Production of Demannosyl-A40926 by a *Nonomuraea* sp. ATCC 39727 Mutant Strain

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Chemical and biological modifications of glycopeptides have long been performed in order to obtain derivatives with improved antimicrobial activity or better pharmacokinetics[1].

Deglycosilated derivatives of several different glycopeptides were produced and used either for biosynthetic or for antimicrobial activity studies[1]. Deglycosilated derivatives were mainly obtained by chemical treatment or by biotransformation with yields of up to 80%[1]. Particularly interesting is the biotransformation process for the yields of transformation and the selectivity of the process. However, this requires a second fermentation step and a second downstream purification. *Nonomuraea* sp. ATCC 39727 (formerly *Actinomadura* sp. ATCC 39727) is the producer of the lipoglycopeptide antibiotic complex A40926[2,3]. A40926 is the natural precursor of the semi-synthetic derivative dalbavancin, which is currently under clinical development at Biosearch Italia SpA. Dalbavancin shows superior activity against Staphylococci and Enterococci, including some VanB isolates, and has excellent pharmacokinetic characteristics[4]. Antibiotic A40926 possesses the typical heptapeptide structure of the D-alanyl-D-alanine binding glycopeptides and is characterized by a *N*-acylaminoglucuronic acid and a mannose moiety bound to the aminoacids number 4 and 7 respectively (Figure 1). Several different related molecules have been identified in the A40926 complex[3,5]. The two major components of the complex are named B0 (formerly B) and B1 and 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).

A40926 aglycone and pseudoaglycones were obtained by chemical hydrolysis and subsequent purification[6]. The A40926 demannosyl derivative showed improved activity against some classes of Staphylococci and was also used as the starting material for the synthesis of several semisynthetic A40926 derivatives[6,7]. It is thus of primary importance the development of a rapid and convenient process for the production of demannosyl-A40926.

In the course of a screening program for *Nonomuraea* sp. ATCC 39727 mutants with desirable characteristics (such as high productivity), we have isolated a clone showing a noteworthy activity in an overlay antimicrobial test. This mutant was able to directly produce demannosyl-A40926 with an abundance of *ca* 95% with respect to the other components of the antibiotic complex. The isolation of this strain allowed demannosyl-A40926 production by a single fermentation/purification process.

Materials and Methods

Culture Media and Fermentation Conditions

The A40926 producer strain *Nonomuraea* sp. ATTC 39727 and its derivative A40DM producing demannosyl-A40926 were maintained as a lyophilised Master Cell Bank (MCB). The A40DM strain was isolated from the parental strain as spontaneous mutant able to produce inhibition when overlaid with *Staphylococcus haemolyticus* 3902 (clinical isolate, teicoplanin resistant). Overlay was performed as follows. *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, deionized 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.

