DEACYLATION OF A21978C, AN ACIDIC LIPOPEPTIDE ANTIBIOTIC COMPLEX, BY *ACTINOPLANES UTAHENSIS*

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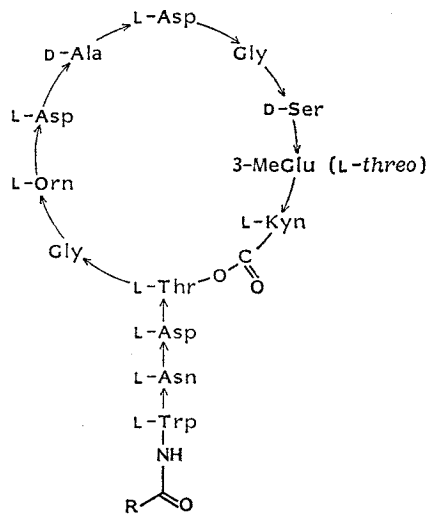
A21978C, produced by *Streptomyces roseosporus* NRRL 11379, is an acidic lipopeptide antibiotic complex that inhibits Gram-positive bacteria. Individual factors of the complex possess an identical peptide core or "nucleus", and are differentiated by the distinctive fatty acid acyl group attached to the *N*-terminus of the nucleus. Certain members of the family Actinoplanaceae deacylated A21978C to yield the unaltered nucleus, which was then reacylated to form new analogs. *Actinoplanes utahensis* NRRL 12052 was the most efficient of these cultures, producing up to 500 μg of nucleus per ml of culture broth per hour. eacylation was also accomplished with semi-pure and *tert*-butoxycarbonyl (*tert*-BOC)-A21978C. In the latter, the ornithine amino group was blocked to prevent formation of diacyl analogs during reacylation. The acylase was an endoenzyme present in submerged cultures of *A. utahensis* from <18 to >168 hours of incubation. Whole cells suspended in phosphate buffer or entrapped in polyacrylamide gel also deacylated A21978C efficiently.

A21978C, produced by *Streptomyces roseosporus* NRRL 11379, is an acidic lipopeptide antibiotic complex that inhibits Gram-positive bacteria[1]. A21978C consists of three major factors; C_1, C_2 and C_3; and three minor factors; C_0, C_4 and C_5. All six factors contain a common, cyclic, polypeptide core, or "nucleus". They differ only by the fatty acid acyl group attached to the nucleus at the *N*-terminal tryptophan residue (Fig. 1). These acyl groups have been identified in A21978C$_1$, C_2 and C_3 as branched-chain fatty acyl units containing 11, 12 and 13 carbon atoms, respectively. A21978C$_0$

Fig. 1. Structure of A21978C major factors.



A21978C$_1$ R=$CH_3CH_2CH(CH_3)(CH_2)_6$–

A21978C$_2$ R=$(CH_3)_2CH(CH_2)_8$–

A21978C$_3$ R=$CH_3CH_2CH(CH_3)(CH_2)_8$–

contains a C-10 fatty acid acyl unit while C_4 and C_5 contain C-12 units[2].

The peptide nucleus of A21978C was desired as an intermediate for chemical reacylation with other side chains in order to study the structure-activity relationships of fatty acid acyl units in the resulting semi-synthetic analogs[3,4]. Because earlier attempts at chemical deacylation of other lipopeptides had resulted in extensive side reactions[2], enzymatic conversion was attempted. The resultant screening revealed that certain members of the family Actinoplanaceae were capable of this conversion, cleaving the acyl side chains from the initial complex of factors to yield a single product, the inactive A21978C nucleus. Another paper provides characterization of the nucleus and describes its use in synthesis of new A21978C analogs[5]. One of these analogs, LY146032, which possesses an *n*-decanoyl side chain, has demonstrated a therapeutic index superior to A21978C and numerous other analogs. This compound, also known as daptomycin, has been widely tested[6~8] and is currently on clinical trial.

Materials and Methods

Culture Growth

Stock Actinoplanaceae cultures, preserved in the vapor phase of liquid nitrogen, were introduced into wide-mouth 250-ml Erlenmeyer flasks containing 50 ml of a medium composed of sucrose 2.0%, pre-cooked oatmeal 2.0%, distiller's grain 0.5%, yeast 0.25%, K_2HPO_4 0.1%, KCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05% and $FeSO_4 \cdot 7H_2O$ 0.0002% in deionized water. After incubation at 30°C for 72 hours on a gyrotatory shaker orbiting at 250 rpm, the resulting mycelial suspension was transferred (2%) into medium PM3. This medium contained sucrose 2.0%, peanut meal 1.0%, K_2HPO_4 0.12%, KH_2PO_4 0.05% and $MgSO_4 \cdot 7H_2O$ 0.025% in tap water. Incubation of both stages was identical except where otherwise noted.

