Berninamycin. 2. Products of Acidic Hydrolysis, Methanolysis, and Acetolysis of Berninamycin A[1]

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Abstract: Berninamycin has been separated into berninamycins A and B by countercurrent distribution and by high-pressure liquid chromatography. Acidic hydrolysis of berninamycin A gives berninamycinic acid and the novel amino acid β-hydroxyvaline, as well as threonine, ammonia, and pyruvic acid. Complete reduction of berninamycin A with sodium borohydride followed by acidic hydrolysis gives β-hydroxyvaline, threonine, butyrine, and alanine; partial reduction with borohydride followed by hydrolysis gives pyruvic and α-ketobutyric acids, plus 2-acetyl- and 2-propionyl-4-[*N*-(1-carboxyethyl)carboxamido]-5-methyloxazoles (**4** and **5**). Methanolysis of berninamycin A under varying conditions gives threonine and β-hydroxyvaline methyl esters, 2-acetyl- and 2-propionyl-4-carbomethoxy-5-methyloxazoles (**6** and **18**) and the two corresponding 4-carboxamidooxazoles (**14** and **15**), and 4-carbomethoxy-5-methyl-2-(1-threoninamido-1-propenyl)oxazole (**17**). Acetolysis of berninamycin A employing hydrochloric acid and glacial acetic acid gives 2-acetyl- and 2-propionyl-4-carboxamido-5-methyloxazoles (**14** and **15**) plus 4-carboxamido-5-methyl-2-[1-(*N*-pyruvylthreoninamido-1-propenyl]oxazole (**19**). Evidence is presented that the 2-acyloxazoles originate from masked dehydroamino acid residues in the intact antibiotic and that five dehydroalanine residues are also present. Structures are assigned to two subunits of the antibiotic ($C_{18}H_{22}N_6O_6$ and $C_{10}H_{10}N_4O_3$) which contain the precursors to the 2-acyloxazoles, and the composition of the antibiotic is discussed, based upon the degradation products described.

Berninamycin is a potentially useful antibiotic by virtue of its specific inhibition of bacterial protein synthesis.[4] Our first report from this laboratory[2] showed that the antibiotic consists of two components, berninamycins A and B, and that the former, major component corresponds to the previously described[4,5] purified berninamycin. Preliminary degradation studies[5] on purified berninamycin (berninamycin A) indicated that the antibiotic is a modified peptide and we have recently assigned[2] structure **1** (Scheme I) to berninamycinic acid, a hydrolysis product of both berninamycins A and B containing approximately one-fourth of the molecular formula of berninamycin A. Berninamycinic acid (**1**) contains the hitherto unreported pyridothiazolopyridinium chromophore.

We wish to report here experimental details of the earlier study[2] and the results of continuing structural studies on berninamycin A carried out in our laboratory. The present report will describe compounds obtained from acidic hydrolysis, methanolysis, and acetolysis of berninamycin A. Of particular significance are several previously unreported oxazole-4-carboxylic acid derivatives, including oligopeptides containing these moieties. Evidence will also be presented for the existence of a number of dehydroamino acid units in berninamycin A, and all the structural units of the antibiotic will be accounted for.

Results

Berninamycins A and B. Crude berninamycin was subjected to countercurrent distribution[6] to give the major component, berninamycin A, along with a second, related antibiotic, berninamycin B, in the approximate ratio 17:1. Berninamycin A is identical with the "berninamycin" obtained originally by column chromatography;[4,5] the chromatographic band containing berninamycin B would have been quite small and was evidently not detected. We have concentrated our efforts on berninamycin A, since it can be obtained in much larger quantities than berninamycin B. Nearly complete separation

Scheme I. Acidic Hydrolysis Products of Berninamycin A (*Isolated as the 2,4-Dinitrophenylhydrazone. † Isolated as the Methyl Ester of the 2,4-Dinitrophenylhydrazone)

