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Biotransformation of the lipoglycopeptide antibiotic A40926 by *Actinoplanes teichomyceticus* cells

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Compound A40926, produced by *Actinomadura* ATCC 39727, is a lipoglycopeptide antibiotic complex which inhibits Gram-positive bacteria and *Neisseria* species. Individual components of the complex have an identical glycopeptide core but differ in the acid chains attached to the amino group of the glucuronic moiety. Suspension cultures and resting cells of *Actinoplanes teichomyceticus* ATCC 31121 were able to deacylate compound A40926 factors to yield the glycopeptide nucleus, which can be then synthetically reacylated to form new analogs. In an optimized fed-batch deacylation process, 0.5 g L^{-1} of compound A40926 was almost completely converted into the deacyl derivative. Under the same conditions, deacylation was also accomplished with *tert*-butoxycarbonyl (*tert*-BOC) A40926, in which the amino group at C15 was blocked to prevent formation of diacyl analogs during reacylation. The deacylase is an endoenzyme whose preliminary characterization is presented.

Keywords: antibiotic; lipoglycopeptide; bioconversion; resting cells; deacylation; A40926

Introduction

Compound A40926 is a lipoglycopeptide antibiotic complex produced by *Actinomadura* sp ATCC 39727. While its activity is in most respects similar to that of other glycopeptides, such as vancomycin and teicoplanin, compound A40926 is much more active than these antibiotics against *Neisseria gonorrhoeae* [7,15]. In fermentation broths, compound A40926 is found as a complex of two main factors (A and B_0) and five minor components (A1, B1, RS1, RS2 and RS3) which have an identical glycopeptide core and differ in the fatty acid chains attached to the amino group of the glucuronic moiety (*n*-undecanoic in A, 10-methyl-undecanoic in B_0, 9-methyl-decanoic in A1, *n*-dodecanoic in B1, 8-methyl-nonanoic in RS1, *n*-decanoic in RS2 and *n*-tridecanoic in RS3) [7,15,16].

Hydrolysis of compound A40926 yields such products as the aglycone and pseudoaglycones, and several synthetic amide derivatives of this antibiotic have been prepared to obtain novel molecules with improved antimicrobial activity [9,11]. The deacylated glycopeptide nucleus of compound A40926 is a desired intermediate for chemical reacylation with other side chains to study the influence of fatty acid acyl groups on structure-activity relationships. Since attempts at chemical deacylation of molecules such as compound A40926 resulted in extensive side-reactions, enzymatic conversion was attempted. The screening of microorganisms able to transform compound A40926 revealed that certain actinomycetes belonging to the *Actinoplanes* genus (*Actinoplanes teichomyceticus* ATCC 31121, *Actinoplanes missouriensis* ATCC 23342 and *Actinoplanes* sp NRRL 3884) cleave the acyl groups from the initial complex of compound A40926 factors to yield the deacylated glycopeptide nucleus [4].

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This paper describes the bioconversion of compound A40926 and of its protected *tert*-BOC derivative into a deacylated nucleus by cells of *Actinoplanes teichomyceticus* ATCC 31121 (Figure 1). The free amino group at C_{15} (methylamine) in compound A40926 was blocked (*tert*-butoxycarbonyl, *tert*-BOC) prior to deacylation, to allow selective reacylation of the amino group of the glucuronic moiety during the synthetic preparation of reacylated derivatives. A preliminary characterization of the deacylating enzyme is also presented.

Materials and methods

A40926 tert-*BOC*

A40926 *tert*-BOC was prepared using Di-tert-butyldicarbonate (Janssen, Geel, Belgium) according to general procedures [1].

Microorganism and growth conditions

Actinoplanes teichomyceticus ATCC 31121 was maintained on slant of ISP no 1 medium [12]. Stock cultures of washed mycelium frozen at −80° C were used as inocula (2.5% v/v) for 100 ml preculture medium S/Bis (glucose 10, bacto peptone 4, bacto yeast extract 4, $MgSO_4 \cdot 7H_2O$ 0.5, K_2HPO_4 4 g L^{-1}) in 500-ml Erlenmeyer flasks. Preculture flasks were incubated for 48–72 h at 28° C on a rotary shaker at

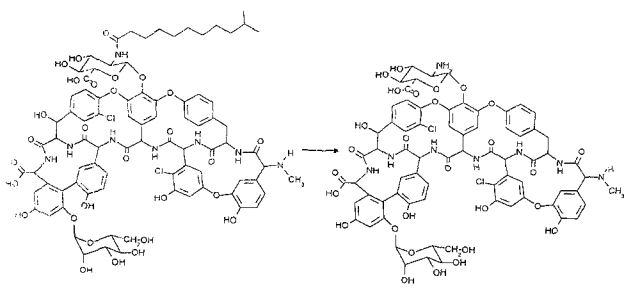

Figure 1 Deacylation of compound A40926 by *A. teichomyceticus* cells

200 rpm. A second and a third preculture stage were introduced for large-scale fermentations.

