# ENZYMATIC AND CHEMICAL MODIFICATIONS OF
# LIPOPEPTIDE ANTIBIOTIC A21978C:
# THE SYNTHESIS AND EVALUATION
# OF DAPTOMYCIN (LY146032)

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The novel lipopeptide antibiotic A21978C complex is active against Gram-positive organisms. This complex consists of a common peptide nucleus with various lipid acyl groups at the N-terminus characteristic of each individual factor. The fatty acid acyl group is removed by incubation of the A21978C complex with *Actinoplanes utahensis* to give the peptide nucleus. This peptide nucleus has the same amino acid sequence as A21978C. New analogs of A21978C were synthesized by acylation of the N-terminus of a *tert*-butoxycarbonyl (*tert*-BOC)-protected nucleus and subsequent deprotection. ^{1}H NMR showed that the newly introduced acyl group was at the desired N-terminus. Three major groups of analogs were synthesized bearing fatty acid acyl, amino-aroyl and extended peptide side chains. Each analog was evaluated for antimicrobial activity and acute toxicity. Of these analogs, the *n*-decanoyl analog of A21978C (LY146032) gave the best survival in the mouse acute toxicity test at a high dose of 1,000 mg/kg, iv and was chosen for further study. This analog has been named daptomycin.

Several classes of antibiotics such as the lipopeptides and the glycopeptides have resisted extensive systematic modification primarily due to their complex chemical structures. However, availability of more precise analytical methods and isolation techniques now make their modification more feasible.

A21978C complex is a family of lipopeptide antibiotics, produced by *Streptomyces roseosporus*.[1] The structures of the major components are shown in Fig. 1. Each member of this family has the same thirteen amino acid peptide core or "nucleus" but differs in the structure of the fatty acid which acylates the N-terminus. A21978C factors $C_1$, $C_2$ and $C_3$ have acyl groups which are composed of branched fatty acids having 11, 12 and 13 carbon atoms, respectively. The A21978C family illustrates some of the common characteristics of the lipopeptide class. The major feature of this class includes a short peptide of 5 to 15 amino acids which may be arranged into a linear form or in a cyclic array, or a combination of both, as they are in A21978C. These amino acids may have unusual structures, such as the D-amino acids L-kynurenine (kyn) and 3-methylglutamic acid residues found in A21978C. The N-terminus of this peptide is invariably N-acylated by a lipid group, usually a fatty acid having 10 to 18 carbon atoms which may be linear or branched. As a class, lipopeptide antibiotics exhibit a wide variety of antimicrobial actions, including activity against Gram-positive and Gram-negative bacteria or fungi. Distinct structural features control the nature of the antimicrobial spectrum.[2] Each of the A21978C factors has excellent activity against Gram-positive bacterial infections (Table 1).[3~5]

Fig. 1. Structure of A21978C.
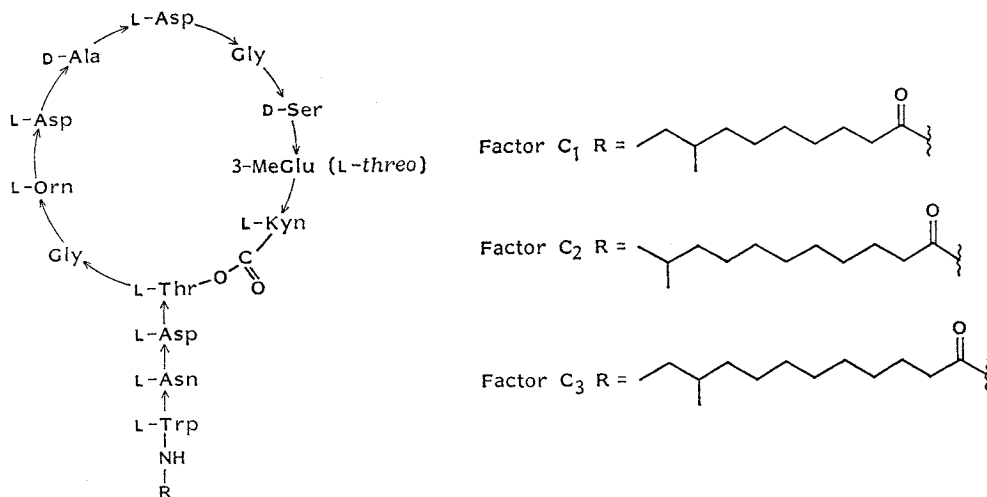


Table 1. *In vivo* activity and acute toxicity of A21978C factors.

| Factor | Mouse ED$_{50}$ (mg/kg×2, sc) | | Mouse toxicity, LD$_{50}$ (mg/kg, iv) |
| --- | --- | --- | --- |
| | *Staphylococcus aureus* | *Streptococcus pyogenes* | |
| A21978C$_1$ | 0.22 | 0.064 | >600 |
| A21978C$_2$ | 0.16 | 0.030 | 175 |
| A21978C$_3$ | 0.08 | 0.032 | 75 |



Daptomycin (LY146032)

