VOL. 56 NO. 9, SEPT. 2003 THE JOURNAL OF ANTIBIOTICS pp. 773 — 782

Production and Characterization of Monochlorinated and Dechlorinated A40926 Derivatives

FABRIZIO BELTRAMETTI*, AMERIGA LAZZARINI, CRISTINA BRUNATI, ALESSANDRA MARAZZI, SRDJAN JOVETIC, ENRICO SELVA and FLAVIA MARINELLI

Vicuron Pharmaceuticals via R. Lepetit, 34, 21040, Gerenzano (Varese) Italy

(Received for publication April 11, 2003)

Nonomuraea sp. ATCC 39727 is the producer of the A40926 complex of lipoglycopeptide antibiotics which contain chlorine atoms in amino acids 3 and 6 of the peptide backbone. Using a classical mutagenesis and selection approach we have isolated a *Nonomuraea* sp. ATCC 39727 mutant strain able to direct production towards new A40926 analogues dechloro-A40926 (DDC) lacking two chlorine atoms and the two monochloro-A40926 (MDC1 and MDC2) that are not produced fermenting the wild type strain. Dechlorinated A40926 derivatives were obtained in considerable amount in a standard fermentation process and were purified and chemically characterized. The dechlorinated A40926 derivatives DDC and MDC2 showed improved antimicrobial activity against coagulase negative staphylococci strains in respect to A40926 complex. Dechlorinated derivatives of the related antibiotic teicoplanin are also reported in the literature and are generally less active than the parental products.

Extensive chemical modifications of glycopeptides have been reported in the literature to obtain derivatives with improved antimicrobial activity or better pharmacokinetics[1]. Some specific modifications in the glycopeptide scaffold are, however, difficult to achieve or involve several complicated chemical steps. Nowadays, several genetic clusters for glycopeptide biosynthesis have been cloned and sequenced and most of the gene functions underlying the biosynthetic machinery have been clarified[2~12]. This has allowed to some extent direct antibiotic modifications by mutation of genes encoding specific functions[2]. However, the approach of direct genetic engineering is often seriously limited by the difficulties encountered in finding suitable cloning vectors and transformation techniques when dealing with poorly characterized microorganisms. In the case here reported, random mutagenesis generated new bioactive molecules modified in the glycopeptide backbone.

A common characteristic of the glycopeptides of the vancomycin group is the presence of 1 or 2 chlorine atoms in different positions in the molecule. The conformation of a glycopeptide nucleus is rigid and dechlorination, which may influence steric aspects, appears interesting in the investigation of structure-activity relationship. Vancomycin and teicoplanin carry two chlorine atoms bound at equivalent positions at the aminoacids number 2 and 6. Dechlorination of vancomycin and teicoplanin were described by HARRIS *et al.*[13] and MALABARBA *et al.*[14]. Two dechlorinated products (one dechloro and one monochloro) were described for teicoplanin. Dechloro teicoplanin derivatives show decreased microbiological activity against some strains of staphylococci and streptococci, in respect to the original antibiotic[14]. Removal of chloro from aminoacid 2 of vancomycin had scarce if any effect on antibacterial activity[15] while dechlorinated derivatives of the vancomycin-like antibiotic A82846B, factors A82846A (lacking chlorine in the position 6) and A82846C (devoid of chlorine) showed increased antimicrobial activity against some strains of staphylococci[15~17]. Indeed, orienticin A, which is the dechlorinated derivative of A82846B lacking chloro from aminoacid 2, showed decreased antimicrobial activity when tested against staphylococci and streptococci[15].

A40926[18,19] is the lipoglycopeptide antibiotic precursor of the semi-synthetic derivative Dalbavancin, which is currently under clinical development at Vicuron Pharmaceuticals. A40926 complex is produced by the actinomycete *Nonomuraea* sp. ATCC 39727 (formerly

* Corresponding author: fbeltrametti@vicuron.it

Actinomadura sp. ATCC 39727). Antibiotic A40926 possesses the typical heptapeptide structure of the D-alanyl-D-alanine binding glycopeptides of the teicoplanin sub-group and is characterized by a *N*-acylaminoglucuronic acid and a mannose moiety bound to the aminoacids number 4 and 7 respectively (Figure 1). Several structural analogues have been identified in the A40926 complex[19,20]. The two major components of the complex, named B0 (formerly B) and B1, are characterized respectively by an *iso*-C12:0 and a n-C12:0 acylic moiety bound to the aminoglucuronic acid of the antibiotic (Figure 1). Two chlorine atoms are present on the aromatic rings of aminoacids 3 and 6 and no data concerning A40926 dechlorinated analogues are available to date.