Biotransformation by suspension cultures in flask

500-ml flasks containing 100 ml of the following media: Medium C (glucose 20, yeast extract 5, asparagine 1.5, $CaCO_2$ 5, NaCl 0.1, $MgSO_4 \cdot 7H_2O$ 0.5, $CaCl_2 \cdot 2H_2O$ 0.1 g L^{-1}, mineral solution 1 ml L^{-1}), Medium T (glucose 5, meat extract 4, yeast extract 1, peptone 4, soybean meal 10, $CaCO_2$ 5, NaCl 2.5 g L^{-1}), AF/MS (glucose 20, yeast extract 2, soybean meal 8, $CaCO_2$ 1, NaCl 4 g L^{-1}), AUR/M (maltose 20, dextrin 10, meat extract 4, yeast extract 2, peptone 4, soybean meal 15, $CaCO_2$ 2 g L^{-1}) were inoculated with 5 ml of preculture. Mineral solution contained (g L^{-1}): 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). Medium T was reported by Singh *et al* [13] as a suitable medium for the fermentation of glycopeptide-producing strains. The other media were developed at the Lepetit Research Center. All media were prepared in distilled water and the pH was corrected to 7.0.

After 24, 48 and 72 h of growth, varying amounts of compound A40926 or A40926 *tert*-BOC sodium salt, previously dissolved in sterile water at a concentration of 50 g L^{-1}, were added to the cultures. Samples of the broth were collected every 24 h to monitor the biotransformation reaction by HPLC analysis. The biomass was measured as the volume of packed mycelium after centrifugation at 1200 × *g* for 10 min in 50-ml graduated conical polypropylene tubes (Falcon, NJ, USA).

Resting cells

After 48 h of growth, washed mycelia were resuspended in the following media: 20 mM Tris-HCl buffer at different pHs (7, 8, 8.5); water or physiological solution; 48-h-old culture broth diluted 1 : 2, 1 : 4, 1 : 8 with sterile water. The ratio of mycelium to medium was unmodified. Substrate was added to the resting cells at a final concentration of 0.5 g L^{-1} and biotransformation was followed by HPLC analysis.

Biotransformation in fermenters

2.4 L and 12 L of preculture were used to inoculate the 70-L and 300-L bioreactors containing 40 L and 200 L of biotransformation medium, respectively. After 48 h growth, the substrate was added to the cultures at a final concentration of 0.5 g L^{-1}. When a stepwise addition of sterile water was introduced in the 40- and 200-L biotransformations, it was started after 24 h from the substrate addition. A total of 20 L and 70 L water were added in 72 h to the 40- and 200-L reactors, respectively. The biotransformations in both fermenters were carried out at 29° C. 70-L and 300-L bioreactors were respectively stirred at 500 rpm and 180 rpm with an aeration rate of 50 L min^{-1} and 100 L min^{-1}.

HPLC analysis

Culture samples of 5 ml were collected and their pH was adjusted to 10 with 1 N NaOH. Mycelium was eliminated by centrifugation and the supernatant fluid was neutralized with 1 N HCl, filtered (0.45 μm) and injected for HPLC analysis. HPLC separations were performed on a 5-μm Hewlett Packard (Amsterdam, The Netherlands) ODS Hypersil column (4.6 × 100 mm) eluted by a linear gradient from 5% phase B (0.2% HCO_2NH_4/CH_3CN 3 : 7) in phase A (0.2% HCO_2NH_4/CH_3CN 9 : 1) to 53% B in 35 min. The flow rate was 1.8 ml min^{-1} and the injection volume was 30 μl. The column effluent was 1.8 ml min^{-1} and the injection volume was 30 μl. The column effluent was monitored at 254 nm. Pure samples of compound A40926 *tert*-BOC were used as external standard.