We noted, however, that the acute toxicity increased with small increases in the chain length of the lipid side chain. This illustrates the functional importance of the side chain to the antimicrobial and toxicological properties of the lipopeptides. The structural diversity of the lipopeptides presents modification opportunities if it is possible to remove the acyl side chain and reintroduce a new acyl side chain. This paper will report the chemical modification of the lipopeptide A21978C to produce the superior analog daptomycin (LY146032).[3,6]
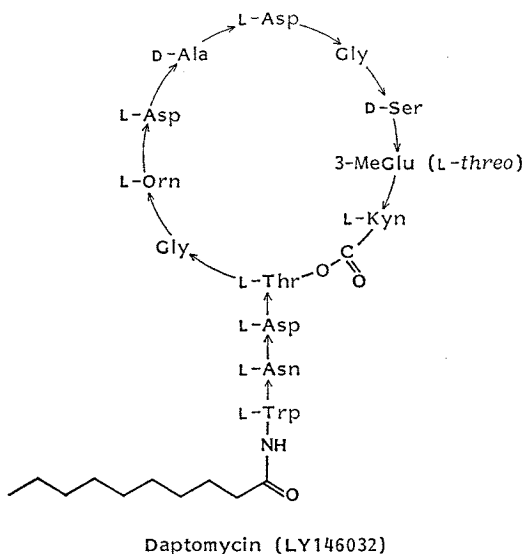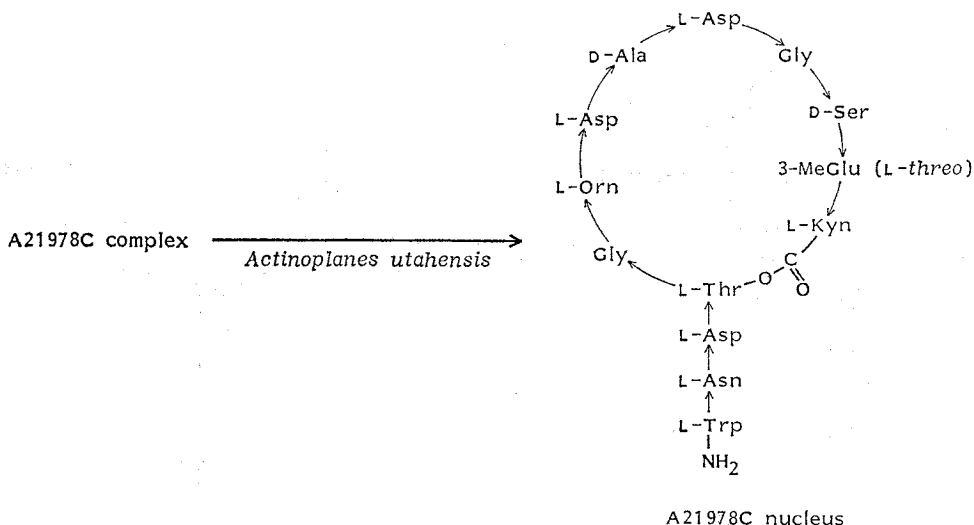
## Deacylation of A21978C

Since the closely related A21978C factors are co-produced in the fermentation, very laborious separation techniques were necessary to separate the desired A21978C$_1$ from the more toxic C$_2$ and C$_3$ factors (Table 1). The solution to this problem was achieved by removing the native acyl side chains to give the common peptide "nucleus" and subsequently reacylating with a desired acyl group to give an analog, and assaying the analogs to find improved therapeutic properties.

Interest in this approach came about as a result of structural studies on A21978C.[1] Sequencing this peptide by the EDMAN method required removal of the acyl group.[7] This was accomplished

Scheme 1.

A21978C complex $\xrightarrow{\quad\textit{Actinoplanes utahensis}\quad}$

L-Asp

D-Ala          Gly

L-Asp          D-Ser

L-Orn          3-MeGlu (L-*threo*)

Gly          L-Kyn

L-Thr—O—C=O

L-Asp

L-Asn

L-Trp

NH$_2$

A21978C nucleus

enzymatically with polymyxin acylase using an acetone powder of *Pseudomonas* species M-6-3.[8]    A similar preparation was used extensively by SHOJI in series of lipopeptide structure studies.[9]    This method generally resulted in production of deacylated peptide in 20~30% yield.   When polymyxin acylase was used to deacylate A21978C, extensive cleavage of the lactone ring was observed.   Therefore, a more selective means to carry out this transformation was required, and a series of organisms was screened for their ability to deacylate A21978C.

A group of *Actinoplanes* cultures known to have penicillin deacylase activity was included in the cultures which were screened.[10]    This group was shown to deactivate A21978C by deacylation.   One culture in particular, *Actinoplanes utahensis* NRRL 12052, was very effective and was used throughout these studies.

When A21978C complex was added to growing cultures of *A. utahensis*, a gradual disappearance of the added antimicrobial activity was observed.   The fermentation was harvested, and the products were purified on Diaion HP-20 resin to give the deacyl-A21978C peptide in 50~60% yield with an efficiency of 70~80%.

Physical and chemical data showed that the product lacked the fatty acid side chain while retaining the peptide structure, which showed no change in the amino acid composition or lactone bond.   The potentiometric titration of the resulting peptide showed the generation of a new amino group at the *N*-terminal amino acid.   This peptide lacked antimicrobial activity.   It was observed that antimicrobial activity could be reconstituted by contact with acylating agents such as acid chlorides.   This property was used to monitor the deacylation by paper chromatography and TLC.   The deacylation reaction mixtures were applied to the chromatographic support and developed with the appropriate solvent. Bioautography at this stage showed that the only bioactive spots belonged to residual starting material (A21978C).   The chromatogram was then sprayed with, or dipped into, a solution of *n*-octanoyl chloride, followed by a solvent wash to remove excess acylating agent and a new bioactive spot was observed at the location of the nucleus.   These observations indicated that the A21978C complex had been converted to an inactive peptide and that this peptide could be reacylated to bioactive compound.

Scheme 2.



A21978C nucleus

Acylating agent
acid halides or
active esters
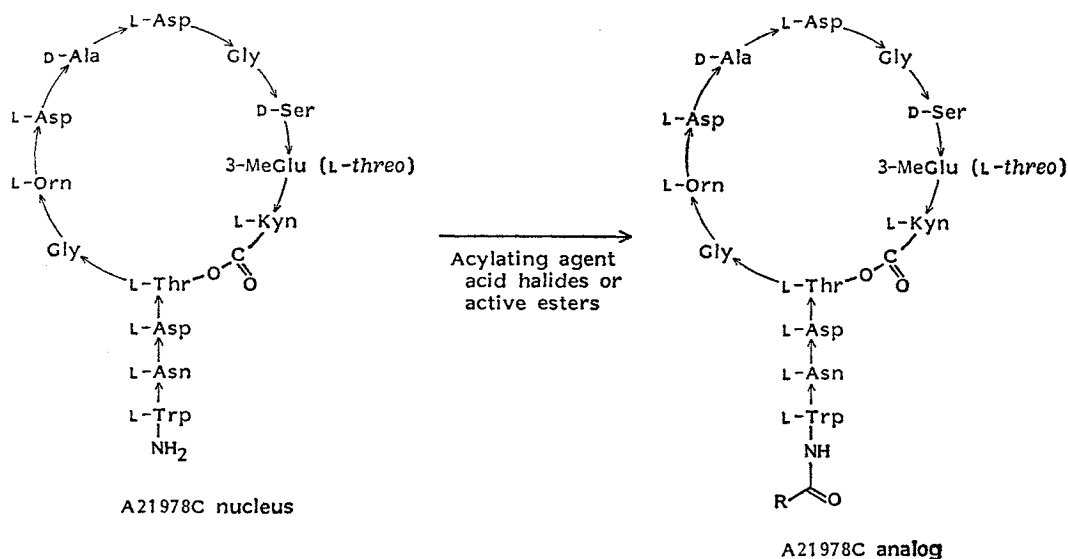
A21978C analog

