A21978C, A COMPLEX OF NEW ACIDIC PEPTIDE ANTIBIOTICS: ISOLATION, CHEMISTRY, AND MASS SPECTRAL STRUCTURE ELUCIDATION

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A21978C, produced by *Streptomyces roseosporus*, NRRL 11379, is a complex of new acidic lipopeptolide antibiotics which inhibits Gram-positive bacteria. HPLC separation of the various components from the purified complex resulted in the isolation of A21978C_1, -C_2 and -C_3 (major components) and -C_4, -C_5 and -C_0 (minor components). Each of these components was fermented with cultures of *Actinoplanes utahensis* (NRRL 12052) to give the identical inactive peptide ("A21978C nucleus") by removal of the fatty acid acyl groups from the *N*-terminus. This peptide was composed of 13 amino acids: L-kynurenine, L-*threo*-3-methylglutamic acid, L-asparagine, L-aspartic acid (3 residues), glycine (2 residues), L-tryptophan, L-ornithine, D-alanine, D-serine and L-threonine. The amino acid sequence was determined using a combination of the Edman degradation and gas chromatography mass spectrum (GC-MS) analysis of appropriately derivatized peptides obtained from partial hydrolysis. Each major component was shown to be acylated with a branched chain fatty acid at the *N*-terminus and the structure of this fatty acid was determined by ^{1}H NMR and mass spectral methods. A structure for A21978C was assigned on the basis of this degradative and physico-chemical information.

A new strain of *Streptomyces roseosporus*, NRRL 11379, was found to produce a series of new antibiotics designated A21978A, -B, -C, -D and -E (see Table 1).[1~3] A21978C, the major component, was highly active against Gram-positive bacteria and was shown to consist of at least six closely related antimicrobial components by high pressure liquid chromatography (HPLC) using a reverse-phase support. The analytical HPLC analysis for the A21978C complex is shown in Fig. 1. This publication will describe structure elucidation of the A21978C antibiotics.

The major elements of this discussion will include 1) isolation and purification of A21978C and its constituent factors; 2) preliminary characterization of the chemical properties of these antibiotics; 3) determination of the structure of the novel constituent amino acids as well as the configuration of all the amino acids; 4) determination of the *N*- and *C*-terminals of the peptide and the positions of the fatty acid acyl groups;

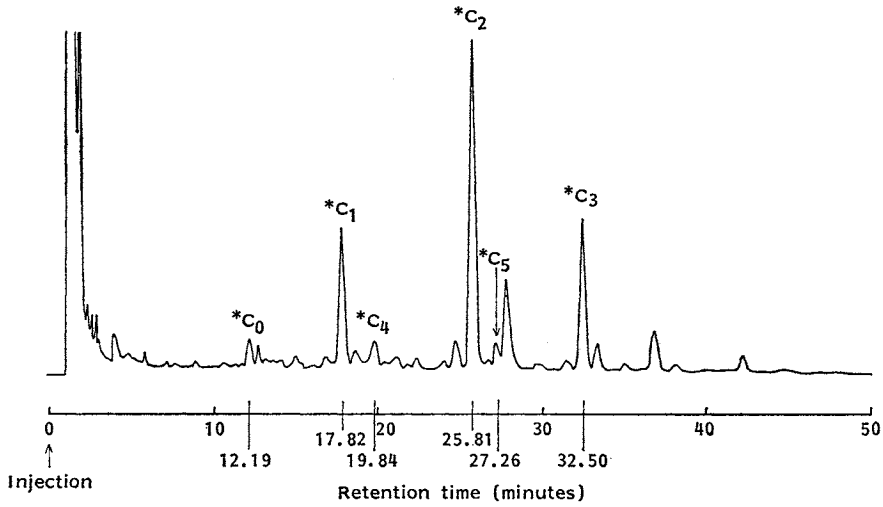
Table 1. Thin-layer chromatography of A21978 factors.

A21978 factor	Rf value*
A	0.66
B	0.57
C complex	0.31
D	0.51
E	0.48

* E. Merck Silica gel plates, MeOH - H_2O (7 : 3), detection organism; *Micrococcus luteus*.

Fig. 1. HPLC separation of the A21978C factors.

This separation utilized an Ultrasphere ODS column (250×4.5 mm) with a solvent gradient consisting of (A) 32% CH_3CN - H_2O containing 1% $NH_4(H_2PO_4)$→(B) 40% CH_3CN - H_2O containing 1% $NH_4(H_2PO_4)$ and detector set at 223 nm.



Scheme 1. Isolation of A21978C complex.

Broth

↓ filtered with 3% filter aid
water wash

Filtrate

↓ adjusted to pH 3.5 with HCl
held 16 hours for complete precipitation
filtered with 0.75% Celite 545

Filter cake

↓ extracted with MeOH, filtered

MeOH extract

↓ 10% water added
adjusted to pH 6.5
concentrated to 1/20 volume *in vacuo*
adjusted pH to 3.0
extracted with butanol

Butanol extract

↓ concentrated *in vacuo* to dryness
dissolved residue in MeOH
added to 30 volumes of acetone
precipitate filtered off and dried

A21978C complex

5) sequence determination of the core A21978C peptide or "nucleus" by gas chromatography mass spectrum (GC-MS) and Edman degradation methods; and 6) structural assignment to the A21978C antibiotics.

Isolation and Purification of the A21978C Antibiotics

A21978C was isolated from filtered broth according to the procedure outlined in Scheme 1. This A21978C complex was further purified and resolved into its various components by the procedure shown in Scheme 2 utilizing IRA-68 resin and silica gel chromatography. The A21978C antibiotics

Scheme 2. Purification of A21978C complex.

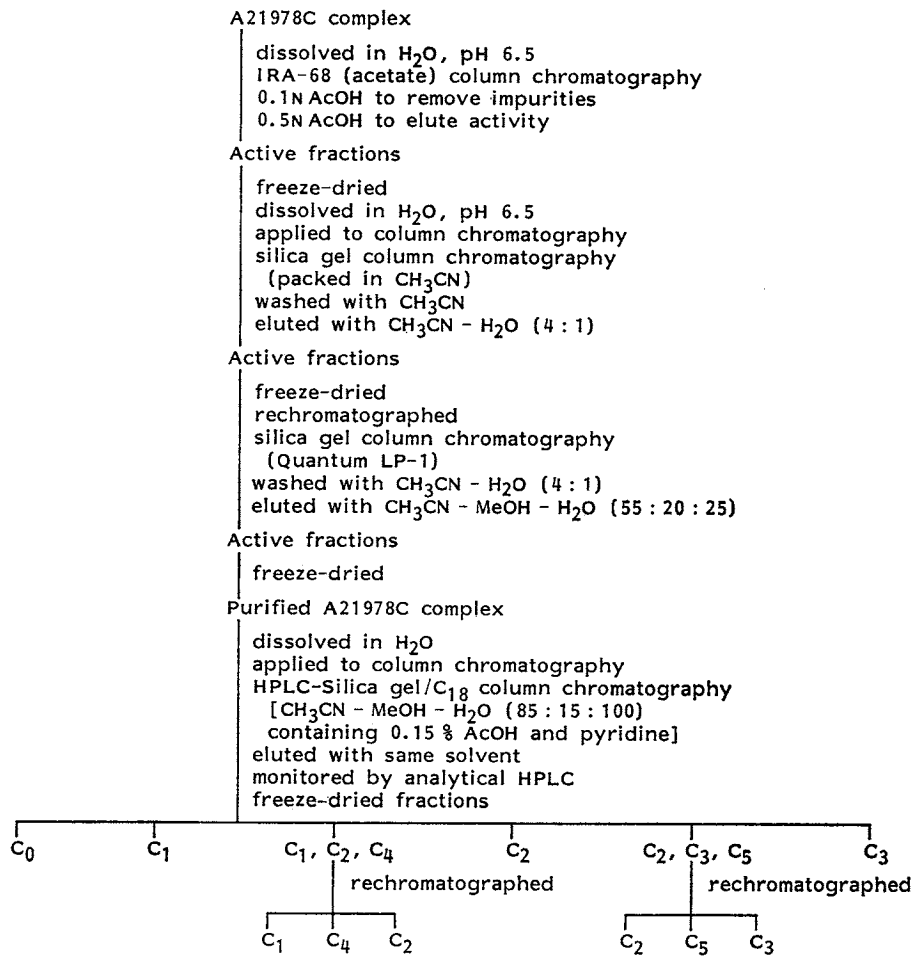


Table 2. Physico-chemical properties of the major A21978C antibiotics.