Enzyme preparation

After 48 h of growth in 100 ml of medium T, mycelium was centrifuged at 1000 × *g* for 15 min at 4° C, washed with 20 ml 10 mM Tris-HCl buffer, pH 8.0, and centrifuged again under the same conditions. The pellet was resuspended in 20 ml 10 mM Tris-HCl buffer and sonicated twice for 1 min at 25° C in a Rapidis Ultrasonic Disintegrator 180 (Ultrasonic Ltd, PBI, Italy), at 30 W power. The sonicated suspension was centrifuged at 15000 × *g* for 30 min and the supernatant phase was filtered through a 0.45-μm pore size Millex GS (Millipore).

The protein concentration in the enzyme extract was estimated by the method of Bradford [5] using the Bio Rad protein dye reagent and following the manufacturer's instructions. Bovine serum albumin (BSA) was used as a protein standard.

Enzyme assay

The activity of the enzyme was determined by monitoring deacyl compound formation with the HPLC procedure previously described. Unless otherwise specified, reaction mixtures of 5 ml contained 0.3 mg ml^{-1} of solubilised proteins and 0.4 mg ml^{-1} of substrate in 10 mM Tris-HCl buffer, pH 8.0.

The reaction mixture was incubated at 30° C for 48 h. The control assay consisted of washed mycelium resuspended in the same buffer with the same substrate concentration. The negative control was the reaction mixture without the enzyme extract. Typically the enzyme solution produced 0.083 mg ml^{-1} of deacyl compound (270 nmoles) under these assay conditions.

Results

Biotransformation by growing and resting cells

To optimize conditions for biotransformation, different concentrations of compound A40926 sodium salt or of its *tert*-BOC derivative (from 0.1 g L^{-1} to 1 g L^{-1}) were added to *Actinoplanes teichomyceticus* flask cultures at different growth phases. The best deacylation yield was achieved when 0.5 g L^{-1} of the substrate was added to 48-h-old *A. teichomyceticus* cultures, ie at the beginning of the stationary phase. *A. teichomyceticus* deacylated compound A40926 *tert*-BOC preparation in the same manner as compound A40926 (data not shown). Deacylation yield and biomass production were followed in four different media (Figure 2 and Table 1). In medium C, in which *A. teichomyceticus* growth was poor, only 23% of the starting material was recovered as deacyl compound after 168 h bio-

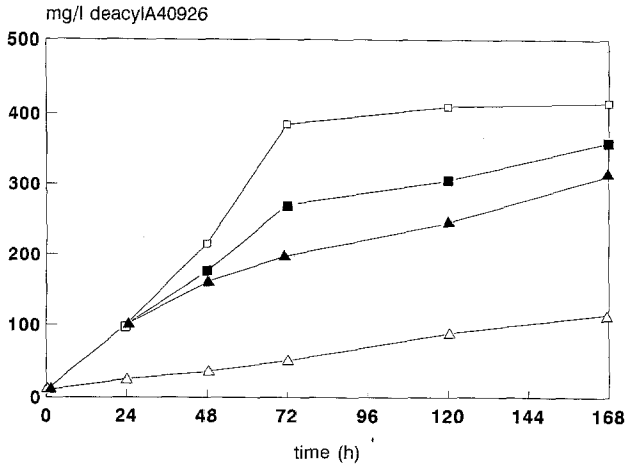

Figure 2 Biotransformation of compounds A40926 or A40926 *tert*-BOC by *A. teichomyceticus* in different media: medium T (▲), medium C (△), AUR/M (□) and AF/MS (■). 0.5 g L^{-1} of substrate was added to the flask cultures after 48 h growth

Table 1 Biomass production after 48 h growth of *A. teichomyceticus* in different media

Medium[a]	Packed mycelium volume[b]
C	2
T	10
AF/MS	16
AUR/M	30

[a]Medium composition as in Materials and Methods
[b]Packed mycelium volume measured as reported in Material and Methods

transformation. A deacylation increase to 60–70% was obtained in media T and AF/MS, in which biomass production was five and eight times higher than in medium C, respectively. In the medium AUR/M in which the biomass production was fifteen-fold higher, the deacylation yield increased up to 80–85% and the kinetics of biotransformation were faster, reaching 77% of conversion at 72 h after the substrate addition.