Isolation of the A21978C nucleus represented a unique opportunity for synthesis of previously unavailable analogs of lipopeptides by selective reacylation. A21978C nucleus has several polar groups, of which four are potentially acylatable: The kynurenine aryl amino group, the serine hydroxyl, the amino side chain at ornithine (Orn), as well as the newly generated terminal amino group at tryptophan.

### Acylation of the A21978C Nucleus

Acylation of the A21978C nucleus was approached by a number of methods. Direct acylation with acyl halides gave a mixture of products. Chromatographic separation showed that these products consisted of a mixture of monoacylated products and minor amounts of diacylated products. The array of products depended somewhat on the reaction conditions as well as the choice of acylating agent. Acyl halides gave a mixture of monoacyl products from which the desired product acylated at the N-terminal Trp residue could be obtained by chromatographic purification. However, appreciable amounts of the monoacyl-adduct acylated at the ornithine side chain were produced along with minor amounts of diacyl products. Use of active esters as the acylating agent presented a better alternative. Active esters of 2,3,5-trichlorophenol (TCP) gave a mixture of mono-acyl products with the desired monoacyl-N-Trp product. Similar results were obtained using active esters of N-hydroxysuccinimide. A greater measure of selectivity was obtained using acid chlorides and controlling the pH between 5.0 and 6.5. These conditions appeared to favor acylation at the N-terminus Trp. Use of active esters of N-hydroxybenztriazole (HBT) similarly gave acylation predominantly at the Trp residue with longer reaction times giving varying amounts of overacylation products. Active esters gave reaction mixtures that favored monoacylation and higher reaction yields (50～60%). However, even under the best of conditions sufficient amounts of the undesired acylation products made the isolation and purification of the desired N-Trp monoacyl product difficult. The selectivity problem was solved by protecting the ornithine side chain in A21978C prior to deacylation.

Acylation at the ornithine side chain was avoided by the use of protecting groups as shown in

Scheme 3.   Preparation of A21978C analogs.



N-Orn-tert-BOC-A21978C nucleus

A21978C analog
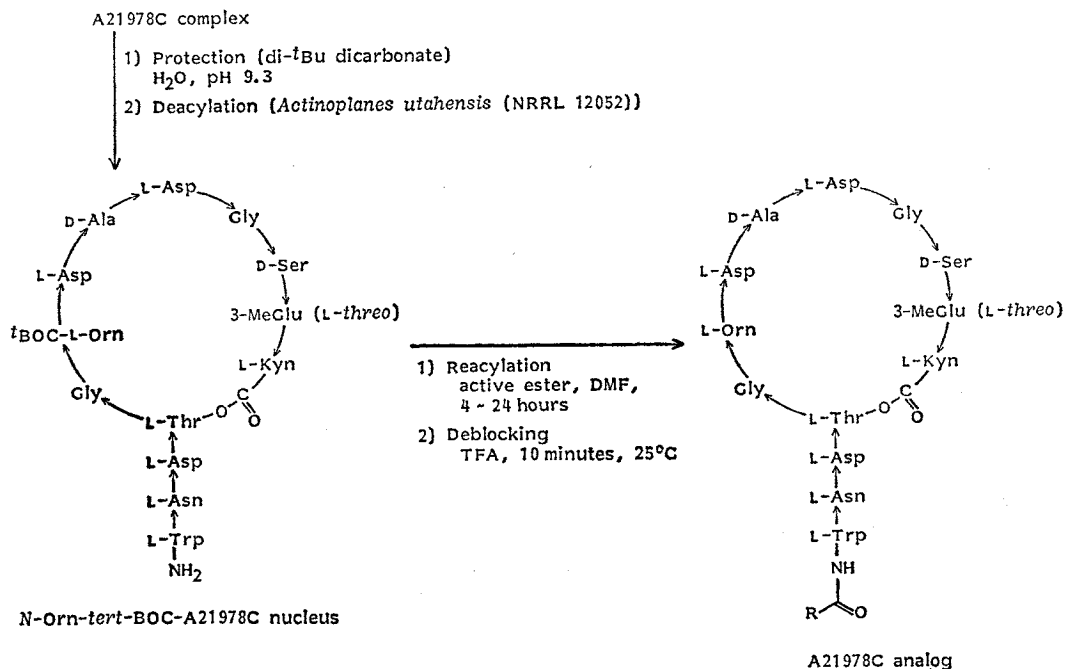
Scheme 3.   The A21978C complex was treated with di-*tert*-butyldicarbonate in aqueous solution at pH 9.3.   This reaction gave an excellent yield of mono-*tert*-butoxycarbonyl (*tert*-BOC)-protected A21978C complex whose physical and chemical properties were consistent for a *tert*-BOC group at the ornithine amino group.   This amino group was, therefore, eliminated as an acylation site and products acylated at the $N$-Trp-terminus would be strongly favored.

This protected antibiotic was added to the *A. utahensis* culture which enzymatically removed the native fatty acid acyl group to give A21978C nucleus bearing a protected ornithine amino group. The *tert*-BOC protecting group survived the conditions employed in the fermentation and in the isolation.   This protected peptide was acylated with TCP esters giving primarily monoacylation products at the desired $N$-terminal position (50~60% yield) with only 5% diacylation occurring.   Deblocking was effectively carried out using trifluoroacetic acid at room temperature for 10 minutes.   This was done with minimal degradation and excellent product purity to yield new analogs of A21978C.