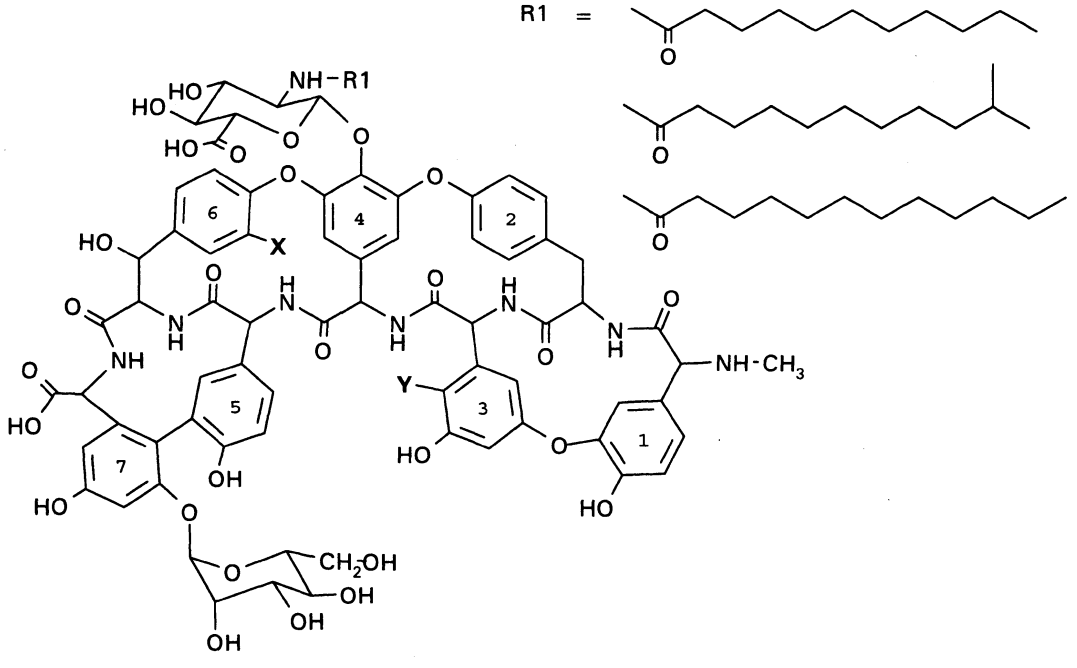
During a screening program for high-producer strains, using mutagenesis and selection, we have isolated a *Nonomuraea* sp. ATCC 39727 mutant producing a mixture of dechloro-A40926 (herein indicated as DDC), the two monochloro-A40926, lacking respectively chlorine at aminoacid 3 (herein indicated as MDC1) and 6 (herein indicated as MDC2) together with smaller amounts of the B0-factor of A40926 itself. DDC and MDC2 compounds showed increased antimicrobial activity against *Staphylococcus haemolyticus* and were then characterized.

Materials and Methods

Culture Media and Fermentation Conditions

The A40926 producer strain *Nonomuraea* sp. ATCC 39727 and its derivative A40DC producing the dechlorinated A40926 derivatives were maintained as a lyophilised Master Cell Bank (MCB). The A40DC strain was isolated from the parental strain after mutagenesis with 1-Methyl-3-nitro-1-nitrosoguanidine (MNNG) as a clone able to give growth inhibition on *Staphylococcus haemolyticus* 3902 (clinical isolate, teicoplanin resistant) in overlay experiments. Overlay was performed as follows.

Fig. 1. Structure of A40926 and dechlorinated A40926 derivatives.



A40926 X=Y=Cl

DDC X=Y=H

MDC1 X=Cl
Y=H

MDC2 X=H
Y=Cl

Nonomuraea was seeded on SM-agar plates (g/liter: glucose 10, Bacto-peptone 4, Bacto-yeast extract 4, $MgSO_4 \cdot 7H_2O$ 0.5, KH_2PO_4 2, K_2HPO_4 4, Bacto-agar 20, deionised water up to) at a density of 200 cfu per plate. After growth, each plate was overlaid with 20 ml of pre-cooled Nutrient agar medium (Difco) inoculated with 10^7 cfu of *S. haemolyticus* 3902 and incubated over night at 37°C. In these conditions *Nonomuraea* sp. ATCC 39727 colonies produce A40926 amounts unable to give inhibition halos on *S. haemolyticus* 3902 while colonies producing different antibiotics or improved A40926 amounts were easily detected.

A Working Cell Bank (WCB) of the strains was prepared as follows. Strains were plated from the lyophilized vials on SM agar medium. The mycelium from a slant was homogenized in 10 ml of saline and inoculated in SM liquid medium (g/liter: glucose 10, Bacto-peptone 4, Bacto-yeast extract 4, $MgSO_4 \cdot 7H_2O$ 0.5, KH_2PO_4 2, K_2HPO_4 4, deionised water up to), grown for 96 hours and stored in 1.5 ml cryo-vials at −80°C. Cryo-vials of the WCB were thawed at room temperature and 2 ml were used to inoculate 100 ml of vegetative medium E25 (g/liter: glucose 25, meat extract 4, yeast autolysate 1, soybean meal 10, peptone 4, NaCl 2.5, $CaCO_3$ 5, deionised water up to) in 500-ml baffled flasks. Strains were grown for 72~96 hours on a rotary shaker at 200 rpm and 28°C. Fermentation was started with a 10% inoculum from the vegetative medium flask in production medium T/2[21] modified according to SELVA *et al.*[22]. A40926, DDC, MDC1 and MDC2 production was estimated at different time intervals of the fermentation as described below.

Production of the Complex of Dechlorinated A40926 in 150 liter-scale Bioreactor

The vegetative pre-culture was prepared by inoculating 1 ml of the WCB described above in 100 ml of vegetative medium E25 followed by incubation at 28°C for 72 hours. Two flasks of the grown pre-culture were used to inoculate a 20 liter Chemap fermenter (Model CF 3000-DCU, Type SG) containing E25 medium. The seed pre-culture was grown for 72 hours at 28°C under following conditions: stirrer speed 600 rpm, aeration rate 0.5 vvm. 150 liters of T/2 production medium were inoculated with the 15 liters pre-culture described above in a 300 L Bioengineering fermenter. The culture was grown 192 hours at 28°C under following çonditions: stirrer speed 180 rpm, aeration rate 0.5 vvm. Samples were collected from the fermentor for analysis and purification.