A21978C factor	Empirical formula	MW	HPLC retention time (minutes)	UV λ_{max}^{EtOH} nm ($E_{1cm}^{1\%}$)
C_1	$C_{73}H_{103}N_{17}O_{26}$	1,633	12.2	223 (307), 260 (62), 280 (30), 360 (33)
C_2	$C_{74}H_{105}N_{17}O_{26}$	1,647	25.8	223 (307), 260 (62), 280 (30), 360 (33)
C_3	$C_{75}H_{107}N_{17}O_{26}$	1,661	32.5	224 (307), 260 (62), 280 (30), 360 (33)

All components were chromatographically homogeneous; molecular formulae were calculated from accurate mass spectral measurement of the composition of A21978C nucleus and on the parent A21978C components.

were effectively separated by reverse phase HPLC (Silica gel-C_{18}) using CH_3CN - MeOH - H_2O (85 : 15 : 100) which contained 0.15% pyridinium acetate. These procedures resulted in the isolation of A21978C_1, -C_2 and -C_3 as major components and A21978C_4, -C_5 and -C_0 as minor constituents (see Table 2).

Preliminary Characterization of A21978C

The individual A21978C factors were each hydrolyzed to give the following ninhydrin-positive amino acids: (number of residues) Asp (4), Ser (1), Thr (1), Gly (2), Ala (1), Orn (1), Trp (1) and two novel amino acids (1 residue each).

The ^{1}H NMR spectra of A21978C$_1$, -C$_2$ and -C$_3$ showed strong methyl and methylene signals at δ 0.98 and $\sim$1.1 ppm, respectively. These signals indicated the possible presence of a fatty acid acyl group, a common feature of many peptide antibiotics.[4,5] Acid hydrolysis (6 N HCl) of the A21978C complex gave an ether extractable fraction which was esterified (CH_2N_2) and shown to be a mixture of C_{11}, C_{12} and C_{13} fatty acid esters by GC-MS analysis. In a similar manner, each A21978C factor was shown to contain a single fatty acid acyl group at the *N*-terminus. Potentiometric titration showed *pKa* values ($\sim$5.8, 7.6 and $\sim$12) that were consistent with the presence of several carboxylic acid groups as well as an aromatic and an aliphatic amino group. These data taken together indicated that the A21978C antibiotics were a family of closely related acidic peptides each bearing a distinctive fatty acid acyl group.

The A21978C factors each had an IR band at 1740 cm^{-1} which shifted to 1720 cm^{-1} when the antibiotic was treated with dilute base (0.1 N NaOH, 20 minutes) at ambient temperatures. This treatment with dilute base resulted in the concomitant loss of antimicrobial activity although there was no change in the amino acid content of the final product. It was concluded that the A21978C antibiotics contain a lactone bond whose integrity was essential for antimicrobial activity. When A21978C was treated with 2,4-dinitrofluorobenzene (DNFB), it was noted that ornithine had reacted through its side-chain amino group and was therefore present as the free amino group in A21978C (*pKa* 7.6).

The oxidation of A21978C with chromic acid destroyed serine but not threonine (amino acid analysis). However, prior treatment of A21978C$_1$ with base resulted in oxidative loss of both threonine and serine. These data supported the presence of a lactone bond involving the threonine hydroxyl group. The presence of a lactone ring at a threonine hydroxyl group was also observed by SHOJI and KATO in their study on the structure of the antibiotic brevistin.[5]

Nature of the Constituent Amino Acids

Since each of the individual A21978C factors had the identical amino acid content as the A21978C complex (amino acid analyzer), it was concluded that each likely had the same peptide core. Of thirteen amino acids, eleven were known to occur widely in peptide antibiotics while two were novel constituents: 3-methylglutamic acid (unknown I) and the fluorescent tryptophan metabolite L-kynurenine (unknown II). Paper chromatography (Whatman I) and TLC (cellulose/aluminum, BPAW) revealed two spots that were Van Urk's spray (blue and yellow) and ninhydrin positive (blue). The slower spot (Van Urk's blue) was identified as L-tryptophan by direct comparison and the faster moving (Van Urk's yellow) was likewise identified as L-kynurenine.[6]

The A21978C antibiotics each had the identical UV chromophore (see Table 2). This spectrum was the sum of the tryptophan and the kynurenine contributions.[7] The presence of L-kynurenine was verified further through the electron impact mass spectrum (EI-MS) of its diacetyl methyl ester, which showed a molecular ion (M^+ *m/z* 306, $C_{15}H_{18}N_2O_5$) and a major fragmentation at *m/z* 162 ($C_9H_8NO_2$). These data corresponded exactly to that expected for the *N,N'*-diacetyl methyl ester of kynurenine and its fragmentation between the side chain methylene and keto groups, respectively.

The isolated amino acid was identical by all chromatographic criteria with authentic kynurenine while molecular rotation verified its configuration.

Chromatograms of A21978C antibiotics were all fluorescent under long wavelength UV light, due primarily to the kynurenine chromophore. The UV spectrum of A21978C included a band at 360 nm (see Table 2) which shifts to 320 nm upon *N*-acetylation.[8] Identical behavior was observed in the UV spectrum of kynurenine and its *N,N'*-acetyl product which led to the conclusion that the kynurenine aryl amino group is present as the free amino group.

The novel aliphatic amino acid was isolated from the C-group (see Experimental section) of amino acids fractionated from the total hydrolysate of A21978C by Amberlite IR-45 (OAc) ion exchange chromatography. This fraction contained primarily aspartic acid and a novel amino acid that chromatographed in a manner similar to glutamic acid (amino acid analyzer) as well as tryptophan. Further chromatography on Amberlite XAD-2 resin removed the tryptophan, and the resulting mixture of acidic amino acid separated on strongly acidic ion exchange resin to give L-aspartic acid and the unknown amino acid. The unknown amino acid was isolated and converted to its phenylthiohydantoin (PTH) derivative. The EI-MS of this derivative showed a series of fragments M^+ *m/z* 278, 260 $(M-18)^+$, 232 $(M-46)^+$, 218 $(M-60)^+$ which were typical of a substituted glutamic acid PTH.[9] The identical fragmentation pattern was obtained from the PTH derivative of authentic 3-methylglutamic acid synthesized by the method of KIM and COCOLAS,[10] confirming the assignment of the methyl group to the 3-position. The synthetic amino acid was obtained as a mixture of *erythro-* and *threo*-racemates.[11] Amino acid analysis of this mixture showed two peaks, the fastest moving corresponding exactly to the unknown amino acid.