### Structural Examination of A21978C Analogs

The number of acyl groups added to A21978C nucleus was determined by integration of the methyl resonances in the NMR spectra of the derivatives.   Both mono-acyl and diacyl analogs were detected.

The sites of acylation for the A21978C derivatives were identified by comparing the 360 MHz $^1$H NMR spectra of the analogs with the spectrum of the underivatized nucleus.   The addition of an acyl group is expected to alter the chemical shifts of protons adjacent to the acylation site; these potential reporter resonances include signals from the $\sigma$ methylene of ornithine, the $\beta$ methylene of serine, the $\alpha$ proton of tryptophan, and (less likely) the ring protons of the kynurenine residue.   Failure of any of these resonances to shift was taken as evidence that acylation did not occur at that site.   These

Table 2.  ¹H NMR data for selected semisynthetic A21978C analogs[a].

| Compound | Acyl group | Chemical shift (ppm) | | | Acylation site |
|---|---|---|---|---|---|
| | | Orn $\delta$-CH$_2$ | Ser $\beta$-CH$_2$ | Trp $\alpha$-CH | |
| A21978C nucleus | None | 2.78 | 3.68 | 3.87 | None |
| tert-BOC-A21978C nucleus | tert-BOC at Orn | 2.97 | 3.71 | | Orn |
| 4 | n-Decanoyl | 2.79 | 3.68 | 4.47 | Trp |
| 5 | n-Undecanoyl | 2.79 | 3.67 | 4.47 | Trp |
| 19 | p-Dodecanoylamidobenzoyl | 3.29 | 3.71 | | Orn |
| 14 | n-Undecanoyl-L-phenylalanyl | 3.03 | | | Orn |
| | Diundecanoyl | 3.02 | 3.71 | 4.46 | Orn, Trp |
| | Didecanoyl | 2.78 | 3.69 | 4.47 | Trp, Kyn |

[a]  Spectra obtained in DMSO solution, 23°C.

results are shown in Table 2.

The ¹H NMR and mass spectral data for the n-decanoyl and n-undecanoyl A21978C analogs (compounds 4 and 5) showed that acylation in these derivatives occurred at the Trp residue at the peptide N-terminal.   Other monoacylation products, formed in minor amounts which were dependent on reaction conditions, were acylated on the Orn σ-amino group.   Diacylated A21978C derivatives, such as the di-undecanoyl analog, had acyl groups at both the Trp and Orn residues.   Although the kynurenine aromatic amino group is potentially acylable, conditions used in this study did not result in extensive acylation at this site.   A minor diacyl product, containing two n-decanoyl groups, was acylated at Trp and Kyn; the UV spectrum of this compound showed a shift in the kynurenine absorbance maximum from 360 nm to 320 nm, and the kynurenine aromatic proton resonances were shifted downfield in the NMR spectrum of the material.

## Synthesis and Evaluation of A21978C Analogs

These methods made it possible to synthesize analogs of the native A21978C antibiotic bearing a large variety of modifications in the acyl group.   These analogs are summarized in Fig. 2.   Table 3 includes some physical criteria for purity.   The finding that subtle structural changes in the acyl group profoundly influenced the toxicity of the various A21978C factors predicted that modification of the side chain would be a fruitful method to design new analogs.   However, there was no systematic way to choose among the possible structural variations.

In the discussion that follows, it will become apparent that there were some minimal structural features that were necessary for good antimicrobial activity.   The chain length of an acyl group of at least four to eight carbon atoms was required to demonstrate detectable activity.   Side chains that had complex structures appeared to require a long alkyl or acyl group somewhere in the overall structure for optimal activity.   It also was possible to elongate the peptide by an amino acid and retain the same activity as long as an acyl side chain was present at the new N-terminus.

Three groups of analogs were synthesized by this method and included those bearing fatty acid acyl-, amino-aroyl-, and extended peptide side chains.   Antimicrobial in vitro and in vivo activity and acute toxicity data for these analogs are shown in Table 4.

The data shown for compounds 1~10, which are analogs bearing fatty acid acyl side chains, in-

Fig. 2.  Structure of A21978C analogs.
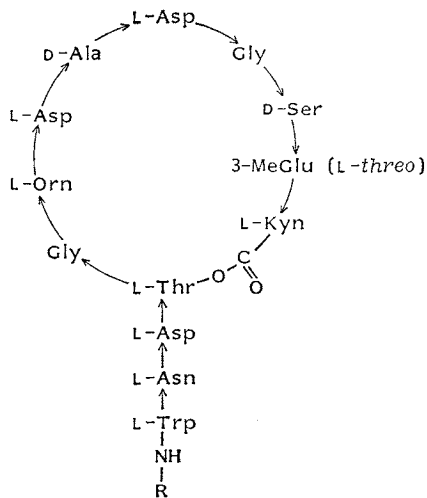


Fatty acid acyl derivatives
1  R=$n$-Heptanoyl
2  R=$n$-Octanoyl
3  R=$n$-Nonanoyl
4  R=$n$-Decanoyl
5  R=$n$-Undecanoyl
6  R=$n$-Dodecanoyl
7  R=$n$-Tridecanoyl
8  R=$n$-Tetradecanoyl
9  R=$n$-Pentadecanoyl
10  R=$n$-Hexadecanoyl

Extended peptide derivatives:
  $\alpha$-$N$-Acylated phenylalanyl side chains
11  R=$n$-Octanoyl-L-phenylalanyl
12  R=$n$-Nonanoyl-L-phenylalanyl
13  R=$n$-Decanoyl-L-phenylalanyl
14  R=$n$-Undecanoyl-L-phenylalanyl
15  R=$n$-Dodecanoyl-L-phenylalanyl
16  R=$n$-Tridecanoyl-L-phenylalanyl
17  R=$n$-Tetradecanoy-L-phenylalanyl
18  R=$n$-Dodecanoyl-D-phenylalanyl

Amino aroyl derivatives
19  R=$p$-Dodecanoylamidobenzoyl
20  R=$p$-Tetradecanoylamidobenzoyl
21  R=$m$-Dodecanoylamidobenzoyl
22  R=$p$-Dodecanoylamidophenacetyl
23  R=$p$-Dodecyloxylbenzoyl