Mutagenesis of Strain ATCC 39727

Mycelium for mutagenesis, was grown in SM liquid medium for 96 hours at 28°C with shaking. Mycelium was then harvested by centrifugation, resuspended in physiologic solution and fragmented by sonication with Vibracell Albra sonicator 400W model, at an intensity of 6 kHz, pulse on, 5 seconds and pulse off, 3 seconds, for a total interval sufficient to give single unbranched hyphae with size ranging from 1 to 5 μm (checked with microscope). The resulting cell suspension was filtered through a 5 μm Durapore membrane filter (Millipore), harvested by centrifugation, resuspended in fresh SM medium and incubated for 3 hours with shaking to revitalize the mycelium. The titre of the culture was determined by plating dilutions on SM agar. Mutagenesis was performed with (MNNG) at different concentrations diluting 1:1 the cell suspension with phosphate buffer 200 mM pH 8 as suggested by DELIC *et al.*[23]. An untreated parallel sample was run as control. The efficiency of the mutagenesis process was estimated by comparing the incidence of streptomycin resistant clones in the mutagenized and in the control sample. The mycelium was either stored in 1 ml aliquots at −80°C or immediately processed in the overlay experiments as above described.

A40926 and Dechlorinated A40926 Derivatives Extraction and Column Purification

Samples were collected from each fermentation at different time intervals and processed for extraction and analysis. The whole bacterial culture was brought to pH 12 with NaOH and filtered through a 0.45 μm durapore membrane filter (Millipore). The filtrated broth was incubated at 50°C for 1 hour and then directly analysed by HPLC as below described.

A40926, DDC, MDC1 and MDC2 were extracted from the broth and purified for LC-MS and antibiotic activity assays as follows. D-alanyl-D-alanine-aminocaproyl Sepharose CL-6B was used as affinity resin as already described[24]. The packed resin was equilibrated with 0.2 M NaCl in 0.05 M NaH_2PO_4 pH 7.5 prior to mixing with pre-filtered fermentation broth prepared as above described and brought at pH 7.5 with HCl 1 M. The antibiotics, selectively bound to the resins were washed with 0.2 M NaCl in 0.05 M NaH_2PO_4 pH 7.5 and eluted with 1.5% NH_4OH. The samples were vacuum dried and resuspended in an appropriate volume of CH_3CN for chemical analysis or DMSO for biological assays.

Extraction and purification from 150 liters tank was performed as follows. The fermentation broth of strain A40DC (150 liters) was brought to pH 11 with the addition

of 400 ml of 10 M NaOH. The broth was transferred to a tank connected to a microfiltration system and was filtered to residual 50 liters. The permeate was adjusted to pH 7.5 with 100 ml of 12 M HCl and then stirred for two days in the presence of 3 liters of Diaion HP20 resin (Mitsubishi Chemicals). The resin was then collected by filtration and was washed sequentially with 20 liters of H_2O : MeOH (7 : 3) (v : v) and with 20 liters of MeOH : H_2O : *n*-BuOH 9 : 1 : 1 (v/v). The antibiotic complex was eluted with 20 liters of 100 mM NH_4OH : acetone 1 : 1. (v/v) mixture. The elute was concentrated under vacuum to 600 ml. By adding under stirring 600 ml of acetone and by adjusting the pH to 5.5 with 3 ml of 12 M HCl, a light precipitate was instantly obtained. This suspension was added to 9 liters of acetone to give a solid precipitate which was filtered and washed with acetone. After drying for 18 hours at 40°C under vacuum, a B0-A40926, DDC, MDC1 and MDC2 complex was obtained with a HPLC titre of 73.2% calculated against a A40926 standard sample. The sample was resuspended in an appropriate volume of CH_3CN for chemical analysis or DMSO for biological assays.

Analytical HPLC

HPLC analyses were performed on a 5 μm particle size Ultrasphere ODS (Beckman) column (4.6×250 mm) eluted at 1 ml/minute flow rate with a 26 minutes linear gradient from 25% to 37% of Phase B. Phase A was 20 mM $HCOONH_4$ pH 4.5 : CH_3CN 95 : 5 (v/v) and Phase B was 20 mM $HCOONH_4$ pH 4.5 : CH_3CN 5 : 95 (v/v) mixture. The chromatography was performed with a Hewlett Packard mod 1100 HPLC system and detection was at 230 nm. As internal standard a sample of A40926 antibiotic was used.

Preparative HPLC

B0-A40926, DDC, MDC1 and MDC2 were separated by preparative HPLC on a 7 μm particle size Lichrosorb RP-8 column (25×250 mm) (MERCK) eluted at 30 ml/minute flow rate with a 26 minutes linear gradient from 25% to 37% of Phase B. Phase A was 20 mM $HCOONH_4$ pH 4.5 : CH_3CN 95 : 5 (v/v) and Phase B was 20 mM $HCOONH_4$ pH 4.5 : CH_3CN 5 : 95 (v/v) mixture. The chromatography was performed with a Hewlett Packard mod 1100 HPLC system and detection was at 230 nm.

A sample of the crude complex was suspended under stirring in 10 ml of 250 mM NH_4OH and was then centrifuged at 3000×*g* for 5 minutes. The supernatant was processed in 200 μl aliquots. The fractions eluted in 37 repeated runs were pooled according to their antibiotic content. The processing of *ca.* 1500 mg of crude extract from bioreactor fermentation yielded 82 mg of DDC and 130 mg MDC2. The pools were freeze-dried and the obtained powder stored at −20°C for further use.

LC-MS

The molecular weights of the A40926 and dechlorinated A40926-derivatives were determined by mass spectrometry using a Finnigan LCQ™ DUO instrument (Thermo Quest). The experimental ESI conditions were as follows: Capillary Temp 250°C, Sheath gas Flow (a.u.) 40, Capillary voltage 46V, Spray voltage 4.7 kV. HPLC conditions were as above described.