Growth Measurements

To determine deacylase activity per unit of biomass at specific points during prolonged incubation, *Actinoplanes utahensis* growth was measured gravimetrically by washing the broth solids twice with deionized water and then drying to constant weight. The weight of insoluble medium components present in the medium prior to inoculation was subtracted from the values obtained for samples taken during the incubation period.

Deacylation Procedure

After *A. utahensis* had been incubated for a period of approximately 60~90 hours in the PM3 medium, a sterile solution of A21978C was pulsed into the broth. Incubation was continued for a period appropriate for deacylation of the level of substrate employed, normally about 1 hour per 500 μg of highly purified substrate per ml of broth. Less pure preparations, containing as little as 20% A21978C, were also successfully deacylated but at a somewhat slower rate. Because the limited aqueous solubility of these impure preparations precluded filter sterilization, they were pulsed into the *A. utahensis* broth as unsterile powders. In order to maintain a constant pH during deacylation in shaken flasks, additional phosphate buffer was incorporated into the broth, to a final concentration of 0.1 M, at the desired pH immediately prior to substrate addition. The additional phosphate was omitted from stirred bioreactors where automatic pH control could be implemented.

Initial Reacylation of A21978C Nucleus

Filter paper discs were saturated with samples believed to contain A21978C nucleus. The discs were then dried, buffered with a 2% solution of $NaHCO_8$, and acylated with an active ester solubilized in petroleum ether.

A21978C *tert*-BOC

Reacylation of the natural A21978C nucleus was desired to yield a single product acylated at the *N*-terminal tryptophan residue. However, an additional acylation frequently occurred at the amino

side chain of the ornithine residue. To prevent formation of diacyl analogs during reacylation of the nucleus, the free ornithine-amino group of A21978C was blocked (*tert*-butoxycarbonyl (*tert*-BOC)) prior to deacylation. *A. utahensis* was subsequently found to deacylate the A21978C *tert*-BOC preparation in the normal manner. The resulting *tert*-BOC nucleus was then reacylated and thereafter deblocked to yield monoacyl analogs of A21978C[3].

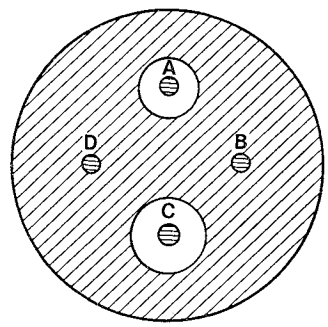
Results and Discussion

Deacylation of A21978C

Removal of the lipid side chains abolished the antibiotic activity of A21978C. Incubation of A21978C with *A. utahensis* consequently resulted in a continuing decline of antimicrobial activity, which allowed the apparent progress of the enzymatic deacylation reaction to be monitored microbiologically. This was accomplished by employing a standard disc-plate agar diffusion procedure to compare the initial antimicrobial activity of the starting substrate with the residual activity after incubation in the presence of *A. utahensis*. Fig. 2 shows filter paper discs impregnated with samples prior to placement on a nutrient agar plate seeded with *Micrococcus luteus*. Pad 'A', surrounded by a large zone of growth inhibition, represents a sample of the *A. utahensis* broth, in which antibiotic activity was not naturally present, immediately after the addition of A21978C. Pad 'B', which has no inhibitory zone, represents a sample of the same broth after an incubation period sufficient for deacylation to occur. The absence of an inhibitory zone indicated that some molecular change had destroyed antibiotic activity. This suggested deacylation, since the nucleus did not possess antimicrobial activity. Acylation of the sample on pad 'B' with hexanoyl chloride restored antimicrobial activity as shown on pad 'C', further suggesting the presence of nucleus capable of being reacylated. Additional evidence was obtained by monitoring samples through descending adsorption chromatography on Whatman No. 1 paper in butanol - pyridine - acetic acid - water (15 : 10 : 3 : 12). Detection by long-wave UV confirmed the conversion of A21978C to a single new component that was further converted to an A21978C-like compound upon reacylation. Pad 'D' is a negative control, demonstrating that hexanoyl chloride did not possess antimicrobial activity in the absence of nucleus.