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

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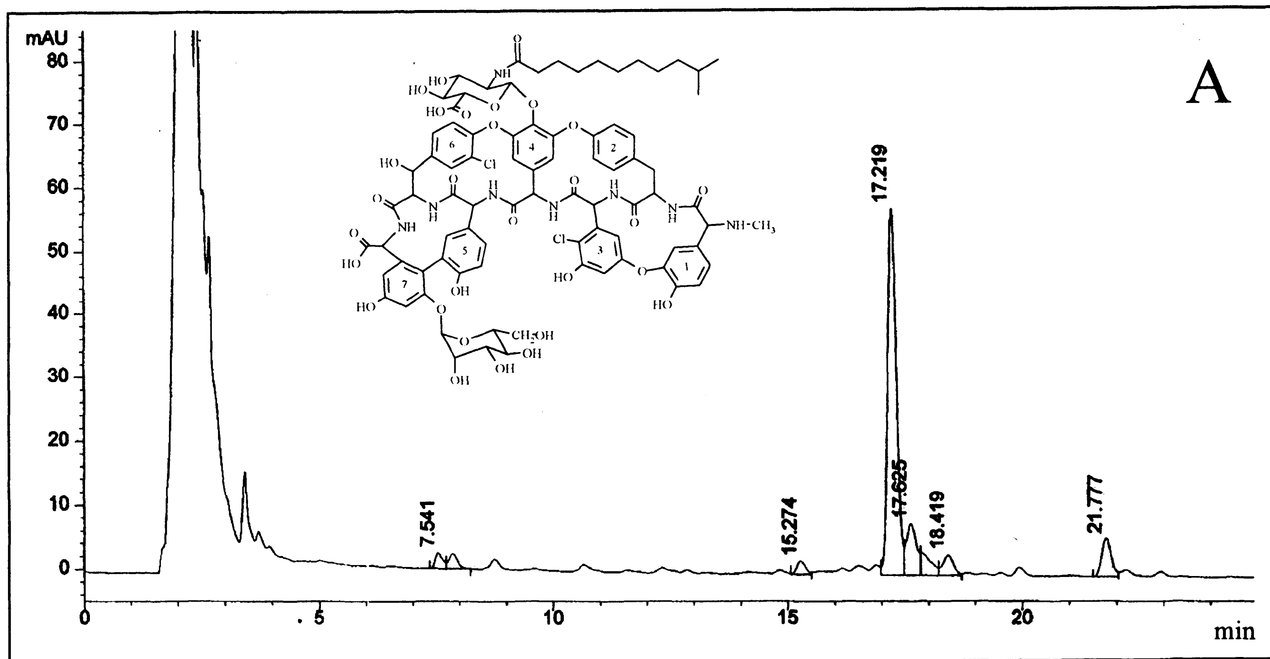
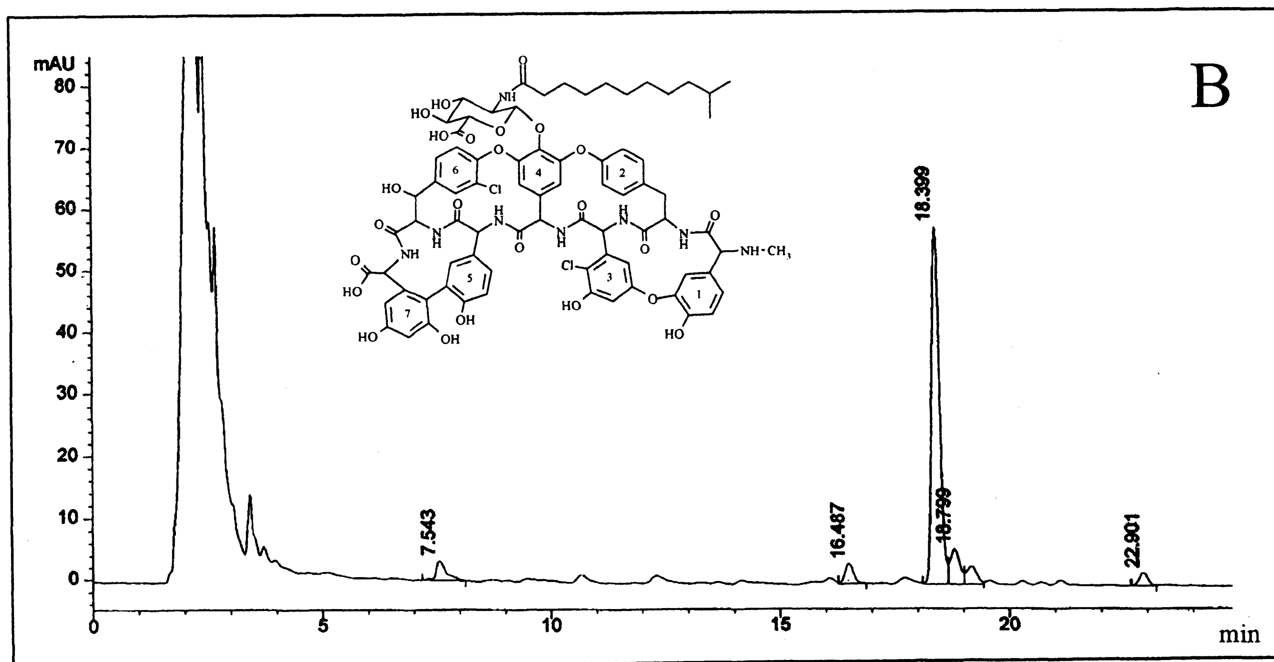
Fig. 1. HPLC analysis of the fermentation broth of strains ATCC39727 (A) and A40DM (B).