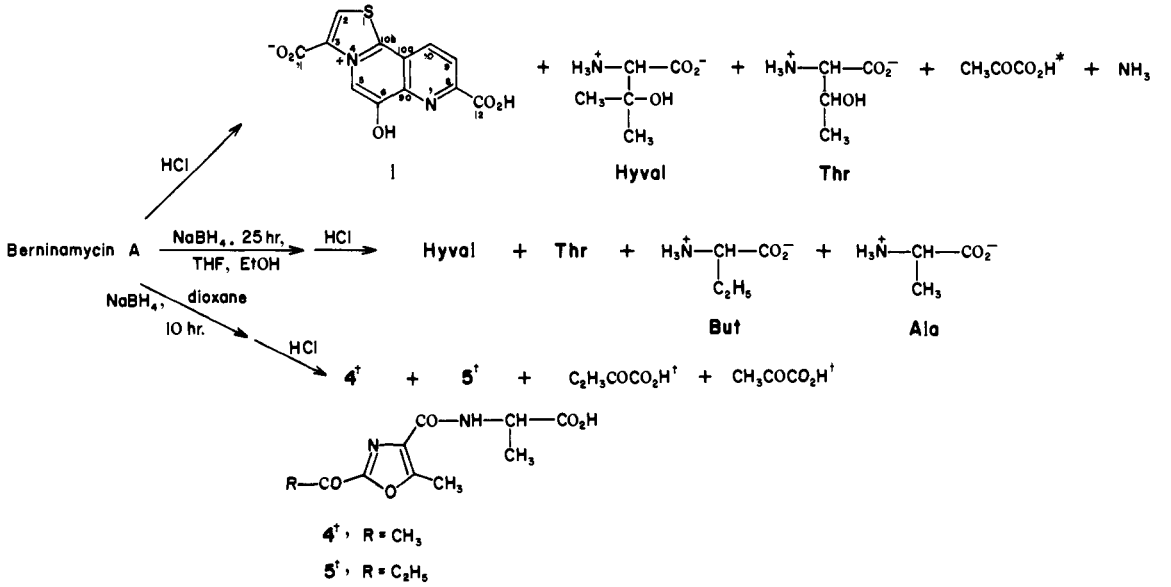


Table I. ^{1}H NMR Spectra of Berninamycin A

Proton position[a]	Chemical shift[g,h] C_5D_5N	Chemical shift[g,h] Me_2SO-d_6
Hyval CH_3 (6 protons)	1.48 (3 H, s), 1.66 (3 H, s)	1.25 (3 H, s), 1.27 (3 H, s)
Hyval H-2	5.36 (1 H, d, J = 6.5 Hz)	4.75 (1 H, d, J = 7 Hz)
Hyval NH	8.93 (1 H, d, J = 6.5 Hz)	8.53 (1 H, d, J = 7 Hz)
Hyval OH	5.73 (1 H, s)	5.23 (1 H, s)
Thr[b] CH_3	1.73 (3 H, d, J = 6.5 Hz)	1.20 (3 H, d, J = 7 Hz)
Thr H-3	5.18 (1 H, d of q, J = 3, 6.5 Hz)	4.36 (1 H, m)
Thr H-2	5.45 (1 H, d of d, J = 3, 7.5 Hz)	4.66 (1 H, d of d, J = 4, 8 Hz)
Thr NH	8.80 (1 H, d, J = 7.5 Hz)	8.03 (1 H, d, J = 7 Hz)
Thr OH		4.98 (1 H, d, J = 7 Hz)
Ox-B[c] H-2γ (CH_3)	1.93 (3 H, d, J = 6.5 Hz)	1.77 (3 H, d, J = 7 Hz)
Ox-B H-2β	6.50 (1 H, q, J = 6.5 Hz)	(5.8)[i]
Ox-B H-5α (CH_3), Ox-A[d] H-5α (CH_3)	2.52 (3 H, s), 2.59 (3 H, s)	2.64 (3 H, s), 2.67 (3 H, s)
Ox-A H-2β, Deala H-β (5 protons); H_{trans}[e]	5.79 (2 H, s), 5.91 (1 H, s), 5.94 (1 H, s), 6.07 (1 H, s), 6.16 (1 H, s)	5.70 (1 H, s), 5.75 (1 H, s), 5.77 (2 H, s), 5.80 (1 H, s), 5.89 (1 H, s)
Ox-A H-2β, Deala H-β (5 protons); H_{cis}[e]	6.77 (1 H, s), 6.82 (1 H, s), 6.83 (2 H, s), 6.91 (1 H, s), 7.02 (1 H, s)	6.09 (1 H, s), 6.20 (1 H, s), 6.41 (1 H, s), 6.48 (1 H, s), 6.52 (1 H, s), 6.55 (1 H, s)
Bmcyl[f] H-9	8.04 (1 H, d, J = 8 Hz)	8.20 (1 H, d, J = 8 Hz)
Bmcyl H-10	8.34 (1 H, d, J = 8 Hz)	8.30 (1 H, d, J = 8 Hz)
Bmcyl H-2	8.57 (1 H, s)	8.52 (1 H, s)
Bmcyl H-5	8.84 (1 H, s)	8.70 (1 H, s)
Ox-A 2α-NH, Ox-B 2α-NH, Bmcyl NH, Deala NH (5 protons)	9.02 (1 H, s), 9.59 (1 H, s), 9.75 (1 H, s), 9.93 (1 H, s), 10.02 (1 H, s), 10.18 (1 H, s), 10.48 (1 H, s), 11.05 (1 H, s)	7.95 (1 H, s), 9.36 (1 H, s), 9.41 (1 H, s), 9.45 (1 H, s), 9.55 (1 H, s), 9.59 (1 H, s), 9.77 (1 H, s), 10.55 (1 H, s)

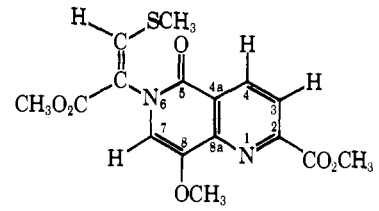
[a] See Scheme V for subunits. [b] Threonyl subunit in g. [c] Dehydrobutyrine-derived oxazole in g. [d] Dehydroalanine-derived oxazole in f. [e] Protons which are trans and cis to the carbonyl or oxazole groups. [f] Berninamycinic acid related subunit in i. [g] In ppm from internal tetramethylsilane. [h] Multiplicities: s = singlet, d = doublet, q = quartet. [i] Multiplet overlapped by Ox-A H_{trans}-2β and Deala H_{trans}-β; position indicated by integration and decoupling of Ox-B H-2γ (CH_3).

of berninamycins A and B in an analytical mode can be achieved easily and quickly by high-pressure liquid chromatography. However, a preparative separation of the antibiotics is not feasible in this way since they are not very soluble in the solvent system employed.

Berninamycins A and B were obtained as neutral, white, microcrystalline solids. Their ultraviolet spectra show increasing absorption from 280 to 200 nm and their infrared spectra contain an especially broad carboxamide band centered at 1670 cm^{-1}. The ^{1}H NMR and ^{13}C NMR spectra of berninamycin A (Tables I and II) contain 50 protons and 51 carbons, while the spectra of berninamycin B contain about 69 protons and 49 carbons. Electron-impact mass spectra of berninamycins A and B show variable peaks in the low molecular weight region due to decomposition and are not amenable to meaningful interpretation.

Elemental analyses indicated the molecular formulas $C_{51}H_{52}N_{14}O_{17}S$ (mol wt, 1164) for berninamycin A and $C_{59}H_{74}N_{14}O_{22}S$ (mol wt, 1362) for berninamycin B. However, the field desorption mass spectrum of berninamycin A indicated a molecular weight of 1146 for berninamycin A, which would correspond to the molecular formula $C_{51}H_{50}N_{14}O_{16}S$, with 1 equiv of water less than the value obtained by elemental analyses.

Hydrolysis of Berninamycin A. Amino Acid Composition. A sample of berninamycin A was hydrolyzed (Scheme I) and automatic amino acid analysis of a portion of the hydrolyzate indicated β-hydroxyvaline,[7] threonine, glycine, and ammonia in the approximate molar ratios 1.0:1.0:0.1:~9, respectively. The remainder of the hydrolyzate was evaporated to dryness and the residue was triturated with dilute acetic acid to give the soluble amino acid fraction. The insoluble residue was removed by filtration and identified as crude berninamycinic acid (**1**, Scheme I). The structures of **1** and tetramethylberninamycinate hydrate (**2**), a derivative of **1** obtained by exhaustive permethylation, were assigned earlier.[2]



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The soluble components were separated on a Dowex 50W-X4 (H^+) column and the four peaks which were detected by ninhydrin assay were identified as L-threonine, L-β-hydroxyvaline,[7] ammonium acetate, and glycine (trace amount), by comparison of their physical properties with those of known samples. L-Threonine and glycine had been identified in a preliminary study.[4]

Dehydroamino Acid Residues. The presence of dehydroamino acid residues in berninamycin A was suggested by the large quantity of ammonia liberated during the acidic hydrolysis of the antibiotic. The other acidic hydrolysis products to be expected from dehydroamino acid residues are α-keto acids and these can be isolated as 2,4-dinitrophenylhydrazones.[8] Accordingly, another sample of berninamycin A was hydrolyzed to yield pyruvic acid 2,4-dinitrophenylhydrazone as the only derivative, in a yield suggesting 5 equiv of pyruvic acid released per mole of the antibiotic (assuming an approximate molecular weight of 1150).