The assignment of the configuration of the particular isomer of 3-methylglutamic acid (3-MeGlu) in A21978C required use of the two enzymes: glutamine synthetase and L-glutamic acid decarboxylase.[12] Of the possible 3-methylglutamic acid isomers, glutamine synthetase had previously been shown to have only D-*threo*-3-methylglutamic acid as a substrate.[12] Therefore, when the diastereomeric mixture of the synthetic 3-methylglutamic acid was treated with glutamine synthetase, a large decrease was observed in the peak height of the most mobile of the two diastereomers in the amino acid analyzer thus locating this as the *threo*-isomer. Since this peak also corresponded to the diastereomer present in A21978C, this antibiotic must therefore contain the *threo*-isomer of 3-methylglutamic acid. Treatment of 3-methylglutamic acid (obtained from A21978C) with L-glutamic acid decarboxylase resulted in 80% of the amino acid being destroyed by the L-amino acid specific enzyme. These experiments taken together allow the assignment of the structure of L-*threo*-3-methylglutamic acid to the amino acid. This amino acid has recently been shown to be a constituent of the antibiotic neopeptin.[13]

Most of the configurations of the amino acid residues in A21978C could be assigned by their reaction with either L- or D-amino acid oxidase (AAO). When the A21978C total hydrolysate was digested with these enzymes, L-AAO destroyed threonine, kynurenine and ornithine, therefore the L-configuration was assigned to those three amino acids. D-AAO destroyed alanine and serine leading to assignment of the D-configuration for these amino acids. Direct isolation and rotation measurements indicated the presence of L-aspartic acid, L-tryptophan and L-kynurenine.

Nature of End Groups and Other Functional Groups

The nature of the *C*- and the *N*-termini in A21978C peptide was determined by a series of en-

zymatic and hydrolytic experiments, respectively. Digestion of A21978C with carboxypeptidase Y (specific for hydrolysis of the *C*-terminal amino acid) under standard conditions did not result in the release of any free amino acids. However, when A21978C was pre-treated with base under mild conditions to open the lactone ring, incubation with this enzyme resulted in the release of L-kynurenine. This experiment confirmed that L-kynurenine was the *C*-terminal amino acid which is released by the enzyme only after the lactone bond to the threonine hydroxyl group has been broken. It had already been established that the β-hydroxyl group of the threonine residue was involved in ester or lactone bond formation. Therefore, the lactone bond in the A21978C molecule links the *C*-terminal L-kynurenine carboxyl group to the threonine hydroxyl group.

Treatment of A21978C with DNFB followed by hydrolysis in 6 N HCl, showed no major loss of any of the constituent amino acids (amino acid analysis) present in the native antibiotic except for ornithine whose position shifts due to reaction of the reagent with the side chain amino group. Therefore, A21978C has no free *N*-terminal amino group except those at the side chains of ornithine and kynurenine and may belong to the lipopeptide family of antibiotics which have an acyl group at the *N*-terminal position.

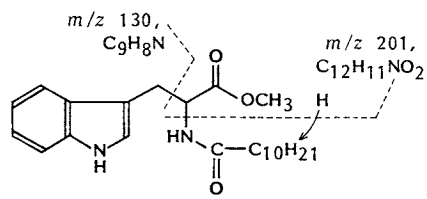
Hydrolysis of A21978C with HCl (0.03 N) caused extensive partial hydrolysis of this antibiotic. It is well established that peptides possessing aspartic acid residues are very susceptible to hydrolysis due to the neighboring group interaction of the β-carboxyl group of the aspartic side chain with the adjacent peptide bond thus promoting cleavage at and about these sites.[14]

Almost half the aspartic acid and alanine present was detected as the free amino acid in 24 hours of partial hydrolysis while half of the alanine present in A21978C was released as the free amino acid. The other amino acids appear in less than 5-mole percent of theory. The high yield of free alanine suggests the presence of an -Asp-Ala-Asp- sequence which would be expected due to increased lability caused by the presence of aspartic acid side chains on each side of this amino acid.

When factors were partially hydrolyzed with 0.03 N HCl, an ether-soluble fraction was formed which was esterified with diazomethane. That obtained from A21978C_1 gave a mass spectra exhibiting a molecular ion at *m/z* 386 as well as fragments at *m/z* 201 and 130. Their elemental composition suggested that this product is an α-*N*-acyl tryptophan methyl ester where the acyl group is $C_{10}H_{21}CO$. The interpretation of this fragmentation is shown in **1**. The mass spectra of authentic α-*N*-acyl tryptophan methyl ester each showed *m/z* 201 and 130 as characteristic fragments as well as a molecular ion which allows determination of the molecular weight of the *N*-acyl group.[15]

The molecular ions of the lipid soluble fractions from factors -C_2 and -C_3 differed from each other by increments of 14 mass units (400 and 414 mass units, respectively) in mass but each had fragments at *m/z* 201 and 130, suggesting that each factor released a characteristic α-*N*-acyl tryptophan fragment suggested that an aspartyl or asparaginyl residue was present adjacent to the tryptophan. This was confirmed by further experiments (see below).

The isolation of α-*N*-acyl-tryptophan from partial hydrolysis of each factor suggested that tryptophan was the *N*-terminal residue and that the terminal amino group was *N*-acylated with fatty acids containing 11, 12 and 13 C-atoms for A21978C_1, -C_2 and -C_3, respectively.



1 M$^+$ *m/z* 386, $C_{23}H_{34}N_2O_3$

A series of α-*N*-acyl-tryptophan methyl esters

bearing straight chain C-11, C-12 and C-13 *N*-acyl groups was synthesized. These derivatives were shown to differ from the corresponding derivatives obtained from A21978C_1, -C_2 and -C_3 by direct comparison of their gas chromatographic retention times indicating that the latter must be branched. The nature of the branching in the fatty acid groups was studied by mass spectral analysis and by ^{1}H NMR. For the identification of the *N*-terminal fatty acid, 4 mg of the antibiotic was hydrolyzed with 2 ml of 6 N HCl for 20 hours at 110°C. The hydrolysate was extracted three times with an equal volume of ether. The pooled extracts were treated with excess diazomethane and the resulting methyl esters analyzed by GC-MS. The spectra showed that the (M^+-29) peak was larger than the (M^+-32) peak for the C-11 and C-13 esters obtained from -C_1 and -C_3 factors which suggested they possessed an *anteiso* branched acyl group.[16] The (M^+-32) fragment was of greater intensity than the (M^+-29) peak for C-12 ester obtained from A21978C_2 which is consistent with a C-12 *iso*-fatty acid. The latter assignment was confirmed by the presence of a doublet in the *C*-methyl region (0.9 ppm) of the ^{1}H NMR of A21978C_2. The -C_1 and -C_3 factors have a multiplet in this region of the spectrum which is consistent with the above assignment.

The sodium salts of the fatty acids derived from a mixture of A21978C_1 -C_2 and -C_3 were dispersed in glycerol and subjected to negative fast atom bombardment (FAB) ionization. The precursor ion ($RCOO^-$) was selected using the first magnet and electrostatic analyzer of a triple sector mass spectrometer having the sectors in the order magnet 1 (B_1)—electrostatic analyzer (E)—magnet 2 (B_2) at a resolution of 2,000. Sufficient argon was introduced into the gas cell between E and B_2 to reduce the precursor ion intensity to about 50% and the products of the collisions of the 8 Kev precursor ions and argon were separated by scanning B_2. The collision induced dissociation (CID) spectra of the carboxylate anions were analyzed by the method of JENSEN.[17] In a typical spectrum a fatty acid carboxylate anion will fragment in increments of 14 mass units (CH_2 group). This fragmentation is observed until a branch point is reached. At this point there is a discontinuity in the spectrum which is observed as a peak of diminished intensity. In this manner it was possible to confirm the branched structures of the A21978C fatty acids to be those assigned above.

These studies demonstrate that the A21978C family of antibiotics have a common peptide core in which each individual member is differentiated by a unique branched-chain fatty acid group which acylates the *N*-terminal-tryptophan residue.