Resting cells of *A. teichomyceticus* in water deacylated 100% of the starting substrate in 120 h, showing that deacylating activity was associated with the mycelium fraction. Only a little activity was detected in the filtered broth, probably due to a residual mycelium contamination or lysis. The complete conversion of the substrate into the deacyl derivatives was also achieved by resuspending resting cells in several buffers at pH varying between 7 and 8.5 and in physiological solution (data not shown). When resting cells were resuspended in the fermentation broth diluted with increasing water percentages, a progressive improvement of the deacylation yield was observed. In the ratio one part fermentation broth to eight parts water, the deacylation yield reached 98% in 120 h. These results showed that the removal of the fermentation broth or its opportune dilution favoured the bioconversion.

Scaling-up of biotransformation

Preliminary scale-up of the initial biotransformation conditions to a 70-L fermenter (40-L fermentation volume) gave a deacylating yield lower than in flasks. Only 38% bioconversion was achieved after 120 h in Medium T. When AUR/M medium was used, the biomass production was four-fold higher than in medium T and 80% deacylation yield was obtained in 96 h. The positive effect of broth dilution observed in flasks was achieved in the 70-L biotransformation by a stepwise addition of sterile water (6.7 L of water per day for a total of 20.1 L in 72 h after substrate addition). Under these conditions, 98% deacylation was reached at 96 h (Figure 3). Further scale-up of the water fed-optimized bioconversion process to the 300-L reactors (200-L working volume) gave an average deacylation yield of 93% ± 2.5 (data not shown).

Characteristics of the deacylase enzyme activity

A soluble crude enzyme preparation containing 20% of the initial mycelium activity was obtained by sonication under the conditions described in Materials and Methods. Longer sonication times as well as the use of Triton X-100 inactivated the solubilized enzyme. By increasing the volume of sonication buffer, a higher protein quantity was extracted from the mycelium but the deacylase specific activity decreased. After all these treatments, about 50% of the initial enzyme activity was retained by the mycelium.

The deacylase activity in this crude preparation increased linearly up to a protein concentration of 1.2 mg ml^{-1}. The dependence of enzyme activity over time was linear, but the reaction proceeded very slowly. As shown in Figure 4, only 18 μM of deacyl compound was obtained after 24 h of incubation and the curve reached a plateau after 30 h. Enzyme activity was stable at 30° C for several days, while it was completely lost after 10 min treatment at 60° C. Freezing and thawing of the solution reduced the activity by half. The enzyme activity was not affected by varying the pH in the range from 6 to 8.5, but was completely lost below pH 5 and above pH 9 (data not shown).

The effects of various cations and metal-chelating agents on deacylase activity are shown in Table 2. Among monovalent ions, only Hg^{+} inhibited the enzyme. All the divalent cations tested affected protein activity. Ca^{2+} and Mg^{2+}

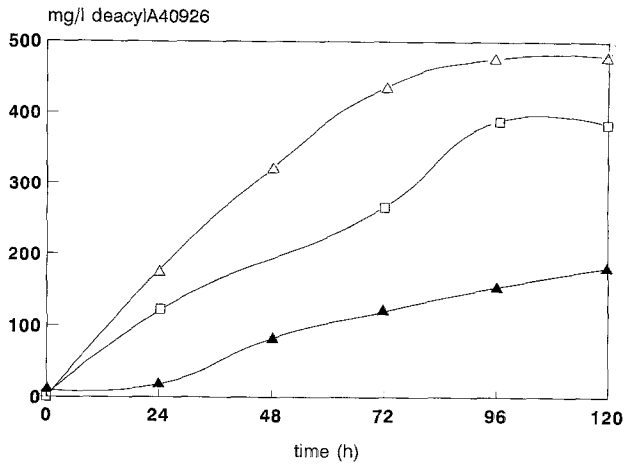

Figure 3 Biotransformation of compound A40926 *tert*-BOC in a 70-L fermenter: medium T (▲), AUR/M medium (□) and AUR/M medium diluted by a stepwise water addition (△) as described in Materials and Methods

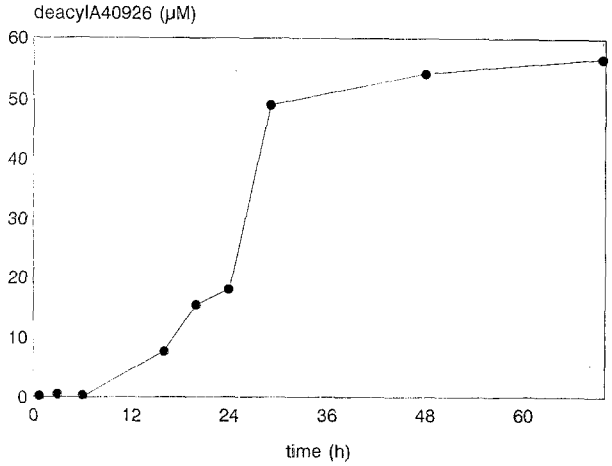

Figure 4 Enzymic conversion of compound A40926 *tert*-BOC. The reaction was carried out at 30° C with a protein concentration of 0.3 mg ml^{-1} as described in Materials and Methods