NMR

DDC and MDC2 were compared with A40926 by ^1H and ^{13}C NMR. For MDC2 a complete assignment of proton and carbon resonance was achieved by two-dimensional homo- and hetero-nuclear NMR experiments. All the spectra were recorded at 40°C in DMSO-d_6 solution (about 20 mg in 0.6 ml) on a Bruker AMX 600 spectrometer. Homonuclear experiments (TOCSY and ROESY) were acquired with a spectral width of 10 ppm. TOCSY experiment was recorded with 512 increments in t_1 and 2048 complex data points in t_2 (40 transients). ROESY experiment was instead recorded with 4096 complex data points in t_2 and 1024 increments in t_1 (32 transients; mixing time 200 milliseconds).

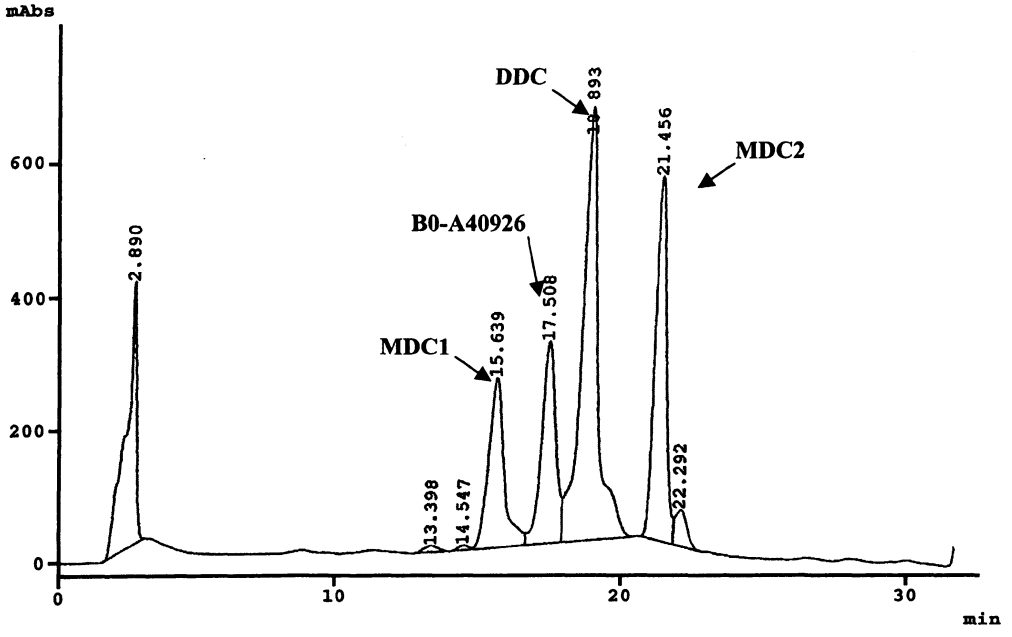
^{13}C-1D spectrum was acquired using a sweep width of 185 ppm; 65000 scans have been accumulated with a relaxation delay of 1.4 seconds.

For HMQC and HMBC spectra 512 increments (64 and 112 respectively) with 2048 complex data points in t_2 have been collected. In HMBC a delay of 70 milliseconds was selected for the development of long-range correlations.

Antimicrobial Activity Assay

The antimicrobial activity of the isolated antibiotics was determined as Minimal Inhibitory Concentration (MIC) by the broth microdilution methodology according to the NCCLS procedures[25] with inocula of approximately 5×10^5 CFU/ml. The media used was the cation-adjusted Mueller-Hinton broth (Difco) supplemented with 30% (vol/vol) fetal calf serum (Difco). All the MICs were read after 24 hours incubation at 37°C. Alternatively, antimicrobial activity was compared in agar diffusion experiments as follows. Serial dilutions of the purified dechloro-A40926s and of a standard of A40926 were spotted on Nutrient agar plates (Difco). Plates were then overlaid with pre-cooled Nutrient agar inoculated with 10^7 cfu/ml of *S. haemolyticus* 3902 and incubated at 37°C for 24 hours. The diameter of the inhibition zone was

Fig. 2. HPLC analysis of the antibiotic complex of strain A40DC after purification with Diaion HP20 resins (see text).



MDC1, monochloro-A40926 (chlorine is bound to aminoacid number 6); B0-A40926, B0 factor of A40926 antibiotic; DDC, dechloro-A40926; MDC2, monochloro-A40926 (chlorine is bound to aminoacid number 3).

correlated with the antibiotic concentration.

Results

Isolation of Mutant A40DC from *Nonomuraea* sp. ATCC 39727

About 12000 clones from a mutagenized sample of *Nonomuraea* sp. ATCC 39727 were plated on SM agar medium and tested by overlay with *S. haemolyticus* 3902 (clinical isolate, teicoplanin resistant). In this condition, the parental strain, which is able to produce the antibiotic A40926, was unable to give inhibition halos on *S. haemolyticus*. This overlay screening was in fact set up to select those mutants producing markedly more antibiotic and/or giving more active components in the A40926 complex. Among the tested clones one able to give an inhibition halo on *S. haemolyticus* 3902 was named A40DC and resulted phenotypically undistinguishable from the parental strain.

Fermentation experiments and subsequent HPLC analysis evidenced the presence of a complex of compounds (Figure 2) not present in the fermentation broth of *Nonomuraea* sp. ATCC 39727 producer of the A40926 complex. In strain A40DC the main peaks were at retention times 15.6 (MDC1), 18.9 (DDC) and 21.5 (MDC2) (Figure 2) while factor B0 (peak retention time of 17.5 minutes) was present in low concentration.