Table 3.  Physical characteristics of A21978C derivatives.

| Compound No. | Extinction coefficients at | | Rf | System | Compound No. | Extinction coefficients at | | Rf | System |
|---|---|---|---|---|---|---|---|---|---|
| | $\lambda_{max}^{EtOH}$ 220 nm | $\lambda_{max}^{EtOH}$ 260 nm | | | | $\lambda_{max}^{EtOH}$ 220 nm | $\lambda_{max}^{EtOH}$ 260 nm | | |
| 1 | 48,100 | 11,400 | 0.85 | a | 13 | 49,181 | 8,465 | 0.52 | b |
| 2 | 46,000 | 10,500 | 0.80 | a | 14 | 41,000 | 10,200 | 0.53 | b |
| 3 | 46,000 | 11,000 | 0.75 | a | 15 | 52,997 | 9,435 | 0.54 | b |
| 4 | 46,500 | 10,000 | 0.70 | a | 16 | 47,025 | 8,823 | 0.56 | b |
| 5 | 46,200 | 10,400 | 0.65 | a | 17 | 48,410 | 9,007 | 0.65 | b |
| 6 | 44,000 | 9,500 | 0.59 | a | 18 | 44,600 | 8,970 | 0.54 | b |
| 7 | 48,500 | 9,800 | 0.53 | a | 19 | 57,000 | 28,531 | 0.34 | b |
| 8 | 48,447 | 9,170 | 0.42 | a | 20 | — | — | 0.83 | b |
| 9 | 50,172 | 9,751 | 0.34 | a | 21 | 58,270 | 19,027 | 0.57 | b |
| 10 | 54,000 | 12,000 | 0.25 | a | 22 | 48,992 | 21,505 | 0.74 | b |
| 11 | 44,504 | 8,899 | 0.48 | b | 23 | 48,440 | 23,514 | 0.78 | b |
| 12 | 47,940 | 9,403 | 0.50 | b | | | | | |

System a:  Rf by reversed phase silica gel TLC (Whatman KC$_{18}$ with fluorescent indicator) using a H$_2$O - MeOH - CH$_3$CN (45 : 15 : 40) with 0.2% pyridine and 0.2% AcOH solvent system.
    System b:  Rf by silica gel TLC (Merck) using a H$_2$O - CH$_3$CN - Me$_2$CO (1 : 2 : 2) solvent system.
    —: Data not available.

dicate that the *in vitro* activity increased with acyl group up to chain length C-12 to C-13. The *in vivo* activity against *Staphylococcus aureus* and *Streptococcus pyogenes* followed a similar relationship. However, the LD$_{50}$ values showed that beyond the C-11 analog there is a definite trend toward increased toxicity. Presence of unsaturation in the side chain did not dramatically change these trends (data

Table 4.  Antimicrobial properties of A21978C derivatives.

| Compound No. | MIC ($\mu$g/ml) | | | ED$_{50}$ (mg/kg$\times$2, mouse, sc) | | LD$_{50}$ (mg/kg, iv) |
| --- | --- | --- | --- | --- | --- | --- |
| | Staphylococcus aureus | Staphylococcus epidermidis | Streptococcus faecalis | S. aureus | Streptococcus pyogenes | |
| A21978C$_1$ | 1 | 1 | 8 | 0.22 | 0.064 | >600 |
| 1 | 8 | 8 | 128 | 2.65 | 1.49 | >600 |
| 2 | 4 | 4 | 64 | 1.4 | 0.65 | >600 |
| 3 | 8 | 8 | 128 | 0.5 | 0.14 | >600 |
| 4 | 0.5 | 0.5 | 16 | 0.11 | 0.03 | 600 |
| 5 | 0.25 | 0.5 | 2.0 | 0.27 | >0.06 | 450 |
| 6 | 0.5 | 0.5 | 4.0 | 0.44 | 0.05 | 144 |
| 7 | 0.125 | 0.5 | 0.03 | 0.54 | 0.046 | 112.5 |
| 8 | 0.5 | 2 | 0.5 | 3.08 | 0.2 | 62.5 |
| 9 | 1 | 4 | 2 | 8.3 | 0.18 | 56.3 |
| 10 | 1 | 2 | 2 | 3.4 | 0.18 | 50 |
| 11 | 4 | 8 | 128 | 2.58 | 1.48 | 450 |
| 12 | 2 | 2 | 32 | 1.38 | 0.59 | >600 |
| 13 | 0.5 | 0.5 | 8 | 0.99 | 0.15 | 600 |
| 14 | 0.5 | 2 | 4 | 1.25 | 0.32 | 400 |
| 15 | 0.5 | 1 | 1 | 2.35 | 0.32 | 250 |
| 16 | 0.5 | 2 | 0.25 | 0.76 | 0.14 | 225 |
| 17 | 0.5 | 4 | 1 | 4.8 | >0.36 | 225 |
| 18 | 0.5 | 4 | 1 | 2.05 | 0.21 | 250 |
| 19 | 2 | 16 | 32 | — | 18.8 | <300 |
| 20 | 1 | 4 | 4 | >18.8 | 1.02 | 250 |
| 21 | 0.5 | 1 | 4 | 1.67 | 0.46 | 277 |
| 22 | 0.5 | 0.5 | 4 | 4.12 | 0.69 | 450 |
| 23 | 1 | 2 | 2 | 3.35 | 0.31 | 67.5 |

—: Data not available.

not shown).

The peptide chain could be elongated by the coupling of an acylated amino acid such as N-acyl-phenylalanine to the N-terminus of the nucleus.  In the phenylalanyl series, the chain length of the N-acyl group was varied from C-8 to C-14.  We observed that the good in vitro activity at the C-10 and higher chain lengths translated adequately in vivo.  Acyl groups with chain lengths from C-9 to C-11 were associated with analogs of lower toxicity.  An additional amino acid did not bestow any therapeutic advantage; this was also the case with the alkyl-amino acids.  The data in Table 4 includes the antimicrobial properties of a number of amino aroyl and aralkyl analogs.  The longer chain lengths in the amide function were associated with good in vitro and in vivo activity and demonstrated the wide variety of structural changes that could be introduced without major changes in the activity spectrum.  The dodecanoylaminophenacetyl derivative had the best activity and the lowest toxicity of this group.  In summary, most of these analogs retained much of the characteristics of the parent A21978C antibiotic with no significant change in the in vitro and in vivo antimicrobial spectra.