An analytical HPLC system was subsequently developed to quantitate deacylation of A21978C and conversion to the common, much more polar, nucleus. This system was also functional with the A21978C *tert*-BOC complex and the *tert*-BOC nucleus (Fig. 3). The quantity of A21978C deacylated or otherwise modified during incubation with *A. utahensis* was determined by subtraction of the residual level from the initial level. Nucleus was quantitated directly.

Fig. 2. Biological monitoring of A21978C deacylation by *Actinoplanes utahensis*.



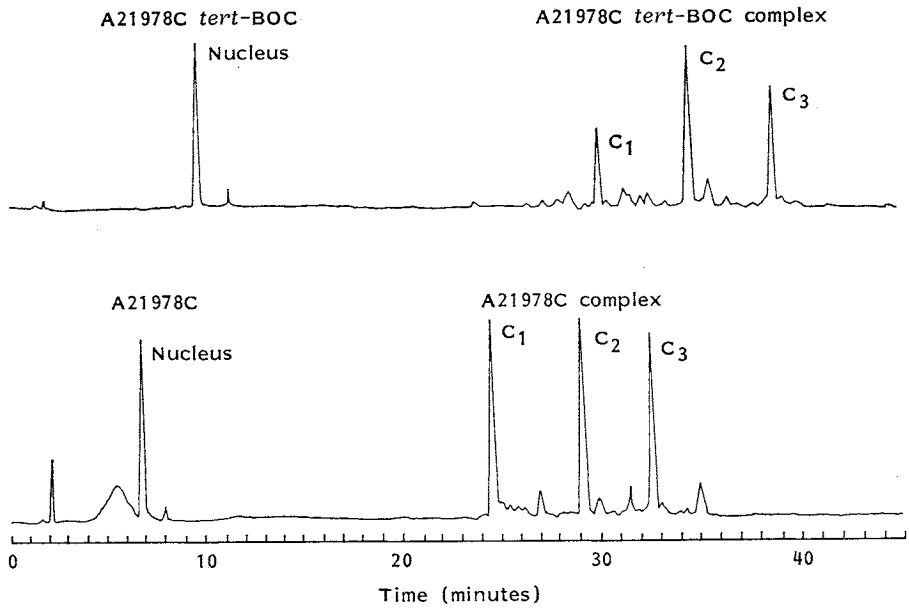
Nutrient agar plate seeded with *Micrococcus luteus*.

Filter paper discs contained; A: A21978C complex, B: A21978C nucleus, C: nucleus acylated with hexanoyl chloride, D: hexanoyl chloride.

Growth Profile of *A. utahensis*

When *A. utahensis* was grown in the PM3 medium, the broth pH initially moved slowly upward from 7.0 to peak at 7.5 during the third and fourth days, then declined to about 6.0,

Fig. 3. HPLC identification of major A21978C and A21978C *tert*-BOC factors and nuclei.

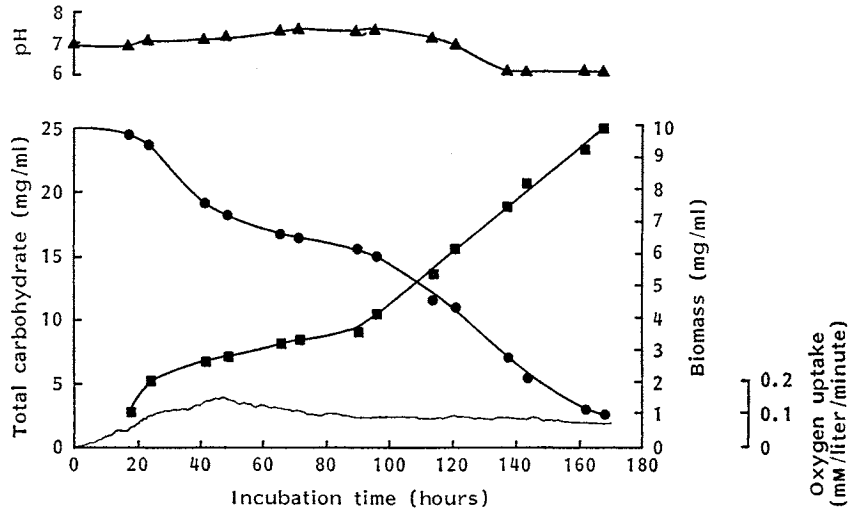


Column: NOVA C18 (Waters Assoc.), flow rate: 1.5 ml/minute, gradient: 5~50% B in 8 minutes, No. 6*, 50~75% B in 12 minutes, No. 4*, 75~100% B in 15 minutes, No. 6*, mobile phase: CH_3CN - H_2O· 0.5% $(NH_4)H_2PO_4$ (w/v); (A) 10 : 90, (B) 45 : 55, detection: 210 nm (UV).