Sodium borohydride has been used previously to reduce the dehydroamino acid residues in thiostrepton,[8] which yielded alanine and butyrine on hydrolysis. Complete reduction of the dehydroamino acid residues in berninamycin A was observed after 25 h at room temperature in a mixture of tetrahydrofuran

Table II. ^{13}C NMR Spectra of Berninamycin A

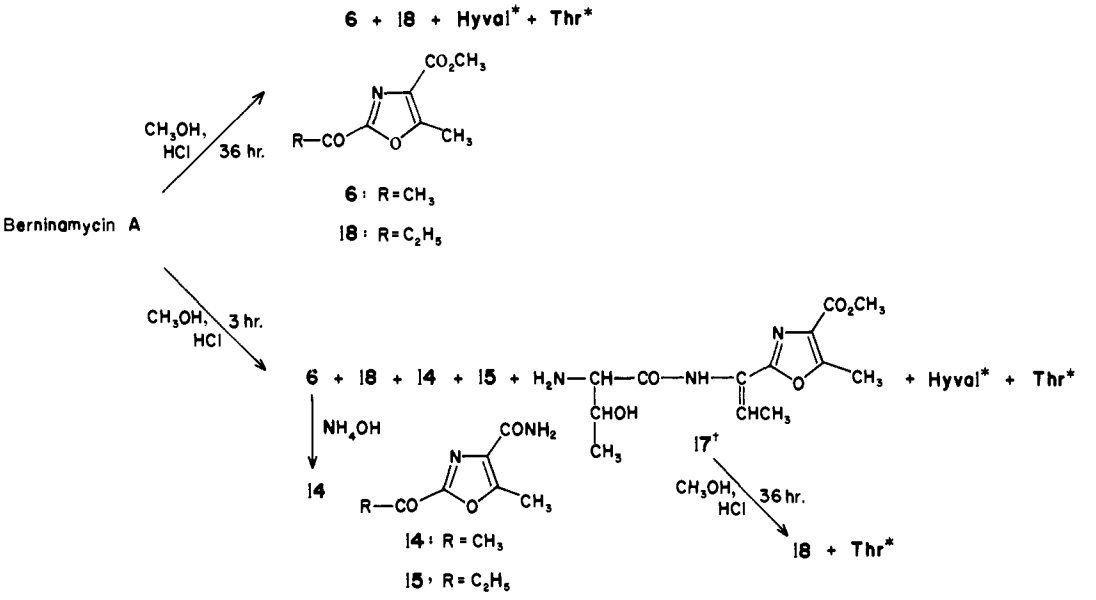
Carbon position[a]	Chemical shift[b,c] and multiplicity[d] in off-resonance decoupled spectrum	
	C_5D_5N	Me_2SO-d_6
$-CH_3$		
Ox-A C-5α, Ox-B C-5α	11.0 q, 11.2 q	11.4 q, 11.5 q
Ox-B C-2γ	13.8 q	13.6 q
Thr	19.6 q	20.4 q
Hyval	26.0 q, 27.6 q	25.9 q, 27.3 q
-CH-NH-		
Thr C-2	58.8 d	57.3 d
Hyval C-2	62.6 d	61.3 d
-CH-OH		
Thr C-3	68.0 d	67.3 d
-C-		
Hyval C-3	72.0 s	70.9 s
$=CH_2$		
Deala C-β (5 carbons)	102.4 m, 103.6 m, 104.4 m, 104.5 m, 105.0 m	105.3 m, 105.7 m, 105.7 m, 105.7 m, 105.7 m
Ox-A C-2β	108.4 m	111.0 m
=CH-S-		
Bmcyl C-2	121.6 d*	122.8 d*
=CH-		
Ox-B C-2β	123.8 d*	126.2 d*
Bmcyl C-9	130.0 d	128.9 d
Bmcyl C-10	140.6 d†	139.0 d†
=CH-N		
Bmcyl C-5	135.2 d†	133.6 d†
=C<		
Bmcyl C-10a	126.6 s	128.2 s††
=C-N		
Deala C-α (5 carbons)	128.4 s, 129.0 s, 129.5 s, 130.2 s, 130.3 s	128.4 s,†† 128.9 s, 129.9 s, 129.0 s, 129.9 s
Ox-A C-4, Ox-B C-4	134.9 s,≠ 135.5 s≠	133.2 s,≠ 134.4 s≠
Ox-B C-2α	135.8 s≠	134.7 s≠
Ox-A C-2α	141.0 s‖	140.2 s‖
Bmcyl C-10b	141.4 s‖	140.6 s‖
Bmcyl C-3	147.3 s‖	146.5 s‖
Bmcyl C-6a	150.1 s#	149.0 s#
Bmcyl C-8	150.9 s#	149.2 s#
Bmcyl C-6	153.6 s#	153.2 s#
=C-O		
Ox-A C-5, Ox-B C-5	155.0 s, 155.5 s	154.2 s, 154.8 s
-N=C-O-		
Ox-A C-2, Ox-B C-2	157.1 s, 158.2 s	156.3 s, 157.8 s
O=C-N-		
Deala C-1 (5 carbons)	160.2 s, 160.4 s, 161.1 s, 162.1 s, 162.7 s	159.2 s, 159.3 s, 159.6 s, 161.2 s, 161.7 s
Ox-A C-4α, Ox-B C-4α	164.0 s,** 165.3 s**	162.8 s,** 163.55**
Bmcyl C-12	166.6 s**	164.8 s**
Thr C-1, Hyval C-1	169.8 s, 170.7 s	168.4 s, 169.0 s
$O=C-O^-$		
Bmcyl C-11	163.6 s**	162.4 s**

[a] See Table I and Scheme V for abbreviations. [b] In ppm from internal tetramethylsilane. [c] Values with the same superscript symbol (*, †, etc.) in the same solvent may be interchanged. [d] Multiplicities in off-resonance decoupled spectra: s = singlet, d = doublet, q = quartet, m = multiplet due to overlapping peaks (should be triplet or quartet).

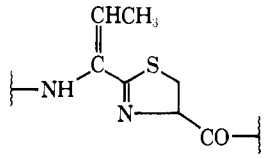
and ethanol. A sample of the crude reduced product was hydrolyzed (Scheme I) and the amino acids (Scheme I), isolated by ion-exchange chromatography as above, were identified as threonine, β-hydroxyvaline, alanine, and butyrine.[9] Quantitation by gas chromatography[10] gave the molar ratio 1.0:0.8:1.1:6.0 for threonine, β-hydroxyvaline, butyrine, and alanine, respectively.