Our criteria for evaluation required that these derivatives have antimicrobial activity and LD$_{50}$ values that were at least comparable to the parent A21978C$_1$ in order to be considered for further study.  The analogs meeting these criteria included the decanoyl (LY146032) (4), and the undecanoyl (5) fatty acid acyl analogs, whose properties closely resembled those of the parent antibiotic.  Two other analogs: The N-decanoylphenylalanyl (13) and the N-dodecanoyl-p-aminophenacetyl (22) com-

Table 5.    Comparative toxicity of A21978C analogs.

| A21978C analog | Mouse acute (1 g/kg, iv) | Rat subchronic (150 or 300 mg/kg/day for 7 days, sc) | |
|---|---|---|---|
| A21978C | 7/10[a] | ↓ | BWG-150, 300[b] |
| | | ↑ | SGPT-300 |
| *n*-Decanoyl (LY146032) | 2/10 | ↑ | SGPT-300 |
| α-*N*-(*n*-Decanoyl)-L-phenylalanyl- | 10/10 | ↓ | BWG-300 |
| | | ↑ | SGPT-150,300 |
| | | ↑ | BUN-300 |
| | | | Nephrotoxicity |
| *N*-(*n*-Dodecanoyl)-*p*-aminophenacetyl- | 9/10 | ↓ | BWG-150, 300 |
| *n*-Undecanoyl- | 10/10 | ↓ | BWG-150, 300 |
| | | ↑ | SGPT-150 |
| | | | Nephrotoxicity |
| | | | Mortality |

[a]   Number dead/number dosed.
[b]   Arrows indicate increase (↑) or decrease (↓) from control values.
BWG: Body weight gain, SGPT: serum glutamyl-pyruvate transferase, BUN: blood urea nitrogen.

pounds were also chosen.  These compounds were compared in a study designed to reveal any important differences in their toxicity profiles.  The mouse survival test studies summarized in Table 5 showed that the decanoyl analog of A21978C gave the best survival while A21978C and the other analogs caused significantly more mortality.  Moreover, the decanoyl analog (4) had only modest toxicity in the rat subchronic study with serum glutamyl-pyruvate transferase (SGPT) elevations at only the higher dose level.  There was no sign of specific organ involvement.  Therefore, the decanoyl-A21978C analog, LY146032, also known as daptomycin, was recognized as a potentially useful analog and is currently undergoing clinical studies.

## Experimental

The following instruments were used in this study: ^{1}H NMR spectrometer Bruker WH360; UV spectrometer, Cary 15; ORD and CD spectropolarimeter, Perkin-Elmer 241 and Jasco J40AS; IR spectrophotometer, Beckman IR426; IR spectrometer, Beckman IR426; high resolution mass spectrometer, Varian MAT731; amino acid analysis, Beckman 120B amino acid analyzer equipped with a Bio-Cal autosample applicator and programmer and single Durrum BC-1A resin; preparative HPLC work was done on a Waters PREP-500 chromatograph.  Amino acids and acid chlorides were obtained from commercial sources.

### Preparation of A21978C Nucleus

The fermentation of A21978C was carried out according to methods outlined elsewhere and resulted in the isolation of A21978C complex.[1]   A fermentation of *A. utahensis* was carried out according to the procedure described in U.S. Patent No. 4,396,543.  After a 66-hour growth period, 100 g of crude A21978C complex was added to the *A. utahensis* culture, and the disappearance of the antimicrobial activity was monitored by assay against *Micrococcus luteus*.

Whole fermentation broth (20 liters) was filtered with a filter aid (Hyflo Super-Cel) and the mycelial cake discarded.  The filtrate was passed through a column containing 1.5 liters of Diaion high porous polymer HP-20 resin.  The effluent was discarded and the column was washed with water to remove residual filtered broth and this effluent also discarded.  The column was eluted with water - $CH_3CN$ mixtures (10 liters each of 95 : 5, 9 : 1 and 4 : 1) collecting 1-liter fractions.  This elution was monitored by analytical HPLC using Silica gel/C18 and a solvent system of water - MeOH (3 : 1) containing 0.2% ammonium acetate and the detector set at 254 nm.  Fractions containing the nucleus were com-

bined, concentrated under reduced pressure to remove $CH_3CN$ and freeze-dried to give purified A21978C nucleus (40.6 g) as a white, amorphous solid which fluoresces under short wavelength UV light: Fast atom bombardment mass spectrum (FAB-MS) $m/z$ 1,466 (M+1), calcd for $C_{62}H_{84}N_{17}O_{25}$: 1,466.58249, found: 1,466.5808; IR (KBr) cm⁻¹ 1655 (s), 1530 (s); UV $\lambda_{max}^{EtOH}$ nm ($\varepsilon$) 223 (41,482), 260 (8,687), 366 (4,000); $pKa$ (66% DMF) 5.2, 6.7, 8.5, 11.1.

### Preparation of the N-Orn-*tert*-BOC-A21978C Complex

A21978C complex, obtained by fermentation by the procedure outlined in U.S. Patent No. 4,208,403, (5 g) was dissolved in water and the pH adjusted from 4.1 to 9.5 with 1 N NaOH (10 ml). Di-*tert*-butyldicarbonate (1 ml) was added with stirring at room temperature for 2 hours. The pH was maintained at 9.5 by the manual addition of 5 N NaOH. The reaction was monitored by TLC (silica gel, $CH_3CN$ - water (7:3 and 8:2) using UV detection). After a brief induction period the reaction became turbid. The reaction was continued until the rate of turbidity increase and base consumption decreased to a minimum. The reaction was allowed to proceed for an additional 90 minutes and the reaction mixture was lyophilized immediately to give 7.1 g of crude N-Orn-*tert*-BOC-A21978C complex.

### Preparation of N-Orn-*tert*-BOC-A21978C Nucleus

The crude N-Orn-*tert*-BOC-A21978C complex (1,200 g) was added to a growing 66-hour *A. utahensis* culture using the fermentation conditions described in the patent cited above. The fermentation was run until assays for antimicrobial activity using *M. luteus* were negative.