The *m/z* values of all ions were measured to better than ± 0.1 unit. Because the mass excess is chiefly due to the large number of hydrogens it does not read a full mass unit for the peptides below $m/z \cong 1{,}800$ and all values were therefore rounded down to the nearest integer. Taking into account the structural elements deduced above for the A21978C antibiotics, it was possible to calculate the empirical formula for the major factors (*e.g.* A21978C_2, $C_{74}H_{104}N_{16}O_{27}$) if it was assumed that all Asx and 3-MeGlx residues are present as carboxylic acids. This molecular weight would be 1,648 mass units for A21978C_2. However, the FAB-MS of A21978C_2 gave an ($M^+ + H$) ion of 1,648 indicating an actual molecular weight of 1,647—one mass unit less than calculated. This observed molecular weight difference could only be accommodated if an asparagine (or a 3-MeGln) replaced one of the aspartic acids or 3-MeGlu residues giving an empirical factor for A21978C_2 of $C_{74}H_{105}N_{17}O_{26}$ (for -C_1 and -C_3 see Table 2).

Amino Acid Sequence Determination and Total Structure of A21978C

A21978C antibiotics were conveniently deacylated by fermentation with *Actinoplanes utahensis*

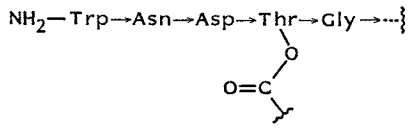
to give a single core peptide (A21978C nucleus) whose chemical properties indicated the generation of a new amino group at tryptophan, loss of the fatty acid acyl group with the lactone bond remaining intact. Each A21978C factor gave the identical peptide. Deacylation also resulted in complete loss of antimicrobial activity. Although deacylation of A21978C could also be accomplished using polymyxin acylase, the required reaction conditions resulted in partial lactone ring rupture.[4,5] Initial work on the sequencing of this peptide was done on the base-treated peptide to remove the lactone bond and thus facilitate interpretation of the Edman degradation results beyond the threonine residue.

This opened-ring peptide was subjected to sequencing by the Edman degradation by both the subtractive and the dansylation methods. Both of these techniques showed a dramatic drop in degradation yields after the second cycle. The dansylation method did reveal a low level of sequencing up to the fourth cycle to give the following partial sequence:

$$NH_2\text{-Trp}\rightarrow\text{Asx}[\rightarrow\text{Asx}\rightarrow\text{Thr}]\rightarrow$$

SHOJI and co-workers had also encountered just such a drop in Edman degradation yields while sequencing the base-treated core peptides from the antibiotics brevistin and cerexin B.[4,5] Their studies showed cessation of sequential degradation when Asn→Asp and -Asn→Asn sequences were encountered. Therefore, it seemed possible that a similar sequence in A21978C was present very close to the *N*-terminus.

Since the mechanism of the degradation method required that all peptide bonds belong to α-amino acids, it was suspected that base treatment caused an $\alpha\rightarrow\beta$ amide bond rearrangement about the asparagine which would not sustain the Edman degradation to the next step. In a third experiment, the Edman degradation was carried out on the A21978C core peptide without prior base treatment, using an automated sequenator to accurately monitor degradation yields.[18] These results showed the degradation to proceed with high yields at each step until the threonine lactone bond was encountered in cycle 4, as would be expected. The degradation could be observed to give glycine PTH in cycle 5 but at reduced yield. These results reveal the following partial structure for the deacylated peptide.



This method clearly assigns asparagine at residue 2 and indicated that this peptide contained only the normal α-bonds at Asn→Asp since good degradation yields were observed. Using this method on A21978C nucleus that had been previously treated with base showed a 70%-drop in degradation yield at the second cycle with essential cessation at the third cycle thereby verifying our initial observations. We attribute this to the effect of base on the Asn→Asp linkage.

This assignment of the asparagine position was confirmed by online mass measurements on fragments observed in the FAB-MS of A21978C_2. The following diagram depicts the limited fragmentation observed at the first three peptide bonds.

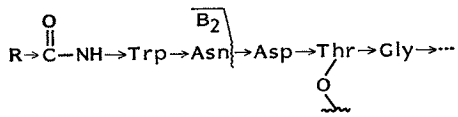


Fig. 2. Total ionization—retention index plot of a 6 N HCl hydrolysate of A21978C$_2$.

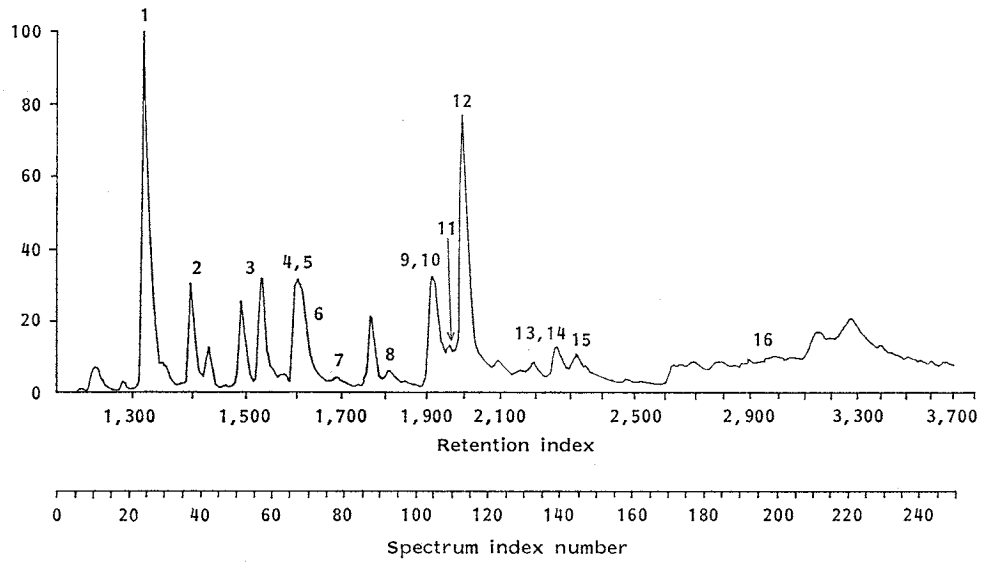


Table 3. Amino acids and peptides identified in 6 N HCl hydrolysates.

		Retention indices	
		Calculated (±5%)	Determined (±1%)
1.	Asx	1,270	1,325
2.	Orn	1,335	1,395
3.	Thr-Gly	1,505	1,533
4.	Asx-Ala	1,605	1,605*
5.	Ala-Asx	1,605	1,605
6.	Asx-Gly	1,610	1,610
7.	Gly-Orn	1,675	1,685
8.	Asx-Thr	1,805	1,810
9.	Ser-MeGlx	1,910	1,915
10.	Asx-Asx	1,910	1,915
11.	Kyn	2,000	1,960
12.	Orn-Asx	1,975	1,990
13.	Trp	2,290	2,255
14.	Asx-Ala-Asx	2,255	2,255*
15.	Orn-Asx-Ala	2,310	2,320
16.	Trp-Asx	2,930	2,985
**	Gly-Ser	1,510	1,490
**	Orn-Asx-Ala-Asx	2,950	3,025

* Peptides used to establish retention index scale.
** Identified in a second hydrolyzate.

Table 4. Amino acids and peptides identified in 0.03 N HCl hydrolysates.