The isolation of butyrine from acidic hydrolysis after reduction indicates a dehydrobutyrine residue in berninamycin A. The ^1H NMR spectrum (C_5D_5N, Table I) of berninamycin A shows a methyl doublet at 1.93 ppm strongly coupled (J = 6.5 Hz) to a one-proton quartet at 6.50 ppm, which can be attributed to the ethylidene group of a dehydrobutyrine residue. On the other hand, α-ketobutyric acid was not detected

Scheme II. Methanolysis Products of Berninamycin A (*Isolated as the Acetyl Derivative of the Methyl Ester. † Isolated as the Acetyl Derivative)



in the hydrolyzate of berninamycin A. Thus, it appears that a masked dehydrobutyrine residue is present. Similar observations were made on thiostrepton, in which a dehydrobutyrine residue appears as part of the dihydrothiazole-4-carboxylic acid unit **3**.[11]



3

The isolation of 5 equiv of pyruvic acid coupled with the isolation of 6 equiv of alanine indicates that five dehydroalanine residues are present in berninamycin A and suggests that the sixth equivalent of alanine is formed from a masked dehydroalanine residue.

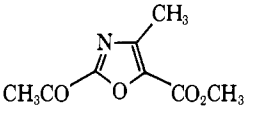
Oxazoles 4 and 5. During investigation of the reduction of berninamycin A with sodium borohydride in dioxane (Scheme I) a precipitate formed after 10 h. Its degree of reduction was determined by hydrolysis to pyruvate, as previously described. Analysis of the crude 2,4-dinitrophenylhydrazones by thin-layer chromatography indicated three compounds and, since an ethyl group was apparent in the ^{1}H NMR spectrum of the crude product, the mixture was treated with methanolic hydrogen chloride to produce a mixture of esters, which could be separated more easily by preparative thin-layer chromatography. The two less polar compounds were identified as the methyl esters of the 2,4-dinitrophenylhydrazones of pyruvic acid and α-ketobutyric acid in a ratio of approximately 1:1.

The third, most polar, fraction, isolated in trace amount, proved to be the most interesting. Its high-resolution mass spectrum indicated a mixture of two homologues, in the ratio of 1:1, with molecular formulas $C_{17}H_{18}N_6O_8$ and $C_{18}H_{20}N_6O_8$. The mixture thus contains dinitrophenylhydrazones of two ketones whose molecular formulas must be $C_{11}H_{14}N_2O_5$ and $C_{12}H_{16}N_2O_5$. In agreement with this conclusion the ^{1}H NMR spectrum contains corresponding peaks for a methyl group (2.57 ppm, s) and an ethyl group (1.23 ppm, t; 2.95 ppm, q). Thus, one compound is the dinitrophenylhydrazone of a methyl ketone and the other that of an ethyl ketone. In light of other, simpler oxazoles (**14, 15**) whose structures will be presented in the next section, structures **4** and **5** (which will also be discussed in detail in the next section) are assigned to the components of the mixture (Scheme I) isolated as 2,4-dinitrophenylhydrazone methyl esters.

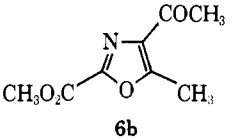
Methanolysis of Berninamycin A. 2-Acetyl-4-carbomethoxy-5-methyloxazole (6). A sample of berninamycin A was heated in a sealed vessel for 36 h at 95 °C in 3 N methanolic hydrogen chloride (Scheme II). The product was evaporated and then distributed between water and chloroform. The water layer was evaporated and the residue was acetylated; traces of the acetyl derivatives of threonine and β-hydroxyvaline methyl esters were detected by mass spectrometry. The chloroform layer afforded one major compound, **6**, a crystalline solid whose molecular formula was established by high-resolution mass spectrometry as $C_8H_9NO_4$.

The ^{1}H NMR spectrum (CCl_4) of **6** has three sharp singlets at 2.60, 2.71, and 3.85 ppm which can be attributed to acetyl, aromatic methyl, and carbomethoxyl groups, respectively. The first and third assignments are supported by the IR spectrum, which contains an aromatic ketone band at 1715 cm^{-1} and an ester band at 1730 cm^{-1}. These three groups account for $C_5H_9O_3$, which leaves C_3NO to complete the molecular formula of $C_8H_9NO_4$. A C_3NO nucleus would be an isoxazole or an oxazole and, since isoxazoles are readily hydrolyzed[12] under the conditions employed, **6** is assigned as an oxazole with acetyl, methyl, and carbomethoxyl substituents. The ultraviolet spectrum (in ethanol), with maxima at 226 and 265 nm, would be appropriate for a substituted oxazole.[12]

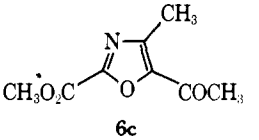
To indicate the juxtaposition of substituents on the oxazole ring, a sample of **6** was reduced with sodium borohydride and then saponified to the hydroxy acid. Since the latter did not lactonize, the acetyl and carbomethoxyl substituents cannot be on adjacent positions of the oxazole. The remaining four possible structures are shown here—**6** in Scheme II, **6a–c** below. In light of the peptide nature of berninamycin A, structure **6** seemed most likely, since it could arise from



6a

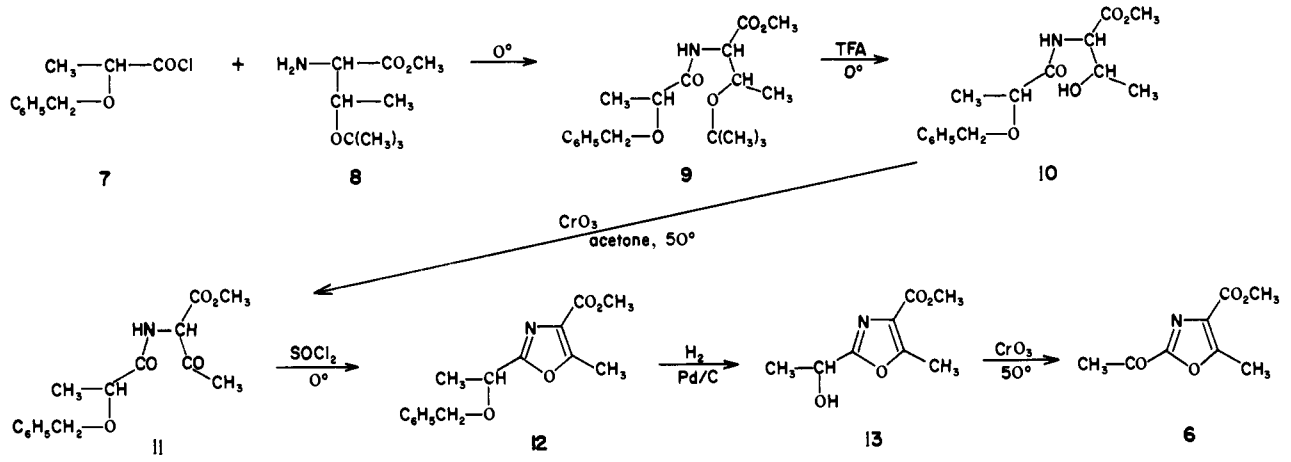


6b



6c

Scheme III. Synthetic Route to 2-Acetyl-4-carbomethoxy-5-methyloxazole (6)



dehydroalanyldehydrothreonine via dehydration. To substantiate this conclusion, a synthesis of **6** was undertaken.

