 250 MHz Bruker spectrometer equipped with an Aspect 3000 computer. (TMS internal standard, δ 0.00; solvent, CD_3SOCD_3).

UV Difference Spectroscopy

Binding constants between peptide and compounds were measured essentially as described by NIETO and PERKINS[13] except that the tandem arrangement of cells was not required since the peptide used had no significant absorption in the range 260 ~ 340 nm.

Experiments were carried out with a Perkin-Elmer model 320 spectrophotometer. Cells with 4 cm light-path filled with 10 ml of solutions containing antibiotic (35×10^{-6} M in 0.02 M Na_2HPO_4, pH 9) were placed in the sample and reference compartments and the difference in absorbance, occurring upon the addition of Ac_2-L-Lys-D-Ala-D-Ala (10 ~ 50 μl of a 4-mM solution) to the sample cell and the same volume of buffer to the reference cell, was measured as 285 ~ 290 nm. Association constants were determined by Scatchard plot analysis.

Microbiological Assay

MIC was determined using the 2-fold dilution method in microtiter system. The media used were: Todd-Hewitt broth (Difco) for Streptococci and Iso-Sensitest broth (Oxoid) for Staphylococci, *Streptococcus faecalis* and Gram-negative bacteria. The final inoculum was about 10^4 cfu/ml. MIC was read as the lowest concentration which showed no visible growth after 18 ~ 24 hours of incubation at 37°C.

Compounds **IV**

Reactions had to be run under a hood and vapors adsorbed by a permanganate solution.

Method A

General procedure.

N^{15}-Carboxy-16,17-didehydro-16-deoxo-16-aminoteicoplanin Aglycone γ-Lactam (**IVc**)

To a solution of N^{15}-(amino(methylthio)methylene)teicoplanin aglycone hydrochloride[7] (**Ic**, 300 mg, 0.229 mmol) in 15 ml of MeOH, TEA (1 ml) was added and the milky mixture was stirred at 45°C for 6 hours. Solvent was evaporated under vacuum, the residue suspended in water at pH 2 and extracted with a mixture EtOAc - BuOH, 8 : 2 (10 ml). The organic solvent was evaporated and the residue treated with Et_2O and filtered, yielding 200 mg of the title compound. Compounds with unsatisfactory purity were purified by column chromatography as reported in the Experimental section.

Method B

16,17-Didehydro-16-deoxo-16-hydroxy-N^{15},O^{16}-(methylaminomethenyl)teicoplanin Aglycone (**IIId**)

To a solution of **Id** hydroiodide (300 mg, 0.212 mmol) in 20 ml CH_3CN and 5 ml water, TEA (2 ml) was added, afterwards the milky mixture was stirred at 40°C for 10 hours, obtaining a complete transformation. Reaction solvent was evaporated under vacuum and the residue, dissolved in CH_3CN - water (8 : 2), was purified on 50 g Silica gel RP-8 column eluting with 25% CH_3CN in water, yielding 100 mg.

N^{15}-Carboxy-16,17-didehydro-16-deoxo-16-methylaminoteicoplanin Aglycone γ-Lactam (**IVd**)

Compound **IIId** was dissolved in MeOH and stirred at 40°C for 6 hours. The HPLC analysis showed the complete transformation of **IIId** into **IVd**, confirmed by adding an authentic sample of **IVd** prepared with Method A.

Acknowledgments

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