		Retention indices	
		Calculated (±5%)	Determined (±1%)
1.	Ser	1,170	1,235
2.	Asx	1,270	1,325
3.	Orn	1,335	1,390
4.	Gly-Ser	1,510	1,490
5.	Thr-Gly	1,505	1,515
6.	Ala-Asx	1,605	1,590
7.	Asx-Ala	1,605	1,590
8.	Gly-Orn	1,675	1,675*
9.	Asx-Asx	1,910	1,900
10.	Ser-MeGlx	1,910	1,900
11.	Kyn	2,000	1,970
12.	Orn-Asx	1,975	1,975*
13.	Gly-Ser-MeGlx	2,250	2,210
14.	Thr-Gly-Orn	2,210	2,240
15.	$C_{11}H_{23}C(O)$-Trp	3,475	3,365

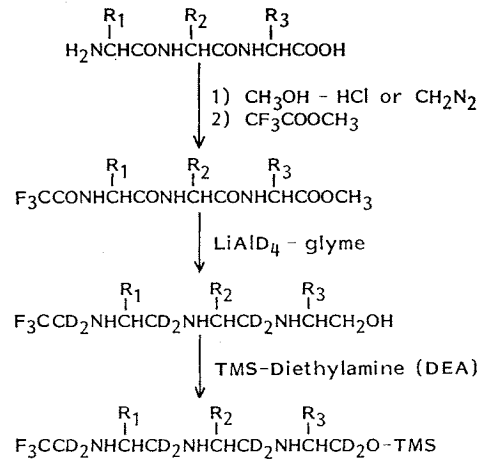
* Peptides used to establish retention index scale.

The mass of the acyl ion B2 was shown to have an accurate mass of *m/z* 483.2963 (483.2971 calculated for $C_{27}H_{39}N_4O_4$). These data indicate that fragment B2 has the correct composition for $C_{11}H_{23}CO$-NH-Trp→Asn$\cdots$. The assignment of the position of the asparagine allows the assignment of the remaining Asx and 3-MeGlx residues to Asp and 3-MeGlu by molecular weight considerations which allows for only one simple amide.

The remainder of the amino acid sequence was determined using GC-MS techniques to identify peptide fragments obtained from partial hydrolysis of A21978C_2 (-C_1 and C_3 gave similar results).

A sample of A21978C_2 (2.6 mg) was partially hydrolyzed for 18 minutes with 6 N HCl at 113°C and derivatized as shown in Scheme 3.[19,20] Approximately 10% of the resulting mixture was subjected to a GC-MS analysis which produced the total ion chromatogram shown in Fig. 2. The sixteen amino acids and peptides identified are listed in Table 3 along with their calculated and determined Kovats retention indices.[20] A second experiment in which the sample was hydrolyzed for 25 minutes yielded the same results and, in addition, the peptides Orn-Asx-Ala-Asx and Gly-Ser were identified. The mass spectrum of Orn-Asx-Ala-Asx is shown in Fig. 3. (This method does not differentiate Asn from Asp).

Scheme 3. Derivatization of peptides for GC-MS.



A 3.3-mg sample of A21978C was hydrolyzed with 0.03 N HCl for 2 hours; half the sample was withdrawn; the remaining sample was hydrolyzed for an additional 3 hours, and the two portions recombined. After derivatization, a GC-MS experiment resulted in a mass resolved total ion chromatogram, in which 15 amino acids and peptides were identified.[21] They are listed in Table 4 along with their observed and calculated retention indices. The distribution of peptides in this hydrolysate was markedly different from the 6 N HCl hydrolysate. As would be expected there was a large amount of aspartic acid, but neither the tripeptide Orn-Asx-Ala nor Asx-Ala-Asx was present. Only small amounts of four Asx containing dipeptides were found. The peptides Thr-Gly-Orn and Gly-Ser-MeGlx, and a C-12 fatty acid derivative of tryptophan, none of which were observed in the 6 N HCl hydrolysate, were all easily identifiable. The spectra of the derivatives from the two tripeptides are shown in Fig. 4.

The position of the kynurenine at the *C*-terminus had been shown by the carboxypeptidase Y reaction discussed above. Therefore, the complete sequence derived from the overlay diagram in

Fig. 3. The mass spectrum of the derivatized Orn-Asx-Ala-Asx peptide.

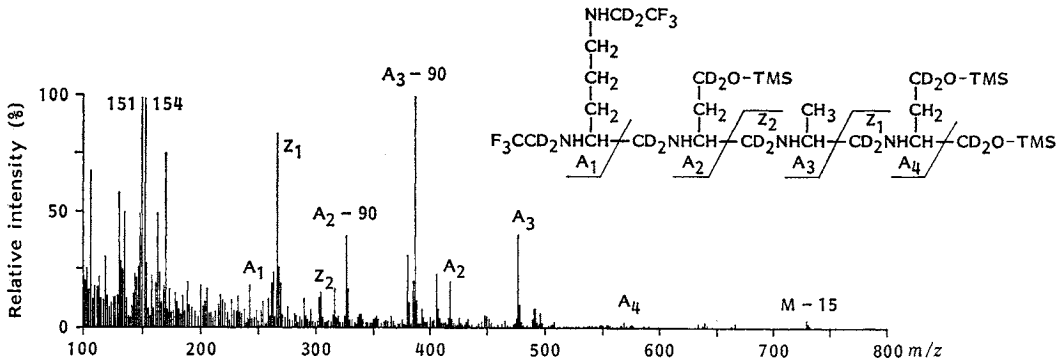


Fig. 4. Mass spectra of derivatized (a) Gly-Ser-MeGlx and (b) Thr-Gly-Orn.

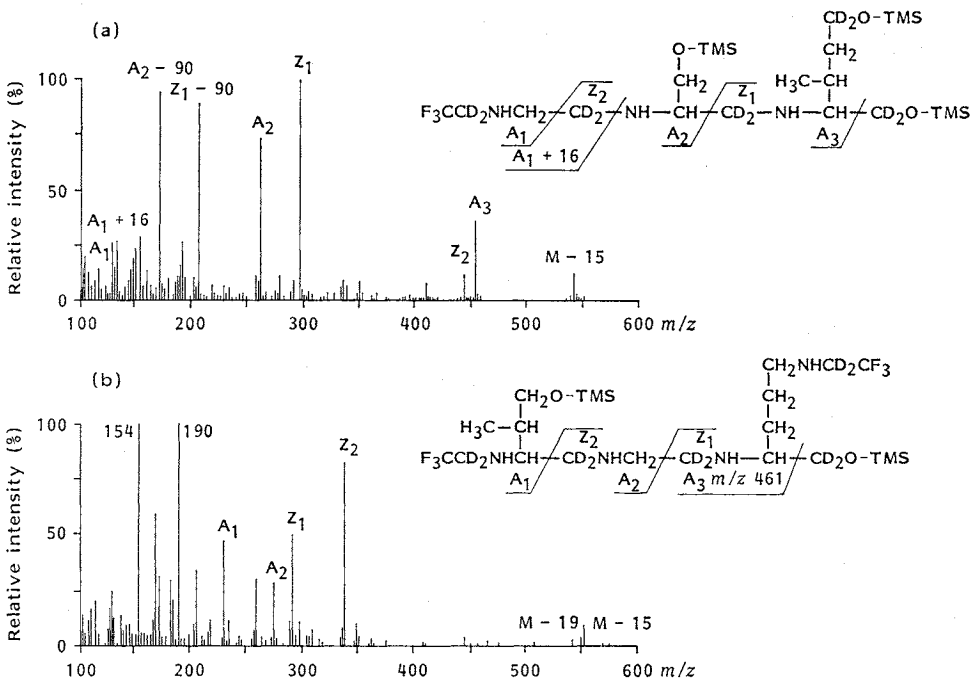


Fig. 5. Peptides identified from partial hydrolysis of A21978C$_2$ by gas chromatographic-mass spectrometric techniques.