Synthesis of the oxazole ring system is most easily accomplished via dehydration of an α-acylamino ketone.[12] Viewing **6** in this light, its logical precursor would be methyl α-pyruvamidoacetoacetate. However, due to the difficulties involved in synthesizing pyruvyl peptides[13] and the need to protect selectively the pyruvyl ketone before dehydration, a route was chosen using a protected lactate residue followed by oxidation (Scheme III).

Racemic *O-tert*-butylthreonine methyl ester (**8**)[14] and racemic α-benzyloxypropionyl chloride (**7**)[15] were condensed, the *tert*-butyl-protecting group was removed[16] from *N*-(α-benzyloxypropionyl)-*O-tert*-butylthreonine methyl ester (**9**), and *N*-(α-benzyloxypropionyl)threonine methyl ester (**10**) was converted to methyl α-(α-benzyloxypropionamido)acetoacetate (**11**) in high yield by oxidation with Jones reagent. The oxidation did not take place in 24 h at room temperature, but it proceeded at a titratable rate at 50 °C. Dehydration of **11** to 2-(1-benzyloxyethyl)-4-carbomethoxy-5-methyloxazole (**12**) was accomplished in thionyl chloride and the benzyl-protecting group was removed by catalytic hydrogenation to afford 4-carbomethoxy-2-(1-hydroxyethyl)-5-methyloxazole (**13**). Oxidation of **13** with Jones reagent at 50 °C yielded **6**, whose spectral parameters were identical with those of the degradation product.

2-Acetyl-4-carboxamido-5-methyloxazole (14). Heating a sample of berninamycin A at reflux for 3 h in 3 N methanolic hydrogen chloride (Scheme II) yielded, in the chloroform layer, the same product previously seen (**6**) plus a new product. The new compound (**14**) has the molecular formula $C_7H_8N_2O_3$ and exhibits ^{1}H NMR singlets at 2.66 and 2.76 ppm, while **6** has the formula $C_8H_9NO_4$ and exhibits singlets at 2.60, 2.71, and 3.85 ppm. The compounds have similar mass spectral fragmentations, except **6** shows loss of methanol from the molecular ion followed by loss of CO, whereas **14** shows loss of ammonia followed by loss of CO. These data argue that the new compound is the amide corresponding to the methyl ester **6**, i.e., that it is 2-acetyl-4-carboxamido-5-methyloxazole (**14**). To confirm this identification, a sample of **6** was converted to **14** by reaction with concentrated ammonium hydroxide. Presumably, **14** is the initial degradation product but is converted to **6** upon prolonged reaction.

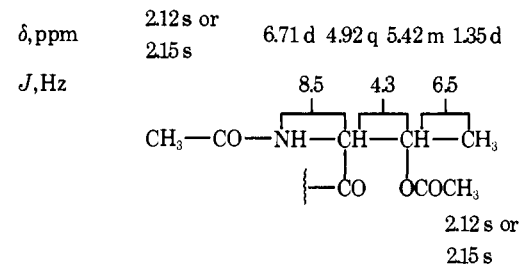
4-Carboxamido-5-methyl-2-propionyloxazole (15). The mass spectra of degradation products **6** and **14** also indicated trace amounts (<5%) of homologues. These apparently differed by a methylene group in the substituent at C-2 or at C-5, since a weak ethyl group (=CCH_2CH_3, 3.00 ppm, q, 2 H; 1.15 ppm, t, 3 H, J = 7.5 Hz) due to the homologue was seen in the ^{1}H NMR spectrum of crude **14** (molar ratio 1:9, respectively). To determine its position, a sample of crude **14** was reduced with sodium borohydride to afford crude 4-carboxamido-2-(1-hydroxyethyl)-5-methyloxazole (**16**). If the homology involved the acetyl substituent at C-2, a large shift in the position of the methylene group and a change in its splitting pattern should be observed on reduction, but if the homology involved the methyl substituent at C-5, little change should be noted. The weak ethyl group absorption in the ^{1}H NMR spectrum of crude **16** appeared at higher field (-$CHOHCH_2CH_3$, 2.12 ppm, m, 2 H; 1.07 ppm, t, 3 H, J = 7 Hz); thus, the homologue was identified as 4-carboxamido-5-methyl-2-propionyloxazole (**15**).

In the previous section, dealing with hydrolysis, structures **4** and **5** (Scheme I) were assigned to the components of the mixture obtained from the hydrolyzate of partially reduced berninamycin A. Evidence leading to these assignments will now be discussed. It was noted above that the mixture isolated consisted of dinitrophenylhydrazones of corresponding methyl and ethyl ketones, suggesting a mixture of homologues related to **14** and **15**. This postulate is substantiated by the ^{1}H NMR spectrum of the mixture which contains an aromatic methyl group's resonance at 2.79 ppm (3 H, s) and by the infrared spectrum of the mixture, which contains an amide carbonyl bond at 1680 cm^{-1}. The remaining portion of both ketones, $C_4H_7O_2$, is accounted for by a carbomethoxyl group (^{1}H NMR 3.72 ppm, 3 H, s; IR 1750 cm^{-1}; mass spectrum, ions at 389.1192 and 375.1057 due to loss of $COOCH_3$) and a -$CHCH_3$ group (^{1}H NMR 4.89, 1 H, q; 1.82, 3 H, d, J = 7 Hz). The latter group must be attached to the carboxamide nitrogen since it is lost as C_2H_5N in the mass spectrum, which contains homologous ions at 346.0802 and 332.0631 [M - $CH_3OOCC(CH_3)HNH$]. Thus, compounds **4** and **5**, isolated as dinitrophenylhydrazone methyl esters, are derivatives of **14** and **15** in which the amide nitrogen is part of an alanine methyl ester moiety.