* Waters Assoc. gradient curve profile number.

Fig. 4. Time course profile of *Actinoplanes utahensis* in PM3 medium.

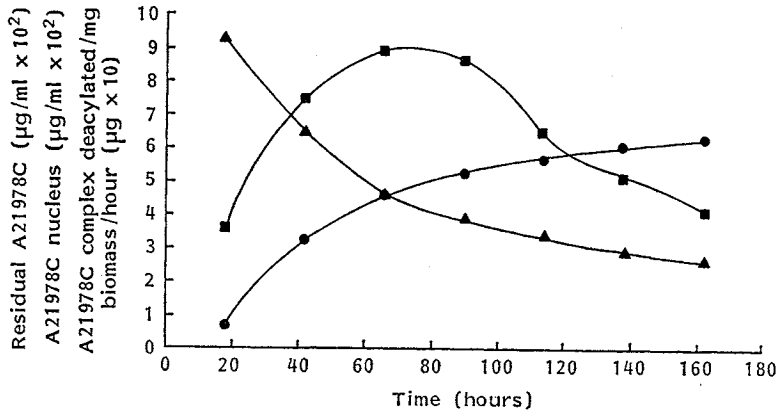
▲ pH, ● total carbohydrate, ■ biomass, — oxygen uptake.



where it remained. The total carbohydrate level declined steadily, though biphasically, displaying an inverse correlation with the biphasic increase in biomass. Oxygen uptake peaked at 0.15 mM/liter/minute at 40~50 hours in stirred bioreactors, then declined slightly and stabilized. Maximum biomass was not achieved until seven or more days of incubation (Fig. 4).

Fig. 5. Effect of *Actinoplanes utahensis* culture age on A21978C deacylation.

■ A21978C deacylated/mg *A. utahensis* biomass, ● A21978C nucleus, ▲ A21978C complex.



Effect of *A. utahensis* Biomass Age on Deacylation Kinetics

The ability of *A. utahensis* to deacylate A21978C was examined daily during a growth period of 7 days. Both deacylation and nucleus formation occurred at the earliest age tested, 18 hours. On a unit volume basis, the rates of both bioactivity reduction and nucleus formation increased daily throughout the period, though the rate of increase was much slower after the third day. When deacylation was calculated on the basis of biomass, however, it became apparent that the enzymatic activity per unit of biomass was greatest at 65~85 hours (Fig. 5). Although the deacylating activity remained present beyond 160 hours, the decline in activity per unit of biomass late in the growth period suggested that synthesis of the enzyme occurred primarily before 65 hours. The existing enzyme was then diluted by additional biomass as growth of *A. utahensis* continued.

Table 1. Effect of *Actinoplanes utahensis* broth pH on A21978C bioactivity reduction and nucleus conversion efficiency.

Broth pH	Bioactivity reduction[a] (μg/ml/hour)	Nucleus produced (μg/ml/hour)	Conversion efficiency[b] (%)
5.0	334	137	41
6.0	391	238	61
6.5	424	330	78
7.0	527	453	86
7.5	592	498	84
8.0	594	493	83
9.0	605	438	72

[a] Initial A21978C concentration was 1,760 μg/ml. Preparation was 40% pure.
[b] 2 hours incubation.

Effect of Broth pH on Bioconversion

The pH of *A. utahensis* broth affected both the deacylation rate of A21978C and the efficiency of nucleus conversion. Antibiotic activity was reduced most rapidly at pH 7.5~9.0 (Table 1). However, alkalinity, particularly above pH 9, was known to hydrolyze the lactone bond of the cyclic peptide[2], which also destroyed antibiotic activity in the absence of deacylation. The ring-opened nucleus could not be recyclized after formation and thus represented an undesirable product. In addition, variable, though ordinarily minor, amounts of non-specific bioactivity degradation occurred under some conditions. Putative deacylation was therefore confirmed by quantitative HPLC measurements of the nucleus actually formed. The highest levels of nucleus were observed at pH 7.5~8.0. Conversion efficiency, calculated quantitatively on the basis of A21978C disappearance vs. actual nucleus

Table 2. Location and nature of deacylase.