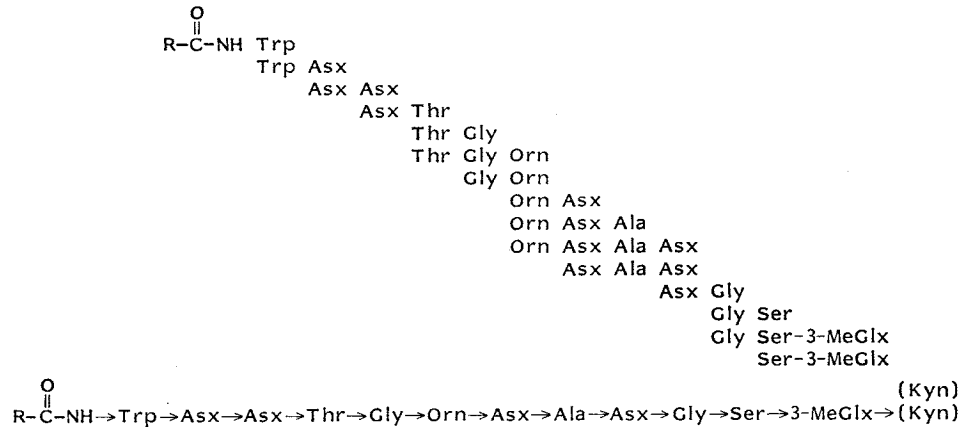
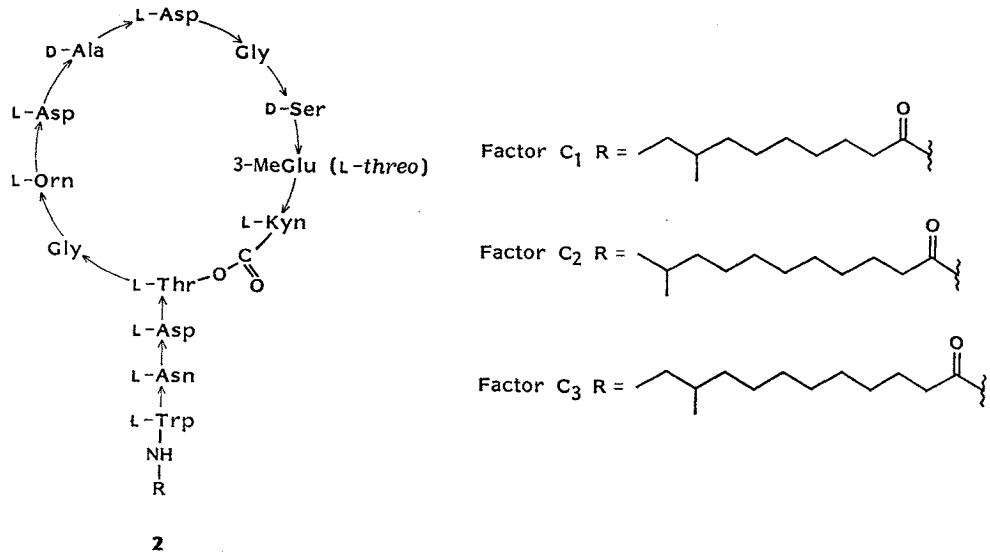


Fig. 5 is consistent with the above data.

It was shown that A21978C contained a lactone bond connecting the *C*-terminus (kynurenine carboxyl group) to the threonine hydroxyl group (see above). Therefore, general structure **2** is assigned to A21978C (Fig. 6).

The A21978C complex of antibiotics has been shown to consist of a family of peptides having the identical peptide core (nucleus) but differing in the nature of the acylating side chain. Therefore, the three major components of this complex, A21978C$_1$, -C$_2$ and -C$_3$, have the same general structure **2** where the *N*-acyl groups are *anteiso*-undecanoyl-, *iso*-dodecanoyl-, and *anteiso*-tridecanoyl, respectively. Minor components A21978C$_0$, -C$_4$ and -C$_5$ also appear to differ in the size and structure of the acyl

Fig. 6. Structure of A21978C.



chains. Studies underway indicate that A21978C$_0$ contains C-10 fatty acids while -C$_4$ and -C$_5$ contain C-12 fatty acids.

Experimental

The following instruments were used in this study: ^{1}H NMR spectrometer, Varian HA100; UV spectrophotometer, Cary 15; ORD and CD spectropolarimeter, Perkin Elmer 241 and Jasco J40AS; IR spectrophotometer, Beckman IR426; high resolution mass spectrometer, Varian MAT731, amino acid analyses, Beckman 120B amino acid analyzer equipped with a Bio-Cal autosample applicator and programmer and single Durrum BC-1A resin; automated peptide sequencing, Beckman Sequencer, Model 890D using the 0.1 M Quadrol program; fast atom bombardment mass spectrometer, ZAB-3F Mass Spectrometer using 8 KeV Xenon Atom Bombardment; GC-MS, Varian MAT 212.

Total Hydrolysis of A21978C

A21978C$_1$ (500 mg) was dissolved in 10 ml of 6 N hydrochloric acid (+4% thioglycolic acid) and placed in five ampoules, sealed and heated at 110°C for 24 hours. The combined solution was extracted with EtOAc and the extract was dried (Na_2SO_4) and evaporated.

The aqueous portion was evaporated under vacuum, diluted with a small quantity of H_2O and applied to an Amberlite IR45 (OAc^-) column (120 ml). The column was eluted with 300 ml of H_2O, 300 ml of 0.3 N NH_4OH and followed by 300 ml of 1 N NH_4OH. The elution was followed by TLC (cellulose/aluminum; butanol - H_2O - pyridine - HOAc, 15 : 12 : 10 : 3; ninhydrin spray), on alternate 10 ml fractions. Three groups of fractions were obtained:

A: H_2O: 85 mg: threonine, alanine and ornithine.

B: 0.1 N NH_4OH: 11 mg: kynurenine (unknown II).

C: 110 mg: aspartic acid, 3-methylglutamic acid (unknown I) and tryptophan.

Group A was chromatographed on Bio Rex 70 (pH 9) and eluted with H_2O (300 ml), followed by 0.001 N NH_4OH (300 ml). The chromatography was carefully monitored by TLC (cellulose/aluminum; butanol - H_2O - pyridine - HOAc, 15 : 12 : 10 : 3; ninhydrin). Two ninhydrin positive bands were eluted. The first (66 mg) was a mixture of threonine, serine, and alanine. These were not separated further. The second band (15 mg) was identified as L-ornithine: $[\alpha]_D^{25}$ +10.1, $[\alpha]_{365}^{25}$ +39.8° (*c* 0.008, 0.5 N HCl); authentic L-ornithine: $[\alpha]_D^{25}$ +22.2° (*c* 0.010, 0.5 N HCl), $[\alpha]_{365}^{25}$ +80.1° (*c* 0.010, 0.5 dm, 0.5 N HCl).

Group C was further purified by chromatography on Amberlite XAD-2 (50 ml). The column was eluted with 600 ml of H_2O followed by 300 ml of 30% MeOH - H_2O. The progress of the chromatography was followed as indicated above. Early fractions contained aspartic acid and 3-methylglutamic acid (unknown I). The later fractions were combined and shown to be L-tryptophan (Van Urk's spray, blue): $[\alpha]_D^{25}$ +9.8° (*c* 0.0038, 0.5 N HCl); authentic D-tryptophan: $[\alpha]_D^{25}$ −4.9° (*c* 0.0004, 0.5 N HCl).