Oxazole 17. The water layer from the 3-h methanolysis was evaporated to dryness and the residue was acetylated. Separation by thin-layer chromatography afforded three compounds. The two less polar compounds were identified as the acetyl derivatives of threonine and β-hydroxyvaline methyl esters, which were also isolated from the 36-h methanolysis above.

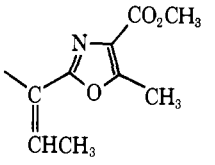
The most polar compound (the diacetyl derivative of **17**) had the molecular formula $C_{17}H_{23}N_3O_7$ (established by high-resolution mass spectrometry). A sample was further methanolyzed under the 36-h conditions. Evaporation of the methanolysis product followed by acetylation gave two main compounds, isolated by thin-layer chromatography. These were identified by mass spectral analysis as 4-carbomethoxy-5-methyl-2-propionyloxazole (**18**), the methyl ester

corresponding to the amide **15,** and diacetylthreonine methyl ester. Combination of the molecular formulas of these two products (**18,** $C_9H_{11}NO_4$; diacetylthreonine methyl ester, $C_9H_{15}NO_5$) indicates addition of 1 mol each of methanol and water and loss of 1 mol of ammonia in their formation from diacetyl **17.** The ^1H NMR spectrum of diacetyl **17** indicates partial structure a (confirmed by decoupling) for the threonine



a

portion of the molecule and, in addition, aromatic methyl (2.64 ppm, s), carbomethoxy (3.94 ppm, s), and ethylidene (=$CHCH_3$; 6.60 ppm, q, 1 H; 1.80 ppm, d, 3 H, J = 8 Hz) groups, which must be in partial structure b. Combining the



b

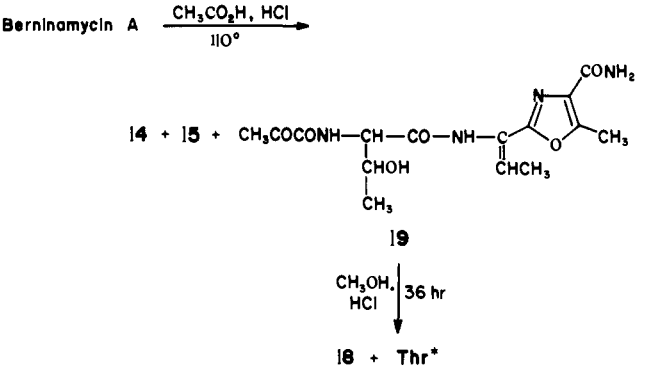
formulas of a ($C_8H_{12}NO_4$) and b ($C_9H_{10}NO_3$) leaves only NH; thus, structure **17** is assigned. The mass spectral fragmentations of diacetyl **17** agree with the formula assigned. Intense ions are found due to loss of acetic acid ($C_{15}H_{19}N_3O_5$) and to cleavage at the amide-threonine carbonyl bond ($C_9H_{12}N_2O_3$).

Hydrochloric Acid–Glacial Acetic Acid Cleavage. Gross and Kiltz[17] found that selective cleavage of subtilin at the dehydroalanine residues of that antibiotic could be achieved with anhydrous hydrochloric acid in glacial acetic acid, which afforded an N-terminal pyruvyl residue and a C-terminal primary amide group. This degradation was performed on berninamycin A (Scheme IV). Of the eight compounds (all more polar than berninamycin A) detected by thin-layer chromatography, three were obtained by preparative thin-layer chromatography in quantities which permitted characterization. The first of these was the methanolysis product **14,** together with a trace of the homologue **15** detected by mass spectrometry.

The second compound, which has been assigned structure **19,** decomposed slowly at room temperature, especially in solution, and, thus, yielded spectral data of poor quality. A sample of **19** was treated with methanolic hydrogen chloride, and the residue upon evaporation of the solvent was acetylated. Preparative thin-layer chromatography of the crude product afforded two major compounds in low yield, diacetylthreonine methyl ester and 2-propionyl-4-carbomethoxy-5-methyloxazole (**18**). Since these same compounds were obtained by methanolysis of the diacetyl derivative of **17,** as previously discussed, it appeared that the new compound (**19**) was quite similar to **17.**

Compound **19** on electron-impact mass spectrometry gave an apparent molecular ion at *m/e* 352.1396 ($C_{15}H_{20}N_4O_6$, C_2H_3O less and one nitrogen more than diacetyl **17**). The IR spectrum of **19** shows a broad carbonyl at 1700 cm^{-1}. The ^1H NMR spectrum of **19** is quite similar to that of diacetyl **17;** the methyl ester and the two acetyl peaks for diacetyl **17** are not apparent in the spectrum of **19** but a new methyl singlet at 2.48 ppm is present. Hydrochloric acid–glacial acetic acid cleavage

Scheme IV. Acetolysis Products of Berninamycin A (*Isolated as the Acetyl Derivative of the Methyl Ester)



should yield a carboxamide at the C-terminal position rather than a carbomethoxyl group and a pyruvyl residue at the N-terminal position rather than an acetyl (from acetylation). The methyl singlet at 2.48 ppm in the ^1H NMR spectrum of **19** can be attributed to such a pyruvyl methyl group. These changes (CH_3O- and CH_3CO- in diacetyl **17** for H_2N- and CH_3COCO- in **19**) plus an extra acetate group (CH_3CO- in diacetyl **17,** H- in **19**) account for the required changes in molecular formula (C_2H_3O less, N more) and structure **19** can be assigned. Examination shows that it contains the same moiety of berninamycin A as found in **17** but that it is differently derivatized due to the nature of the hydrochloric acid–glacial acetic acid degradation.

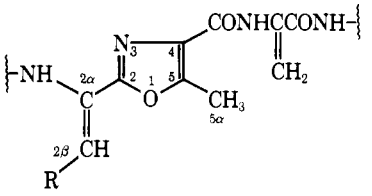
The third compound isolated was more difficult to work with than **19** and was obtained in low yield. Preliminary characterization indicated that it was similar to **19,** but considerably larger, the electron-impact spectrum indicating an apparent molecular ion at *m/e* 622.1594 ($C_{27}H_{26}N_8O_8S$). Additional attempts to purify and obtain satisfactory characterization of this larger compound were unsuccessful.