The chemical structures of the B0-A40926 and B0-demannosyl-A40926 are reported.

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[8] modified according to SELVA *et al.*[9]. A40926 and demannosyl-A40926 production

was estimated at different time intervals of the fermentation as described below.

A40926 and Demannosyl-A40926 Extraction and Column Purification

Samples were collected from each fermentation flask at different time intervals and processed for A40926 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 and demannosyl-A40926 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[10]. The packed resin was equilibrated with 0.2 M NaCl in 0.05 M NaH_2PO_4 pH 7.5 prior to mixing with 1 liter of 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.

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-minute 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 254 nm. As internal standard an authentic sample of A40926 antibiotic was used.

LC-MS

The molecular weights of the A40926 and demannosyl-A40926 products 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 46 V, Spray voltage 4.7 kV. HPLC conditions were as above described.

Microbiological Activity Assay

The microbiological activity of the isolated antibiotics was compared in endpoint experiments as follows. Serial dilutions of the purified demannosyl-A40926 and of a real 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 first dilution of the antibiotic showing no inhibition on *S. haemolyticus* was considered as the endpoint of inhibition.

Results and Discussion

Clones of *Nonomuraea* sp. ATCC 39727 were overlaid with *S. haemolyticus* 3902 (clinical isolate, teicoplanin resistant) at a concentration of 10^7 CFU/ml. 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. The A40DM strain was isolated from the *Nonomuraea* sp. strain ATCC 39727 as spontaneous mutant able to produce inhibition when overlaid with *S. haemolyticus*. *Nonomuraea* sp. ATCC 39727 and the derivative A40DM were fermented as described in the section Materials and Methods. The fermentation broth of both clones was basified to pH 12, filtered and analysed by HPLC as above described. As shown in Figure 1, *Nonomuraea* sp. ATCC 39727 produced the typical HPLC profile of A40926 antibiotic complex with the main peak represented by the B0 component showing a retention time of 17.22 minutes. The fermentation broth of strain A40DM showed a similar HPLC profile where, however, the main peak resulted delayed of 1.2 minutes (retention time 18.40 minutes). The HPLC retention time of the main peak of the fermentation broth of strain A40DM was identical to that of an authentic sample of demannosyl-A40926[6]. The main peaks of either the ATCC 39727 and A40DM fermentation broths were purified and analysed by LC-MS. The peak of ATCC 39727 (namely B0-A40926) showed a *m/z* of the mono protonated product corresponding to 1733.3 while for A40DM *m/z* was 1571.3 suggesting that the B0 component of the A40926 antibiotic lacked the mannose residue. This hypothesis was confirmed by the identity of the *m/z* fragmentation profile of the A40DM product with that of an authentic demannosyl-A40926 sample (data not shown)[6].

To test the possibility of using strain A40DM in a standard fermentation process for the production of demannosyl-A40926, we performed fermentation and analysis of production in either strain ATCC 39727 and

A40DM. The kinetics of demannosyl-A40926 production was similar to that of the A40926 antibiotic complex. The amount of demannosyl-A40926 produced was comparable to A40926 production in the parental strain. This suggested that the glycosilation process occurred in the last phases of A40926 biosynthesis as demonstrated in the case of the similar glycopeptide teicoplanin[11].

It was already reported the improved activity of semi-synthetic demannosyl-A40926 against *S. haemolyticus*[6]. The microbiological activity of demannosyl-A40926 was tested by agar diffusion as described in the section Material and Methods and showed an endpoint of 10 μg/ml *vs* 100 μg/ml for a A40926 standard sample. This confirmed the previously reported improved activity of demannosyl-A40926 against coagulase negative Staphylococci.

The biosynthesis of glycopeptide aglycones has always been considered of primary importance due to their antimicrobial activity and to the fact that aglycones are a convenient starting material for chemical modifications. The strain herein described is a good source for the one step production of demannosyl-A40926. Furthermore, the occurrence in the fermentation broth of demannosyl-A40926, which is identical in the rest of the structure to the A40926 molecule, indicates that coupling of mannose to A40926 occurs in the last phase of the biosynthesis. This is also consistent with the similarities in the production kinetics of either A40926 or demannosyl-A40926.

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