Chemical Characterization of Dechlorinated A40926 Derivatives

The fermentation broth of strain A40DC was purified by binding to D-alanyl-D-alanine-aminocaproyl Sepharose CL-6B resins and the mixture eluted from the resin maintained the complex composition as in the fermentation broth. The sample was analysed by LC-MS and the fractions showed a *m/z* main peak of 1697 (MDC1), 1732.5 (B0-A40926), 1663 (DDC) and 1697 (MDC2) respectively. The loss of 35 mass units in samples MDC1 and MDC2 was consistent with loss of one chlorine atom from either aminoacids 3 or 6 in the B0-A40926 molecule and loss of 70 mass units in DDC suggested the loss of both chlorine atoms. In *Nonomuraea* sp. ATCC 39727 fermentations, the formulation of the medium T/2 (see Materials and Methods) boost the production of B0 factor (*iso*-C12:0 *N*-

Table 1. ^{1}H and ^{13}C-NMR assignments of MDC2.

position	δ proton (ppm)	δ Carbon (ppm)	position	δ proton (ppm)	δ Carbon (ppm)
w1	n.a.	-	OH(5d)	n.a.	-
CH_3	2.33	33.82	w6	6.47	-
x1	4.32	65.92	x6	4.14	61.86
y1	-	n.a.	y6	-	167.4
1a	-	128.85	z6	5.10	71.85
1b	6.60	115.7	OH(z6)	n.a.	-
1c	-	142.56	6a	-	139.0
1d	-	146.4	6b	7.45	126.4
1e	6.95	117.3	6c	7.07	122.2
1f	7.04	124.4	6d	-	154.1
OH(1d)	n.a.	-	6e	7.04	127.12
w2	7.55	-	6f	7.42	127.4
x2	4.90	54.24	w7	8.36	-
y2	-	n.a.	x7	4.44	57.24
z2	2.81; 3.29	37.32	y7	-	173.0
2a	-	n.a.	7a	-	136.81
2b	7.59	130.8	7b	-	119.9
2c	7.16	122.6	7c	-	154.2
2d	-	154.4	7d	6.74	100.7
2e	7.04	120.2	7e	-	157.0
2f	7.07	130.1	7f	6.54	108.1
w3	7.57	-	OH(7e)	n.a.	-
x3	6.06	53.85	AG1	5.44	101.0
y3	-	166.9	AG2	3.69	55.95
3a	-	138.2	AG3	3.60	73.81
3b	-	113.9	AG4	n.a.	n.a.
3c	-	n.a.	AG5	n.a.	n.a.
3d	6.72	107.2	COOH (AG5)	-	n.a.
3e	-	154.3	AG-NH	7.75	-
3f	6.50	106.5	OH(AG3)	n.a.	-
OH(3c)	n.a.	-	OH(AG4)	n.a.	-
w4	7.53	-	FA1	-	172.2
x4	5.60	54.32	FA2	2.06	35.85
y4	-	169.2	FA3	1.46	25.08
4a	-	133.0	FA4-FA8	1.14-1.24	28.44-29.09
4b	5.85	108.0	FA9	1.12	38.27
4c	-	152.5	FA10	1.50	27.15
4d	-	n.a.	FA11-FA12	0.85	22.3
4e	-	n.a.	M1	5.28	96.6
4f	5.33	106.0	M2	n.a.	n.a.
w5	8.52	-	M3	3.51	69.66
x5	4.56	53.05	M4	3.48	66.25
y5	-	n.a.	M5	3.49	73.45
5a	-	n.a.	M6	3.50	60.75
5b	6.69	125.5	OH(M2)	n.a.	-
5c	-	120.8	OH(M3)	n.a.	-
5d	-	154.9	OH(M4)	n.a.	-
5e	7.17	123.9	OH(M6)	n.a.	-
5f	7.14	135.6			

n.a. = not attributed

acyl residue) of the A40926 complex and drastically reduces the proportion of the other *N*-acyl derivatives. A similar situation was observed in strain A40DC where dechlorinated derivatives with *iso*-C12:0 *N*-acyl residues were predominant in the complex (Figure 2).

DDC and MDC2 factors resulted active in the agar diffusion test against *S. haemolyticus* whereas MDC1 and B0 showed a fainter inhibition in this assay. DDC and MDC2 were separated by preparative HPLC.

The proton and carbon NMR signals of MDC2 showed shifts typical for a glycopeptide molecule and in particular their value is fairly the same observed in A40926. As in A40926, the chlorinated ring 3 showed the distinctive chemical shift at 113.9 ppm for carbon 3b that gave clear long-range correlation with the protons 3d, 3f, x3. Proton 3f shows strong ROEs to 1b, and w3. Ring 6, which was chlorinated as well in A40926, in MDC2 was characterized by a four-proton spin system that excluded the presence of chlorine. ROEs and C–H long-range carried out the correct assignment of these protons. In detail, the quaternary carbon 6a was identified by long-range correlation to z6; others J^3(C, H) correlations to 6a are 6c and 6e. The proton 6b gives ROEs to z6, x6 and long-range correlation with carbon 6f. Proton 6c instead shows J^3(C, H) correlations to 6e. This confirmed in MDC2 the hypothesis of the loss of chlorine atom from the aminoacid number 6 in respect to the A40926 molecule. The proton and carbon assignments of MDC2 are reported in Table 1 according to the nomenclature initially proposed by BARNA *et al.*[26] for teicoplanin (Figure 3).