Fermentation broth (100 liters) was filtered using filter aid (Hyflo Super-Cel) and the filtrate passed over a column containing 7.5 liters of Diaion HP-20 resin. The column was washed with water (38 liters) followed water - $CH_3CN$ mixtures, 95:5 (40 liters), 9:1 (40 liters) and 85:15 (100 liters). The elution was monitored by analytical HPLC using Silica gel/C18 column with UV detection (280 nm). Fractions containing the nucleus were combined, concentrated under vacuum to remove solvent and freeze-dried to give 298 g of crude N-Orn-*tert*-BOC-A21978C nucleus.

This N-Orn-*tert*-BOC-A21978C nucleus preparation (30 g) was dissolved in water - $CH_3CN$ (9:1) containing 0.2% AcOH and 0.8% pyridine and chromatographed on a 4.7×192-cm steel column containing Silica gel (Quantum LP-1)/C18 equilibrated in the same solvent. The column was developed under pressure with water - $CH_3CN$ - MeOH (80:15:5) containing 0.2% AcOH and 0.8% pyridine collecting 350 ml fractions. The progress of the chromatography was monitored by analytical HPLC. Fractions containing the product were concentrated under vacuum and freeze-dried to give purified N-Orn-*tert*-BOC nucleus (18.4 g, 90% purity) as an amorphous solid which fluoresced under short wave length UV light: FAB-MS $m/z$ 1,566 (M+1), calcd for $C_{67}H_{92}N_{17}O_{27}$: 1,566.634860, found: 1,566.6342; IR (KBr) cm⁻¹ 1710 (sh), 1660, 1530; UV $\lambda_{max}^{90\% EtOH}$ nm ($\varepsilon$) 220 (42,000), 260 (10,600), 366 (~4,000); HPLC retention time: 6 minutes on 4.6×300 mm column of Silica gel/C18 using a water - $CH_3CN$ - MeOH (80:15:5) solvent containing 0.2% ammonium acetate at a flow rate of 2 ml/minute with UV detection at 280 nm.

### General Procedure for the Preparation of N-Alkanoyl-amino Acids

The appropriate alkanoyl acid chloride was added dropwise to a solution of the amino acid (1:1 mol ratio) in pyridine to bring the final concentration of reactants to 0.1 to 0.2 M. The reaction mixture was stirred for 3 to 6 hours at ambient temperature and poured into water to give a precipitate. The product was collected by filtration and crystallized from MeOH.

### General Procedure for Preparation of 2,4,5-Trichlorophenyl Active Esters

(A): The following preparation of 2,4,5-trichlorophenyl *n*-decanoate illustrates use of acid chlorides for the synthesis of active esters: A solution of decanoyl chloride (5.6 ml) and 2,4,5-TCP (5.6 g) in ether (1 liter) and pyridine (120 ml) was stirred for 4 hours. The reaction mixture was filtered and the solvents removed under reduced pressure. The crude active ester was purified on a silica gel column (Woehlm) using toluene as the eluent. Fractions were monitored by TLC and appropriate fractions were pooled to yield the desired 2,4,5-trichlorophenyl *n*-decanoate (10.4 g).

(B) The N,N'-Dicyclohexylcarbodiimide Method for Active Ester Preparation: The following

procedure illustrates the synthesis of active esters using N,N'-dicyclohexylcarbodiimide as the coupling agent:

A solution of N-(n-decanoyl)-L-phenylalanine (31.9 g, 0.1 mol) and 2,4,5-TCP (19.7 g, 0.1 mol) in 1 liter of anhydrous ether was treated with N,N'-dicyclohexylcarbodiimide (20.6 g, 0.1 mol). The reaction was stirred overnight at room temperature and the precipitate of N,N'-dicyclohexylurea (DCU) was removed by filtration. The filtrate was concentrated to dryness under reduced pressure. Trituration of the residue with ether, removal of the solids DCU by filtration and evaporation of the solvent under reduced pressure gave a solid. This solid was crystallized from $CH_3CN$ to give N-(n-decanoyl)-L-phenylalanyl 2,4,5-trichlorophenylate, mp 122~124°C.

### Preparation of N-Trp-[N-(n-decanoyl)-L-phenylalanyl]-N-Orn-tert-BOC-A21978C Nucleus

The following procedure serves to illustrate the general method used here for the acylation of the N-Orn-tert-BOC-A21978C nucleus at the N-terminal amino group of the latter peptide.

A solution of N-(n-decanoyl)-L-phenylalanyl-2,4,5-trichlorophenylate (1.0 g, 0.02 mol) and N-Orn-tert-BOC-A21978C nucleus (10 g, 0.006 mol) in anhydrous dimethylformamide (1 liter) was stirred at room temperature for 96 hours under an inert atmosphere of nitrogen. The solvent was removed under reduced pressure and the residue stirred (2 hours) with 1 liter of diethyl ether - $CHCl_3$ (4:1) mixture to remove the halophenol biproducts. The solid residue was collected by filtration (10.3 g) and purified using preparative HPLC (Waters PREP LC/System 500 unit equipped with Pre-Pak-500/C18 column as the stationary phase). Isocratic elution was carried out with a water - MeOH - $CH_3CN$ solvent system (2:1:2) collecting 250-ml samples every 60 seconds. The desired material eluted after 2 liters of eluent had been used and was identified by reversed phase TLC (Silica gel/C18, water - MeOH - $CH_3CN$ (3:3:4), detected by Van Urk's spray reagent (yellow)). Combined fractions were reanalyzed by the TLC system described above and by bioautography (silica gel TLC, $CH_3CN$ - $Me_2CO$ - water (2:2:1) vs. M. luteus as the detecting organism). The procedure gave 6.02 g of N-Trp-[N-(n-decanoyl)-L-phenylalanyl]-N-Orn-tert-BOC-A21978C nucleus.