Discussion

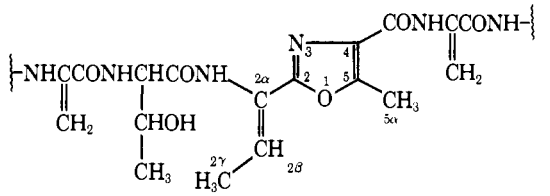
Origin of the Oxazoles. The isolation of homologous oxazoles **14** and **15** (as well as their methyl ester counterparts **6** and **18**) after methanolysis of berninamycin A suggests that two similar oxazole precursors exist in the intact antibiotic. The aromatic methyl of **14** is found at 2.66 ppm and that of **17** at 2.64 ppm. There are two (and only two) methyl singlets, at 2.52 and 2.59 ppm, in the ^1H NMR spectrum of berninamycin A (Table I); these can be assigned to the aromatic methyl groups present in the two oxazoles, thus arguing that both oxazole ring systems exist as such in the antibiotic. The 2-acetyl and 2-propionyl groups in **14** and **15,** however, must arise during methanolysis. There are no ketone carbonyl bands in the IR spectrum of berninamycin A, and the requisite methyl ketone and ethyl ketone resonances are not present in the ^1H NMR spectrum. Thus, the 2-acetyl and 2-propionyl groups in the oxazoles must be present in some masked form in the antibiotic.

Careful examination shows C-2 in oxazole **14** is the carboxyl carbon of a masked pyruvyl residue and C-2 in **15** is the carboxyl carbon of a masked α-ketobutyryl residue. In view of the number of dehydroamino acid residues in berninamycin A, it seems reasonable that the ketone groups in oxazoles **14** and **15** result from acidic hydrolysis of the corresponding masked dehydroamino acid residues which are part of the oxazoles in the intact antibiotic. The primary amide groups in **14** and **15** also probably result from similar hydrolytic cleavage of a dehydroamino acid residue to which each is attached. Compounds **4** and **5,** arising from hydrolysis following partial reduction, contain alanine residues at the C-terminal positions of the homologous oxazoles, indicating that the corresponding dehydroamino acids at these positions in the intact antibiotic are dehydroalanine residues.

The precursors to oxazoles **14** and **15** in berninamycin A can thus be depicted by partial structures c and d. Furthermore, isolation of **17** and **19** confirms partial structure d and allows its extension by the addition of threonine and dehydroalanine (pyruvyl) residues to substructure e, a sizable unit of intact berninamycin A.



c, R = -H
d, R = -CH_3



e

Some discrepancy exists between the number of dehydroalanine residues in berninamycin A suggested by the isolation of 5 equiv of pyruvic acid from the hydrolyzate of the intact antibiotic and that suggested by the isolation of 6 equiv of alanine (plus 1 equiv of butyrine) from the hydrolyzate of borohydride-reduced berninamycin A. Since the butyrine (1 equiv) in the hydrolyzate of the reduced product apparently arises from reduction or cleavage of the oxazole precursor shown in e, it seems reasonable that the corresponding oxazole precursor in c should react similarly and afford a saturated amino acid (alanine) upon hydrolysis of the reduced product. This additional equivalent of alanine reconciles the results obtained from the pyruvate analysis and those obtained by the hydrolysis of the reduced antibiotic and indicates that five intact dehydroalanine residues are present in berninamycin A, three of which are contained in partial structures c and e.

Composition of Berninamycin A. Berninamycinic acid (**1**) is a major degradation product of the antibiotic. In discussing the composition of subunits, one must first establish whether or not the novel zwitterion in berninamycinic acid [composed of the quaternary nitrogen (N-4) and the carboxylate anion (C-11)] exists in the intact antibiotic. An examination of the ^{1}H NMR data indicates that the zwitterion is present in berninamycin A. There are four aromatic protons in the ^{1}H NMR spectrum (D_2O, pH 9) of sodium berninamycinate, doublets at 8.00 and 8.41 ppm (J = 8 Hz) and singlets at 8.39 and 9.21 ppm. The doublets correspond to the protons at C-9 and C-10 and the singlets to those at C-2 and C-5. Investigation of substituted pyridines and quinolines[18] indicates that the proton at the carbon para to the ring nitrogen appears at lower field than the proton at the meta carbon, and thus C-9 and C-10 can be assigned to the resonances at 8.00 and 8.41 ppm, respectively.

The remaining resonances at 8.39 and 9.21 ppm can be differentiated since the resonance at 9.21 ppm shifts with change in pH and slowly disappears upon prolonged standing in basic deuterium oxide, thus must be H-5 (ortho to a phenol). The C-2 proton can then be assigned to the resonance at 8.39 ppm.

In the ^{1}H NMR spectrum of berninamycin A (C_5D_5N) there are also four aromatic protons, doublets at 8.04 and 8.34 ppm (J = 8 Hz) and singlets at 8.57 and 8.84 ppm. These correspond to the aromatic protons in the berninamycinic acid moiety in berninamycin A and they have almost identical chemical shifts. The proton at C-2 in the thiazolium ring in berninamycinic acid should be highly sensitive to loss of the quaternary center, since this would lead to dearomatization of the thiazolium ring and result in a large upfield shift of the C-2 proton. Since the chemical shift of the proton at C-2 in sodium berninamycinate (D_2O, 8.39 ppm) is quite similar to that in the intact antibiotic (C_5D_5N, 8.57 ppm), it appears that the thiazolium ring in berninamycinic acid exists as such in berninamycin A.

The quaternary nitrogen present in berninamycin A needs a counterion, since the antibiotic is neutral. If berninamycin A were a salt resulting from an inorganic counterion, such a salt should be titratable, whereas berninamycin is not. Therefore, a carboxylate group at either C-11 or C-12 is presumed to be the counterion for the quaternary nitrogen, as in berninamycinic acid. These carboxylate anions can be differentiated on the basis of titration data. The carboxyl group at C-12 in berninamycinic acid has pK_a = 5.8, while the carboxyl group at C-11 has $pK_a < 2$.[19] Since berninamycin A is neutral, or at least does not have $pK_a > 2$, the C-12 carboxyl group cannot be free and is, as discussed below, present as an amide. Thus, the C-11 carboxylate ($pK_a < 2$) is the counterion for the quaternary nitrogen. The points of attachment to the berninamycinic acid moiety must be the carboxyl at C-12 and the substituent at C-6.