The mixture of aspartic acid and 3-MeGlu was chromatographed on 50 ml of Bio Rad AG50W-X4 (200~400 mesh) in H_2O. The column was eluted sequentially with 300 ml of H_2O, 300 ml of 0.1 N HCl and 0.2 N HCl until ninhydrin positive material no longer was eluted. Each fraction was monitored as above. Two distinct bands eluted. The first was recrystallized twice from EtOH - H_2O and was identified as L-aspartic acid: $[\alpha]_D^{25}$ +16.06°, $[\alpha]_{365}^{25}$ +68.79° (*c* 0.0033, 5 N HCl) values for authentic L-aspartic acid: $[\alpha]_D^{25}$ +23.9°, $[\alpha]_{365}^{25}$ +82.5° (*c* 0.0098, 0.5 N HCl).

The second band was identified as 3-MeGlu (see text) unknown I but could not be induced to crystallize.

Enzymatic Digestion of A21978C_1 Total Hydrolysates with L- and D-Amino Acid Oxidase

A21978C_1 (1 mg) was dissolved in 300 μl of 6 N hydrochloric acid and divided equally among three ampoules, sealed and hydrolyzed for 21 hours at 110°C. The samples were dried under N_2 stream and over KOH - $CaSO_4$ under high vacuum. Enzyme solutions were prepared as follows; L-amino acid oxidase (400 μg) in 3.2 M ammonium sulfate was centrifuged for 20 minutes at 4°C at 2,000 rpm. The supernatant was pipetted and discarded. The enzyme was dissolved in 0.4 M Tris-HCl buffer, pH 7.8 (400 μl).

D-Amino acid oxidase (500 μg) in 3.2 M ammonium sulfate was centrifuged (2,000 rpm) for 20 minutes at 4°C. After removing the supernatant the precipitated enzyme was dissolved in 500 μl of 0.1 M sodium pyrophosphate, 15 μg/ml FAD (pH 8.3) buffer.

Ampoules of the A21978C_1 hydrolysate were digested with 100 μl of D-AAO for 17 hours at 37°C, each tube covered with plastic films having been punctured for O_2 equilibration.

Each digest sample was dried under N_2, at 4 hours under vacuum over KOH - $CaSO_4$. The digests were then analyzed for free amino acids along with the undigested sample (control) in the amino acid analyzer using the lithium citrate system. The following table summarizes these results.

	Control	L-AAO	D-AAO
		nmol	
Asp	0.689*	0.564*	0.656*
Thr	0.140	0.098	0.118
Ser	0.120	0.112	Trace
3-MeGlu	0.147	0.134*	0.132*
Gly	0.327*	0.298*	0.303*
Ala	0.164	0.148*	0.014
Kyn	0.160	0.006	0.089
Orn	0.143	0.013	0.124

* Not a substrate.

The A21978C_2 and -C_3 factors gave identical results to the above.

Enzymatic Digestion of 3-Methylglutamic Acid with Glutamine Synthetase and L-Glutamate Decarboxylase

The 3-MeGlu obtained from A21978C_2 was dissolved in 400 μl of glass distilled H_2O to give 20 μmol/ml. Ten μl (200 nmol) of this solution was dried under N_2 and stored in a freezer as a control.

A fresh solution of L-glutamate decarboxylase Type V (Sigma) was prepared by dissolving 90 μg of the enzyme in 100 μl of a 0.1-M pyridine - HCl - 0.1 M NaCl buffer (pH 4.5). A 10-μl sample of the above 3-MeGlu solution was dried thoroughly under N_2 to which was added 50 μl of the above L-glutamate decarboxylase solution. This solution was covered with a perforated plastic film and al-

lowed to incubate for 22 hours at 37°C. The reaction solution was dried under N_2 stream, then high vacuum, and analyzed for 3-MeGlu by amino acid analysis. The L-glutamic acid decarboxylase Type V lowered the 3-MeGlu content to 0.012 μmol from 0.056 μmol in the control sample.

To another digest tube was added imidazole buffer (30 μl), $MgCl_2$, ATP, 2-mercaptoethanol (10 μg each), hydroxylamine-HCl (20 μg), 10 μl (200 nmol) of the 3-MeGlu preparation and glutamine synthetase (10 μl, 1.33 U). The mixture was allowed to incubate for 22 hours at 37°C. After drying in N_2 stream and under vacuum, the resulting mixture was analyzed for 3-MeGlu content by the amino acid analyzer (lithium citrate buffer). Analysis showed no significant consumption of 3-MeGlu over control.

Preparation of 3-Methylglutamic Acid

3-Methylglutamic acid was prepared from diethyl acetamidomalonate and ethyl acetonate by the method published earlier by KIM and COCOLAS.[10] Amino acid analysis of the 3-MeGlu synthesized showed that two diastereomers, A and B, had formed which eluted chromatographic separation (retention times: A (3,076 seconds) and B (3,299 seconds). The diastereomer with the shortest retention time corresponds exactly to the 3-MeGlu diastereomer obtained from A21978C. Digestion of this mixture with glutamine synthetase (see previous experiment) which is specific for D-*threo*-3-MeGlu caused a 50 percent decrease in the area under the peak at 3,076 seconds. These data identify the A peak as the *threo*-diastereomer.

The absence of a reaction of the 3-MeGlu from A21978C with glutamine synthetase is evidence that it has the L-*threo*-configuration. Its reaction with L-glutamate decarboxylase confirms the L-configuration.

PTH Derivative of A21978C Unknown Amino Acid I

Unknown amino acid I (2 mg) was dissolved in 2 ml of pyridine - H_2O (1 : 1), heated in a water bath at 40°C and the pH of the solution adjusted to 8.6 with dilute NaOH. Phenylisothiocyanate (0.05 ml) was added and the pH held at 8.6 by addition of 0.1 N NaOH. The reaction was extracted twice with toluene and the cooled aqueous was acidified to pH 1 (0.1 N HCl) and the solvents removed under vacuum. The residue was suspended in 1 N HCl and heated to 80°C for 10 minutes then cooled. After extracting the reaction mixture three times with EtOAc, the organic extracts were evaporated to dryness and the mass spectral analysis (EI-MS) showed the following fragments: *m/z* 278 $(M)^+$, 260 (M−18), 232, 218, 206, 135 and 119. The PTH of unknown I was methylated with CH_2N_2 and the resulting product was examined by EI-MS: *m/z* 292 (M^+), 278, 260, 244, 232, 218, 206, 202, 135 and 119. Peak matching results are discussed in the main body of this report. Synthesis of the PTH derivative of synthetic 3-MeGlu gave a product with the identical mass spectrum (EI-MS).

Partial Hydrolysis of A21978C

(a) Ether Soluble Components: A solution of A21978C_1 (9 mg) was dissolved in 0.03 N HCl (1 ml), sealed in an ampoule and heated at 110°C for 24 hours. After cooling, the solution was extracted twice with Et_2O, dried over Na_2SO_4 and evaporated to dryness. The residue was methylated in the usual manner with CH_2N_2 and examined by GC-MS analysis. The GC-MS showed the ether extract to consist of one major component whose mass spectrum consisted of the following fragments: *m/z* 386 $(M^+, C_{23}H_{34}N_2O_3)$, 201 $(C_{12}H_{11}NO_2)$ and 130 (C_9H_8N). (Peak matching determined in the EI-MS). This fragmentation pattern is consistent with an 2-*N*-undecanoyltryptophan methyl ester (see text).

Hydrolysis and methylation of factors A21978C_2 and -C_3 by this procedure showed similar results with each showing a *m/z* 201 and 130 fragments but had different retention times (GC) and molecular ions: $(M^+$ 400 and 414).

Authentic *n*-undecanoyl, *n*-dodecanoyl and *n*-tridecanoyl α-*N*-amides of tryptophan methyl ester (prepared from appropriate acyl chlorides) differed substantially in their retention times from the derivatives obtained from A21978C_1, -C_2 and -C_3, respectively.