Actinoplanes utahensis biomass	Aqueous suspending agent	Nucleus produced[a] (μg/ml/hour)
—	Uninoculated PM3 medium	0
Whole cells	PM3 medium	382
—	PM3, cells removed at 72 hours	23
Whole cells	Phosphate buffer	341
Disrupted cell pellet	Fresh phosphate buffer	359
—	Supernatant buffer from cell disruption	38

[a] Initial A21978C concentration was 5 mg/ml. 6 hours incubation.

produced, was slightly greater at pH 7.0. Because the nucleus was most stable under neutral or slightly acidic conditions, pH 7.0 was selected as the standard condition for deacylation.

Deacylase Characteristics

In order to determine whether the deacylase was a cellular enzyme or an exoenzyme, A21978C was pulsed into several reaction mixtures. Bioconversion to nucleus did not occur in the uninoculated PM3 medium (Table 2). The standard whole-broth culture, *A. utahensis* in the PM3 medium in which it had been grown for three days, produced a normal level of nucleus. The same PM3 medium in which the culture had been grown but from which the mycelia had been removed, produced 6% of the nucleus level observed when the *A. utahensis* cells were present. Washed whole cells suspended in phosphate buffer produced normal levels of nucleus, indicating that the deacylase was a cellular enzyme.

Washed *A. utahensis* mycelia were fractionated by sonication in phosphate buffer and sedimented by centrifugation at $10,000 \times g$. The cell pellet, resuspended in fresh buffer, produced control levels of nucleus while the supernate from the sonicated cells produced a very low level of nucleus. These data indicated that the deacylase was a particulate enzyme, possibly associated with the cellular membrane.

The deacylase was also apparently a constitutive enzyme produced in a broad variety of complex, as well as synthetic, media over a temperature range of <25 to >40°C. Although the rate of deacylation varied with incubation temperature, nucleus conversion efficiency was similar over the same temperature range. Deacylation of A21978C proceeded rapidly, with efficient conversion to nucleus, under a wide variety of conditions. Refrigerated mycelia of *A. utahensis* retained deacylase activity for periods in excess of 6 weeks.

Deacylation of Other Actinoplanaceae

Screening of numerous cultures representing several microbial families indicated that additional members of the family Actinoplanaceae also deacylated A21978C. These cultures were grown under the conditions described for *A. utahensis* to obtain direct quantitative comparisons. *A. utahensis* and *Actinoplanes missouriensis* were markedly superior to the remaining cultures in the rate of bioactivity reduction (Table 3). Four of the five cultures were similar in nucleus conversion efficiency, with poor conversion efficiency being demonstrated only by *Actinoplanes* sp. NRRL 12065. *A. utahensis* was the most efficient of the group, demonstrating both the most rapid rate of A21978C disappearance and the highest percentage of nucleus conversion.

Table 3. Deacylation of A21978C by various members of the family Actinoplanaceae.

Culture	Bioactivity reduction[a] (μg/ml/hour)	Conversion efficiency[b] (%)
Actinoplanes utahensis NRRL 12052	616	93
A. missouriensis NRRL 12053	504	90
Actinoplanes sp. NRRL 8122	258	84
Actinoplanes sp. NRRL 12065	273	21
Streptosporangium roseum NRRL 12064	291	88

[a] Initial A21978C concentration was 2 mg/ml. Pure substrate.
[b] After 2 hours incubation.

Deacylation by Immobilized Cells

The excellent stability of the deacylating enzyme and the aqueous solubility of A21978C suggested immobilization of *A. utahensis* mycelia as a potential method of continuous nucleus production. Whole cells were subsequently entrapped in polyacrylamide gel[9] and loaded into a column. A21978C was solubilized in 0.1 M phosphate buffer at pH 7.0. The column was equilibrated and then permitted to flow at a rate providing enzyme-substrate exposure equivalent to that in shaken flasks during batch experiments. Normal disappearance of the A21978C bioactivity began immediately and was followed later by the appearance of nucleus. Conversion efficiency was similar to that observed with free mycelia suspended in phosphate buffer.

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