Although NMR analysis was not performed, we suppose that MDC1 represented the alternative monochlorinated isomer. Thus, in MDC1 the chlorine bound to aminoacid number 3 was presumably lost while chlorination on aminoacid number 6 was retained.

Antimicrobial Activity of DDC, and MDC2

MDC2 and DDC showed improved activity against coagulase negative staphylococci when compared with A40926. The antimicrobial activity against coagulase positive staphylococci showed instead a slight decrease when compared with antibiotic A40926. No inhibition was observed against VanA enterococci, as for the A40926 antibiotic itself (Table 2).

Fig. 3. Structure of MDC2 as determined by NMR analysis.

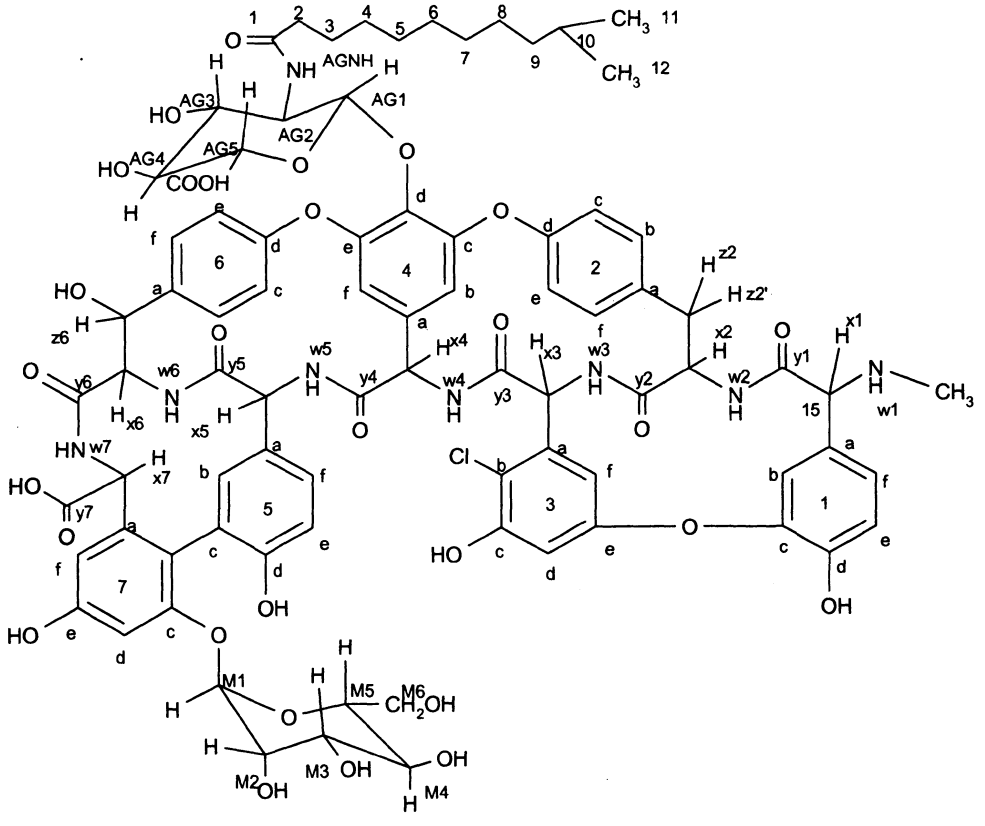


Table 2. Antimicrobial activity of A40926 and dechlorinated A40926 derivatives DDC and MDC2.

Microorganism	MIC (μg/ml)			
	A40926	DDC	MDC2	VANCOMYCIN
Staphylococcus aureus Smith Met-S	0.5	1	2	0.5
S. aureus Met-S 3982[a]	0.25	1	1	2
S. aureus Met-S 3989[a]	0.125	0.5	0.5	1
S. aureus Met-S 3988[a]	0.25	0.5	1	1
S. aureus TOUR	0.125	0.125	0.25	0.5
S. aureus Met-R 3818[a]	4	1	1	1
S. aureus Met-R 1096[a]	0.25	0.5	0.5	1
S. aureus Met-R 1526[a]	0.25	0.5	1	1
S. aureus Met-R 1400[a]	0.25	1	1	1
S. aureus Met-R 613[a]	0.25	0.5	0.5	0.5
S. aureus VISA Met-R 3797[a]	4	8	4	8
S. aureus VISA 3798[a]	4	8	4	8
S. haemolyticus Met-R 1729[a]	64	2	4	2
S. haemolyticus Met-S 1730[a]	16	2	4	2
S. haemolyticus 602[a]	>64	16	8	4
S. haemolyticus 3902[a]	64	8	8	2
S. epidermidis ATCC12228	32	1	0.25	2
S. epidermidis 533[a]	16	0.125	0.125	4
Streptococcus pyogenes C203[b]	≤0.06	≤0.06	≤0.06	0.5
S. pneumoniae Pen-I 2868[a]	0.5	0.25	0.5	0.125
S. pneumoniae UC41	0.125	0.125	0.125	0.5
Enterococcus faecalis VanS 1139[a]	0.125	0.125	0.25	2
E. faecalis (isogenic of L 560) 559[a]	0.125	0.25	0.5	0.5
E. faecalis VanA 560[a]	>64	>64	>64	>64
E. faecalis Van A 1841[a]	64	>64	>64	>64
E. faecium (isogenic of L569) 568[a]	0.5	1	2	1
E. faecium Van-A 569[a]	>64	>64	>64	>64
E. faecium VanA 2215[a]	64	>64	>64	>64
E. durans VanA 1618[a]	>128	>64	>64	>64
Escherichia coli SKF12140	>64	>64	>64	>64
Candida albicans SKF2270	>64	>64	>64	>64

[a] Clinical isolate [b] SKF 13400

The antimicrobial activity of vancomycin is reported as reference.