Table 2 Effects of ions on deacylase activity

Compound added	Concentration (mM)[a]	Relative activity (%)[b]
Na^+	0.1	100
	10	100
K^+	0.1	100
	10	100
Hg^+	0.1	75
	10	48
Hg^{2+}	0.1	60
	1	1
Cu^{2+}	0.1	133
Zn^{2+}	0.1	40
	1	20
Ca^{2+}	0.1	80
	10	80
Mg^{2+}	0.1	80
	10	80
Fe^{3+}	0.1	100
	1	90
	10	0

[a]Different concentrations were tested in the range between 0.1 and 10 mM. Only concentrations showing significant effects are reported
[b]Relative activity is expressed as a percentage of the enzyme activity measured as described in Materials and Methods

slightly reduced the enzyme activity whereas a marked inhibition was caused by Zn^{2+} and Hg^{2+}. The deacylating activity was enhanced by Cu^{2+} 0.1 mM. The positive effect of Cu^{2+} was also shown by the inhibitory effect of EDTA. Fe^{3+} exhibited an inhibitory effect on enzyme activity. We also investigated the effect of dithiothreitol (DTT), since this reducing agent is commonly used to stabilise crude protein extracts. No protection effect was observed, but a slight decrease in activity was obtained at 5 mM, indicating a possible involvement of a sulfhydryl group in the mechanism of action of the enzyme.

Discussion

Biotransformation is one of the widely practised approaches for structural modification of complex natural compounds. Among the known microbial transformations of antibiotics, enzyme-catalyzed acylation and deacylation of β-lactams, macrolides and aminoglycosides have been often described as advantageous in comparison to organic synthesis reactions [see 10 for references]. The most famous application is the industrial use of penicillin acylases for the production of 6-aminopenicillanic acid (6-APA), a key-intermediate of various clinically important semi-synthetic derivatives [14]. Deacylating capability is widely represented among the members of the *Actinoplanes* genus. *Actinoplanes utahensis* is reported to deacylate different antibiotics such as A21978C [2], echinocandin B [3] and penicillins G, K and V [8]. Leucomycin A5 and ardicins have been deacylated by *Actinoplanes missouriensis* [13] and *A. teichomyceticus* [6] respectively.

Actinoplanes teichomyceticus ATCC 31121, *Actinoplanes missouriensis* ATCC 23342 and *Actinoplanes* sp NRRL 3884 deacylated the lipoglicopeptide A40926 [4]. *Actinoplanes teichomyceticus* is the most efficient of these microorganisms. Its growing cultures and resting cells are able to almost completely convert 0.5 g L^{-1} of compound A40926 as well as its protected *tert*-BOC derivative. Two critical factors affecting the deacylation yield have been identified during the optimization of a scalable batch process. Since the deacylase is an endoenzyme, biomass productivity is a relevant parameter. Another important factor is the removal of the fermentation broth in flasks or its dilution with water in fermenters, which suggests the presence of either better substrates or inhibitors of the deacylase activity.

The characteristics of the *A. teichomyceticus* deacylase seem similar to those of the deacylating activities so far described in the *Actinoplanes* genus [2,3,6,8,13], which are endoenzymes associated with membrane integrity and with a rather broad-substrate specificity. The basic physiological function of these enzymes in the producer cells does not seem to be correlated with the deacylation reactions of glycopeptides. To this purpose an interesting result is that *A. teichomyceticus* does not deacylate teicoplanin, a glycopeptide antibiotic which it produces [6]. The low specificity for substrates and the unclear physiological role of such enzymes does not represent an obstacle to their industrial application, if appropriate processes are developed. This is widely demonstrated by the industrial use of immobilized penicillin acylase for which the reaction forming 6-APA is considered a side-reaction of the deacylase enzyme without any physiological relevance [14].

The optimized fed-batch process of the deacylation described in this paper has been successfully used to produce about 200 grams of *tert*-BOC deacyl compound used for the preparation of new semi-synthetic analogs, whose therapeutic index is under evaluation.

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