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

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(Received for publication March 7, 1988)

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. Eaclylation 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 bacteria1). A21978C consists of three major factors; C1, C2 and C3; and three minor factors; C0, C4 and C5. 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 A21978C1, C2 and C3 as branched-chain fatty acyl units containing 11, 12 and 13 carbon atoms, respectively. A21978C0

Fig. 1. Structure of A21978C major factors.

A21978C1: R=CH3(CH2)11-CH3
A21978C2: R=(CH2)12CH(CH3)1-CH3
A21978C3: R=CH3(CH2)13CH(CH3)1-CH3
contains a C-10 fatty acid acyl unit while C4 and C5 contain C-12 units. 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. Because earlier attempts at chemical deacylation of other lipopeptides had resulted in extensive side reactions, 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. 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 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%, K2HPO4 0.1%, KCl 0.05%, MgSO4·7H2O 0.05%, and FeSO4·7H2O 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%, K2HPO4 0.12%, KH2PO4 0.05% and MgSO4·7H2O 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 NaHCO3, 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 prepara-
tion in the normal manner. The resulting tert-BOC nucleus was then reacylated and thereafter de-
blocked to yield monoacyl analogs of A21978C3).

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 micro-
biologically. 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 in-
cubation 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 antimi-
crobial 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 chromato-
graphy on Whatman No. 1 paper in butanol - pyridine - acetic acid - water (15:10:3:12). Detec-
tion 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 subsequent-
ly 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 incuba-
tion with A. utahensis was determined by subtrac-
tion of the residual level from the initial level. 
Nucleus was quantitated directly.

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. 2. Biological monitoring of A21978C deacyla-
tion by Actinoplanes utahensis.

Nutrient agar plate seeded with Micrococcus 
luteus.
Filter paper discs contained; A: A21978C com-
plex, B: A21978C nucleus, C: nucleus acylated 
with hexanoyl chloride, D: hexanoyl chloride.
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: CH3CN - H2O - 0.5% (NH4)2HPO4 (w/v); (A) 10 : 90, (B) 45 : 55, detection: 210 nm (UV).

* Waters Assoc. gradient curve profile number.

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).
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.

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 peptide⁶, 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.

<table>
<thead>
<tr>
<th>Actinoplanes utahensis biomass</th>
<th>Aqueous suspending agent</th>
<th>Nucleus produceda (μg/ml/hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>Uninoculated PM3 medium</td>
<td>0</td>
</tr>
<tr>
<td>Whole cells</td>
<td>PM3 medium</td>
<td>382</td>
</tr>
<tr>
<td>—</td>
<td>PM3, cells removed at 72 hours</td>
<td>23</td>
</tr>
<tr>
<td>Whole cells</td>
<td>Phosphate buffer</td>
<td>341</td>
</tr>
<tr>
<td>Disrupted cell pellet</td>
<td>Fresh phosphate buffer</td>
<td>359</td>
</tr>
<tr>
<td>—</td>
<td>Supernatant buffer from cell disruption</td>
<td>38</td>
</tr>
</tbody>
</table>

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 x 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 Actinoplanaeeae.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Bioactivity reduction (µg/ml/hour)</th>
<th>Conversion efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Actinoplanes utahensis</em> NRRL 12052</td>
<td>616</td>
<td>93</td>
</tr>
<tr>
<td><em>A. missouriensis</em> NRRL 12053</td>
<td>504</td>
<td>90</td>
</tr>
<tr>
<td><em>Actinoplanes</em> sp. NRRL 8122</td>
<td>258</td>
<td>84</td>
</tr>
<tr>
<td><em>Actinoplanes</em> sp. NRRL 12065</td>
<td>273</td>
<td>21</td>
</tr>
<tr>
<td><em>Streptosporangium roseum</em> NRRL 12064</td>
<td>291</td>
<td>88</td>
</tr>
</tbody>
</table>

* Initial A21978C concentration was 2 mg/ml. Pure substrate.
* 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

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 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.

Acknowledgments

The authors thank the many members of the Lilly Research Laboratories who contributed to this investigation. We especially thank R. Miller, D. R. Berry and D. M. Berry for invaluable analytical HPLC assistance and G. M. Clem and R. W. Wetzel for skillful technical assistance.

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