Discussion

The A40DC strain was isolated from the *Nonomuraea* sp. strain ATCC 39727 by mutagenesis and selection. This mutant was detectable by overlay with *S. haemolyticus* 3902 (clinical isolate, teicoplanin resistant) due to the stable production of A40926 dechlorinated derivatives. PUK *et al.*[6] reported in the glycopetide balhimycin producer strain *Amycolatopsis mediterranei* DSM5908 the presence of a single halogenase enzyme responsible for the chlorination of both aromatic rings of amino acids 2 and 6. The

inactivation of the halogenase gene yielded a dechlorinated derivative that was identical to balhimycin in the rest of the structure suggesting that halogenation was not a precondition for subsequent biosynthesis steps. A similar situation was observed in *Nonomuraea* sp. In fact, the dechlorinated derivatives present in the fermentation broths of strain A40DC consisted of two monochloro-, the dechloro- and the B0-A40926 molecules, thus suggesting the presence of a single, partially inactivated halogenase enzyme able to yield all the possible combinations in the loss of the two chlorine atoms. Furthermore, the occurrence in the fermentation broth of *Nonomuraea* sp. A40DC of a dechloro mixture, which is identical in the rest of the structure to the A40926 antibiotic, suggested that biosynthesis occurs independently from the presence of chlorine in the molecule.

We have found that two out of three dechlorinated A40926 derivatives (DDC and MDC2) have improved microbiological activity against some clinically relevant microorganisms. In the cases of the corresponding teicoplanin derivatives, MALABARBA *et al.*[14] reported that the loss of chlorine bound at aminoacid number 2 or of both chlorines of teicoplanin decreased the binding affinity for the D-alanyl-D-alanine target of the antibiotic. This was correlated with a consequent marked reduction of *in vitro* and *in vivo* antimicrobial activity. HARRIS *et al.*[13] reported similar results for vancomycin and suggested that chlorine contributes to the stability and the specificity of the binding to the target.

We have evidenced by MS studies a dimerizing capacity of dechlore A40926 (DDC) improved with respect to that of A40926 (M. FEROGGIO, unpublished result). This dimerization capacity is peculiar of some glycopetides and was extensively studied as a determinant of their antimicrobial effectiveness[27]. In the case of DDC molecules the influence of the loss of the bulky, electronegative chlorine could facilitate the dimerizing reaction resulting in an increase of antimicrobial activity.

Acknowledgments

We are grateful to CLAUDIO QUARTA and GIANCARLO LANCINI for helpful discussion, to MARINA FEROGGIO for kindly sharing unpublished results and to ROBERTA ROSSI, ARMANDO GRIGOLETTO, MARCO PEDROLI, and LUC JAQUET for skillful technical assistance.

References

1) LANCINI, G. C. & B. CAVALERI: Glycopeptide Antibiotics (Dalbaheptides). *In* Biotechnology (2nd edition). *Ed.* H. J. REHM & G. REED, pp. 369~395, Vol. 7, 1997
2) PELZER, S.; R. SUSSMUTH, D. HECKMANN, J. RECKTENWALD, P. HUBER, G. JUNG & W. WOHLLEBEN: Identification and analysis of the balhimycin biosynthetic gene cluster and its use for manipulating glycopeptide biosynthesis in *Amycolatopsis mediterranei* DSM5908. Antimicrob. Agents Chemother. 43: 1565~1573, 1999
3) VAN WAGENINGEN, A. M.; P. N. KIRKPATRICK, D. H. WILLIAMS, B. R. HARRIS, J. K. KERSHAW, N. J. LENNARD, M. JONES, S. J. JONES & P. J. SOLENBERG: Sequencing and analysis of genes involved in the biosynthesis of a vancomycin group antibiotic. Chem. Biol. 5: 155~162, 1998
4) RECKTENWALD, J.; R. SHAWKY, O. PUK, F. PFENNIG, U. KELLER, W. WOHLLEBEN & S. PELZER: Nonribosomal biosynthesis of vancomycin-type antibiotics: a heptapeptide backbone and eight peptide synthetase modules. Microbiology 148: 1105~1118, 2002
5) POOTOOLAL, J.; M. G. THOMAS, C. G. MARSHALL, J. M. NEU, B. K. HUBBARD, C. T. WALSH & G. D. WRIGHT: Assembling the glycopeptide antibiotic scaffold: The biosynthesis of A47934 from *Streptomyces toyocaensis* NRRL15009. Proc. Natl. Acad. Sci. USA 99: 8962~8967, 2002
6) PUK, O.; P. HUBER, D. BISCHOFF, J. RECKTENWALD, G. JUNG, R. D. SUSSMUTH, K. H. VAN PEE, W. WOHLLEBEN & S. PELZER: Glycopeptide biosynthesis in *Amycolatopsis mediterranei* DSM5908: function of a halogenase and a haloperoxidase/perhydrolase. Chem. Biol. 9: 225~235, 2002
7) PFEIFER, V.; G. J. NICHOLSON, J. RIES, J. RECKTENWALD, A. B. SCHEFER, R. M. SHAWKY, J. SCHRODER, W. WOHLLEBEN & S. PELZER: A polyketide synthase in glycopeptide biosynthesis: the biosynthesis of the non-proteinogenic amino acid (*S*)-3,5-dihydroxyphenylglycine. J. Biol. Chem. 276: 38370~38377, 2001
8) PAVLOV, A. Y.; M. N. PREOBRAZHENSKAYA, A. MALABARBA & R. CIABATTI: Synthesis and antibacterial activity of derivatives of the glycopeptide antibiotic A-40926 *N*-alkylated at the aminoglucuronyl moiety. J. Antibiotics 51: 525~527, 1998
9) HUBBARD, B. K.; M. G. THOMAS & C. T. WALSH: Biosynthesis of L-*p*-hydroxyphenylglycine, a non-proteinogenic amino acid constituent of peptide antibiotics. Chem. Biol. 7: 931~942, 2000
10) HERMANN, R.; F. RIPAMONTI, G. ROMANO, E. RESTELLI, P. FERRARI, B. P. GOLDSTEIN, M. BERTI & R. CIABATTI: Synthesis and antibacterial activity of derivatives of the glycopeptide antibiotic A-40926 and its aglycone. J. Antibiotics 49: 1236~1248, 1996
11) ECKSTEIN, T. M.; F. S. SILBAQ, D. CHATTERJEE, N. J. KELLY, P. J. BRENNAN & J. T. BELISLE: Identification and recombinant expression of a *Mycobacterium avium* rhamnosyltransferase gene (*rtfA*) involved in glycopeptidolipid biosynthesis. J. Bacteriol. 180: 5567~5573, 1998
12) CHEN, H.; M. G. THOMAS, B. K. HUBBARD, H. C. LOSEY, C. T. WALSH & M. D. BURKART: Deoxysugars in glycopeptide antibiotics: enzymatic synthesis of TDP-L-epivancosamine in chloroeremomycin biosynthesis. Proc. Natl. Acad. Sci. USA 97: 11942~11947, 2000