### Preparation of N-Trp-[N-(n-decanoyl)-L-phenylalanyl]A21978C Nucleus

The following procedure outlines the procedure used to deblock the N-Orn-tert-BOC-A21978C analog. A flask (100-ml) (was cooled in an ice bath to 5°C), containing N-Trp-[N-(n-decanoyl)-L-phenylalanyl]-N-Orn-tert-BOC-A21978C nucleus (6.03 g, 0.008 mol) and anhydrous FTA containing 2% anisole (50 ml) was added in one portion. After the mixture went into solution (2 minutes) the reaction was stirred for 10 minutes under nitrogen and evaporated under reduced pressure at temperatures below 40°C. The semi-solid residue was triturated twice with ether - methylene chloride (4:1), two 100-ml portions, and the residual solid was isolated by filtration. This solid was washed with ether to give the TFA salt. This salt was dissolved in water (50 ml) and the solution was adjusted to pH 5.4 with pyridine to give 6.1 g of a white solid after lyophilization to remove the pyridinium trifluoroacetate. The white solid was dissolved in 35 ml of MeOH and purified by preparative HPLC using a Waters PREP 500 equipped with a reversed phase C18 Silica gel column and eluting with a step gradient of water - MeOH - $CH_3CN$ containing 0.1% pyridinium acetate in ratios of 3:1:2, 2:1:2 and 1:2:2, and collecting 250-ml fractions every 60 seconds. The progress of the chromatography was followed by reversed phase TLC as with the chromatography of the protected analog (see above). The desired N-Trp-[N-(n-decanoyl)-L-phenylalanyl]A21978C nucleus was eluted with the 2:1:2 solvent and the combined fractions gave 2.23 g of a cream colored product after lyophilization.

### Procedures for Direct Acylation of A21978C Nucleus

Method A – Modified Schotten-Baumann Reaction: The acylation of the A21978C nucleus with alkanoyl or alkenoyl chlorides was carried out according to this general procedure. The A21978C nucleus (2.0 g, 1.35 mmol) was dissolved in 200 ml of pyridine - water (9:1). The acylating agent (18~20 mmol excess acid chloride dissolved in 15 ml $Me_2CO$) is added dropwise over 1~3 hours and the reaction allowed to stir for an additional 3-hour at ambient temperatures. The acetone was removed under reduced pressure. The aqueous phase which remained was diluted with 200 ml of water and the pH of the solution was adjusted to pH 3 to 3.4 with glacial acetic acid. The solution

was washed eight times with ethyl ether and the aqueous layer was lyophilized.

The crude acylated nucleus was purified by preparative HPLC. The product was dissolved in $4 \sim 6$ ml of water or the eluting solvent and injected onto a stainless steel column ($84 \times 1.6$ cm) packed with a LP-1/C18 support. The column was eluted with a solvent system of the following composition; water - MeOH - $CH_3CN$ - pyridine - AcOH ($45:15:40:2:2$) at $105 \sim 140$ kg/cm$^2$ pressure and at a flow rate of $10 \sim 12$ ml/minute using a Milton-Roy LDC duplex pump and a detector set at 280 nm. The fractions were combined on the basis of TLC using reversed phase plates (Whatman KC18) and the same solvent used in the HPLC separation. The product was detected on these plates with UV light. Purity of the combined desired fractions were determined by analytical HPLC (C18 Micro-bondapak, Waters Co.), with the solvent system used in the preparative separation using a UV monitor set at 280 nm. The appropriate fractions were concentrated under reduced pressure and lyophilized. Purity ranged from $85 \sim 90\%$ and was assessed by HPLC, TLC and UV spectra.

Method B – 2,4,5-Trichlorophenyl Active Ester Method: A solution of lauric acid (200 mg, 1 mmol) and 2,4,5-TCP (220 mg, 1.1 mmol) in ether (20 ml) was stirred for 1 hour and 206 mg of $N,N'$-dicyclohexylcarbodiimide was added in one portion. The reaction mixture was stirred for 24 hours at ambient temperatures under an inert atmosphere and filtered to remove the precipitated DCU. The precipitate was washed with ether and the filtrate was evaporated to dryness under reduced pressure. The residue was triturated three times with ether and filtered to remove residual DCU. The filtrate was concentrated to dryness to give a gum (482 mg) which crystallized from petroleum ether-B - ether ($10:1$) to give 220 mg ($51\%$) of lauroyl 2,4,5-trichlorophenylate as white needles, mp $59 \sim 60°C$.

A solution of A21978C nucleus (50 mg, 0.031 mmol), lauroyl 2,4,5-trichlorophenolate (12 mg, 0.032 mmol) in 6 ml of DMF was stirred for 3 hours at room temperature and overnight at 50°C under nitrogen. The reaction mixture was concentrated under vacuum to 1/4 volume controlling the temperature below 55°C and diluting with excess water and $CHCl_3$. The aqueous layer was washed twice with $CHCl_3$ and concentrated under vacuum without the use of heat to remove volatiles to give a yellow powder which showed that $30\%$ of A21978C nucleus was still present. The reaction was examined by TLC (reversed phase Whatman KC18 Silica gel plates, $75\%$ MeOH - $H_2O$, $0.1\%$ pyridinium acetate, Von Urk's or Ehrlich's spray) and showed a series of three spots in the lower third of the plate. The central major spot had the identical Rf as the analog prepared by the SCHOTTEN-BAUMAN method. The other spots were not identified.

### Methods for Evaluation of Antibiotic and Toxicity Parameters

*In vitro* antimicrobial assays were done by the standard agar dilution method using Mueller-Hinton agar supplemented with $1\%$ supplement C (Difco Laboratories, Detroit, Michigan). Mouse protection tests (ED$_{50}$) were run using 5 groups of 8 mice per group ($19 \sim 21$ g and random sex). The mice were inoculated with the infecting organism by the intraperitoneal route. The mice were treated with the appropriate dilution of the antibiotic at 1 and 5 hours post-infection by the subcutaneous route and observed for 7 days. All of the infected control animals died during this period. The ED$_{50}$ was calculated by the REED-MUENCH method.[11] Acute toxicity, as expressed by the LD$_{50}$ value, was determined using mice ($19 \sim 21$ g, random sex and four per group). These animals were administered the antibiotic by the intraperitoneal route and observed for 7 days. LD$_{50}$ values were calculated by the REED-MUENCH method.[11]

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