(b) Peptide Fragments: Ten sealed ampoules containing 10 mg/ml of A21978C_1 in 0.03 N HCl were heated to 105°C. Individual ampoules were opened at 4, 8, 12, 24 and 51 hours. After

evaporation of the solvents the samples were analyzed for free amino acids (amino acid analyzer) using lithium citrate buffer. This experiment showed a rapid release of aspartic acid (50% in 24 hours) and alanine (50% in 24 hours). Lower levels of free glycine, ornithine and kynurenine (10%) were observed. Amino acid analysis showed the presence of a large number of unidentified ninhydrin positive peptides. Total hydrolysis (6 N HCl, 110°C) of the partial hydrolysate had the same amino acid composition as obtained from total hydrolysis of A21978C.

Chromic Acid Oxidation of A21978C and Base Treated A21978C

A 6-mg sample of A21978C was added to a solution of CrO_3 (160 mg) in 0.1 ml of pyridine and 3 ml of HOAc and allowed to stand for 20 hours at room temperature. A second sample of A21978C (2 mg) that had been deactivated with dilute base was also oxidized under identical conditions. Each sample was diluted with excess water at the end of the reaction period and extracted with butanol. These extracts were evaporated, lyophilized, totally hydrolyzed (6 N HCl, 8 hours) and analyzed for free amino acids (amino acid analyzer) with the following results:

Oxidation Product from	Asp	Thr	Ser	Gly	Ala	3-MeGlu
	nmol					
A21978C	4.0	0.55	Trace	1.64	0.82	0.55
A21978C (base treated)	3.56	Trace	Trace	1.92	1.0	0.71

Preparation of A21978C Nucleus

A fermentation of *Actinoplanes utahensis* NRRL 12052 was carried out using the conditions outlined in U.S. 4,537,717.[22] After 48 hours purified A21978C complex (50 g) was added and incubated for 16 hours. The broth filtrate was passed over a column containing 3.1 liters of Diaion HP-20 resin. The column was eluted with 10 volumes of water followed by CH_3CN - H_2O (95 : 5). This elution was monitored by analytical HPLC using silica gel/C_{18} substrate and a solvent system of H_2O - MeOH (3 : 1) containing 0.1% NH_4OAc. After collecting 24 liters, the eluting solvent was changed to H_2O - CH_3CN (9 : 1). Fractions containing the nucleus were eluted with this system. After combining and concentrating under vacuum to remove CH_3CN, the residue was lyophilized to give A21978C nucleus (partially pure) (24 g). This material was dissolved in 400 ml of H_2O and pumped onto a 4.7×192 cm steel column containing 3.3 liters of silica gel (Quantum LP-1)/C_{18} packed in H_2O - MeOH - CH_3CN (8 : 1 : 1), 0.2% HOAc, 0.8% pyridine. The column was developed at 140 kg/cm^2 and the eluate monitored at 280 nm. Fractions containing the nucleus were freeze dried to give 14 g of A21978C nucleus: ($C_{62}H_{83}N_{17}O_{25}$).

Edman Degradation - Dansylation of A21978C Nucleus

A solution of A21978C nucleus (1.1 mg, 0.5 μmol) in 400 μl of 0.4 M dimethylallylamine - trifluoroacetate (DMAA - TFA) buffer (pH 9.2) was treated with 40 μl of phenylisothiocyanate for 1 hour at 50°C (sand bath) under an atmosphere of N_2. After cooling, the solvents were removed under a stream of N_2 and extracted three times with Et_2O, centrifuging each time. The combined extracts were evaporated under N_2 stream and redissolved in 400 μl of the above-mentioned buffer. After removing 20 μl of this solution for dansylation, the remainder was subjected to a second Edman degradation by the above procedure. Dansylation was performed by the addition of 200 μl of 12 mM dansyl (DNS) chloride in acetone to each aliquot and allowing reaction to occur at room temperature overnight in the dark. The residue after evaporation was dissolved in 1 ml of 6 N HCl in a sealed ampoule and hydrolyzed at 105°C for 3 hours. After evaporating to dryness under vacuum the residue was dissolved in a small amount of pyridine - acetone (1 : 1) and applied to a silica gel plate for two-dimensional TLC using the following solvent systems:

Solvent I: Et_2O - CH_3OH - formic acid, 100 : 5 : 1.

Solvent II: Acetone - NH_4OH, 85 : 15.

The DNS-amino acids were detected under UV light and identified by comparison with authentic DNS-amino acids. The degradation of A21978C appeared to slow after the second cycle and es-

sentially ceased by the fifth cycle. The following partial sequence was deduced by this method:

NH_2-Trp-Asx-Asx-Thr ..... (see text)

Edman Degradation of A21978C Nucleus

A solution of A21978C nucleus (27 mg) in 1.5 ml of 0.4 M DMAA - TFA buffer (pH 9.4) was treated with 0.15 ml of phenylisothiocyanate under N_2 in a centrifuge tube that was sealed with plastic film. The reaction was maintained at 50°C for 60 minutes. Solvents were evaporated under N_2 and 0.2 ml of trifluoroacetic acid was added. The reaction was heated to 50°C for 15 minutes, cooled and evaporated to dryness. After three extractions with dichloroethane the residue was redissolved in the above buffer. A small amount was submitted for hydrolysis and amino acid analysis while the remainder was dissolved in DMAA - TFA buffer (1 ml) and the above procedure repeated. The amino acid analysis results from each cycle show that the Edman deacylation of A21978C slows after the second cycle but small decreases in amino acids were observed into cycle 4. These results are shown in the following table:

	Trp	Asx	Thr	Ala
		nmol		
Original peptide	0.62	3.80	0.88	1.0
Cycle 1	Trace	3.84	0.90	1.0
Cycle 2	—	3.06	0.88	1.0
Cycle 3	—	2.74	0.77	1.0
Cycle 4	—	2.66	0.56	1.0
Cycle 5	—	2.77	0.58	1.0

Reaction of A21978C with 2,4-Dinitrofluorobenzene

Base-treated A21978C (8 mg) was dissolved in 2 ml of EtOH - H_2O (2:1) to which 8 mg of $NaHCO_3$ and 0.2 ml of 5% ethanolic 2,4-dinitrofluorobenzene (w/v) was added. After 2 hours the reaction mixture was extracted with Et_2O, acidified and re-extracted with Et_2O. The aqueous layer was extracted with butanol and this extract evaporated to dryness to give a yellow foam which did not give a positive ninhydrin test and showed all amino acids present in the original A21978C with the exception of ornithine upon amino acid analysis.

Sodium Borohydride Reduction of A21978C

A solution of A21978C (4.0 mg) in 2.5 ml of pyridine - H_2O was treated with 40 mg of $NaBH_4$ at 0°C and then left in the refrigerator for 3 days. The reaction was diluted with HOAc - H_2O and extracted with butanol. The solvent was evaporated and amino acid analysis of the resulting product showed all the constituent amino acids of A21978C except kynurenine.

Determination of the *C*-Terminal Amino Acid in A21978C Using Carboxypeptidase Y

Base-treated A21978C was digested with carboxypeptidase Y using the conditions outlined by HAYASHI *et al.*[28] Samples of the digest were withdrawn after 10 minutes, 90 minutes and 24 hours, and analyzed for free amino acids in the amino acid analyzer. A steady increase in free kynurenine was observed going from 0.02 μmol released after 10 minutes, 0.109 μmol at 90 minutes to 0.125 μmol after 24 hours. L-Kynurenine was the only amino acid liberated with this enzyme. No substantial amino acid release was observed with this enzyme when A21978C was not initially treated with base.

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