13) HARRIS, M. C.; K. RAJAMOORTHI, H. KOPECKA & T. M. HARRIS: The role of the chlorine substituents in the antiobiotic Vancomycin: preparation and characterization of mono- and dideclorovancomycin. J. Am. Chem. Soc. 107: 6652~6658, 1985
14) MALABARBA, A.; F. SPREAFICO, P. FERRARI, J. KETTENRING, P. STRAZZOLINI, G. TARZIA, R. PALLANZA, M. BERTI & B. CAVALLERI: Dechloro teicoplanin antibiotics. J. Antibiotics 42: 1684~1697, 1989
15) NAGARAJAN, R.: Structure-activity relationships of vancomycin antibiotics. *In* Glycopetide antibiotics. *Ed.* R. NAGARAJAN, pp. 195~218, Vol. 63, Marcel Dekker Inc., New York, 1994
16) COOPER, R. D. G.; B. E. HUFF, T. I. NICAS, J. T. QUATROCHE, M. J. RODRIGUEZ, N. J. SNYDER, M. A. STASZAK, R. C. THOMPSON, S. C. WILKIE & M. J. ZWEIFEL (Eli Lilly & Co.): Glycopeptide antibiotic derivatives. U.S. 5,840,684, November 24, 1998
17) NAGARAJAN, R.; D. M. BERRY & A. A. SCHABEL: The structural relationships of A82846B and its hydrolysis products with chloroorienticins A, B and C. J. Antibiotics 42: 1438~1440, 1989
18) GOLDSTEIN, B. P.; E. SELVA, L. GASTALDO, M. BERTI, R. PALLANZA, F. RIPAMONTI, P. FERRARI, M. DENARO, V. ARIOLI & G. CASSANI: A40926, a new glycopeptide antibiotic with anti-*Neisseria* activity. Antimicrob. Agents Chemother. 31: 1961~1966, 1987
19) ZERILLI, L. F.; D. M. EDWARDS, A. BORGHI, G. G. GALLO, E. SELVA, M. DENARO & G. C. LANCINI: Determination of the acyl moieties of the antibiotic complex A40926 and their relation with the membrane lipids of the producer strain. Rapid Commun. Mass Spectrom. 6: 109~114, 1992
20) WALTHO, J. P.; D. H. WILLIAMS, E. SELVA & P. J. FERRARI: Structure elucidation of the glycopeptide antibiotic complex A40926. Chem. Soc. Perkin Trans. 1: 2103~2107, 1987
21) QUARTA, C.; A. BORGHI, L. F. ZERILLI, M. T. DE PIETRO, P. FERRARI, A. TRANI & G. C. LANCINI: Isolation and structure determination of a novel complex of the teicoplanin family. J. Antibiotics 49: 644~650, 1996
22) SELVA, E.; L. GASTALDO, D. M., G. CASSANI & F. PARENTI (Biosearch Italia S.p.A.): Method for selectively enhancing the production of factors A or B.sub.0 of antibiotic A 40926 complex. U.S. 5,843,679, December 1, 1998
23) DELIC, V.; D. A. HOPWOOD & E. J. FRIEND: Mutangenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) in *Streptomyces coelicolor.* Mutat. Res. 9: 167~182, 1970
24) RIVA, E.; M. ZANOL, E. SELVA & A. BORGHI: Column purification and HPLC determination of teicoplanin and A40926. Chromatographia 24: 295~301, 1987
25) M7-A, N. D.: Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 17, 1997
26) BARNA, J. C. J.; D. H. WILLIAMS, D. J. M. STONE, T. W. C. LEUNG & D. M. DODDRELL: Structure elucidation of the teicoplanin antibiotics. J. Am. Chem. Soc. 106: 4895~4902, 1984
27) BEAUREGARD, D. A.; D. H. WILLIAMS, M. N. GWYNN & D. J. KNOWLES: Dimerization and membrane anchors in extracellular targeting of vancomycin group antibiotics. Antimicrob. Agents Chemother. 39: 781~785, 1995