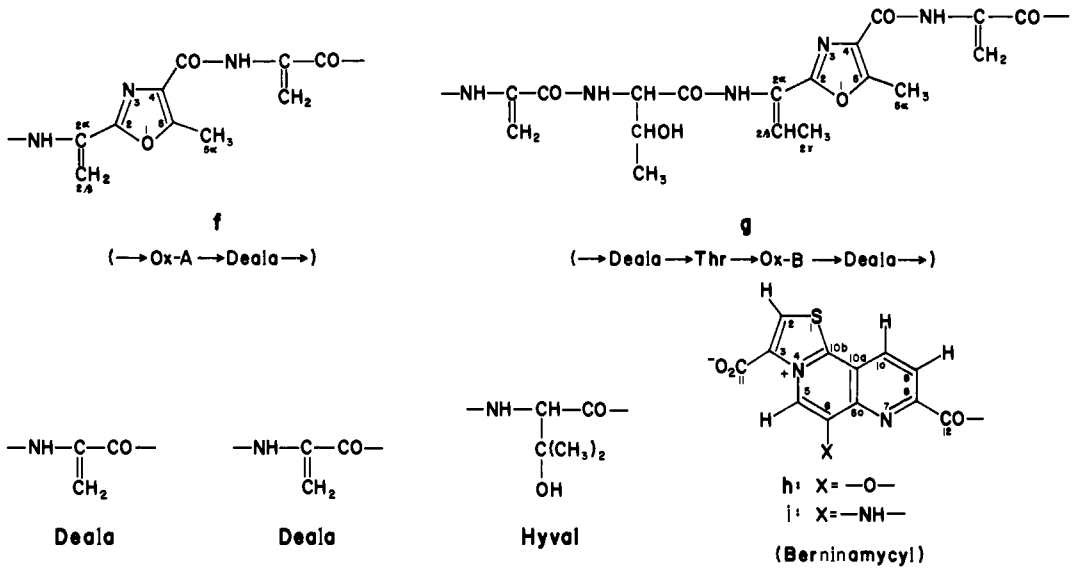
The degradation studies described in this report indicate that 1 equiv each of threonine, β-hydroxyvaline, the dehydroalanine-derived oxazole (found in **4, 6,** and **14**), the dehydrobutyrine-derived oxazole (found in **5, 15, 17, 18,** and **19**), and berninamycinic acid (**1**), plus 5 equiv of dehydroalanine, is present in the antibiotic. If these units are joined in the antibiotic by peptide bonds with loss of water, then these components (Scheme V), excluding berninamycinic acid, account for the empirical formula $C_{39}H_{45}N_{11}O_{13}$ (threonine, $C_4H_7NO_2$; β-hydroxyvaline, $C_5H_9NO_2$; dehydroalanine-derived oxazole, $C_7H_6N_2O_2$; dehydrobutyrine-derived oxazole, $C_8H_8N_2O_2$; five dehydroalanines, $5 \times C_3H_3NO = C_{15}H_{15}N_5O_5$).

Viewed in the same light involving loss of 1 mol of water, berninamycinic acid (**1**) would contribute $C_{12}H_4N_2O_4S$ (subunit h), bringing the total combined subunit formula to $C_{51}H_{49}N_{13}O_{17}S$, NH_3 less than the formula indicated by elemental analyses ($C_{51}H_{52}N_{14}O_{17}S$) or NH less and O more than the formula indicated by the field-desorption mass spectrum ($C_{51}H_{50}N_{14}O_{16}S$).

Regardless of which molecular formula for berninamycin A is correct, one nitrogen atom is missing from the subunits shown in Scheme V. This could be in a C-terminal carboxamide group. If that were the case and all of the subunits of Scheme V were then linked together, the berninamycyl residue would of necessity have either a free phenolic hydroxyl at C-6 or an acyloxy group at C-6. However, berninamycin A does not give a positive ferric chloride test (while berninamycinic acid does), and there is no aryl ester (or any other ester) carbonyl band in the infrared spectrum of berninamycin A (phenyl acetate absorbs at 1760 cm^{-1}). Thus, the C-6 hydroxyl of berninamycinic acid appears to be neither free nor acylated. An attractive alternative postulate is that the C-6 oxygen of berninamycinic acid does not exist in berninamycin A but is introduced by replacing a nitrogen during hydrolysis of berninamycin A. This would be reasonable for an amide (or amine) on an electron-deficient aromatic ring.[20] The middle ring of berninamycinic acid is electron deficient and might be expected to undergo such a reaction with relative ease. However, since this is the first reported occurrence of the novel ring system, there are no available model compounds with which to test the hypothesis adequately.

In the event of a nitrogen at C-6 of berninamycinic acid, the appropriate subunit for berninamycinic acid in berninamycin A would be i ($C_{12}H_5N_3O_3S$), which would make the combined

Scheme V. Subunits of Berninamycin A



subunit formula $C_{51}H_{50}N_{14}O_{16}S$, in agreement with the field-desorption mass spectrum. That formula would require a cyclic linkage of all the subunits f, g, i, Deala, Deala, and Hyval. The alternative formula from microanalyses ($C_{51}H_{52}N_{14}O_{17}S$) would require a C-terminal carboxyl group (from any unit) and an N-terminal amino group (from i or Hyval). Berninamycin A is neutral and there is no evidence for a free amino group (negative ninhydrin test) or a free carboxyl group (negative diazomethane test). Thus, a cyclic formula involving the six subunits in Scheme V is indicated for berninamycin A. Complete structural assignment of the antibiotic awaits additional evidence linking these subunits.

^{1}H NMR Spectrum of Berninamycin A. The subunits of Scheme V are in accord with the ^{1}H and ^{13}C NMR spectra of the antibiotic. The total ^{1}H NMR spectrum can be assigned with respect to the subunits, as can the unique resonances in the ^{13}C NMR spectra. The remaining ^{13}C NMR resonances are reasonable for the carbons which are not specifically assigned.

The assignment of the ^{1}H NMR spectrum of berninamycin A is shown in Table I. The individual peaks were assigned by correlating the chemical shifts observed for the degradation fragments with the spectrum of the intact antibiotic. The threonine and β-hydroxyvaline assignments were verified by spin decoupling, as were the olefinic side chain in the dehydrobutyrine-derived oxazole B (part of g) and the AB quartet in the berninamycinic acid moiety. Amide protons are observed in the region 9–11 ppm and their assignments were confirmed by their disappearance after shaking with deuterium oxide.

The only remaining peaks in the spectrum of berninamycin A are 11 singlets which integrate for 12 protons. They appear in two groups in the regions 5.7–6.2 ppm (six protons) and 6.1–7.0 ppm (six protons). These shifts are quite close to those observed for the vinylic protons in α-acetamidoacrylic acid [*N*-acetyldehydroalanine, which is found at 2.04 (3 H, s), 5.74 (1 H, s), and 6.32 ppm (1 H, s) in Me_2SO-d_6] and are therefore assigned as the vinylic protons which are trans (~5.8 ppm) and cis (~6.7 ppm) to the carbonyl groups in the five dehydroalanine residues and the one dehydroalanine-derived oxazole (part of f) in berninamycin A.

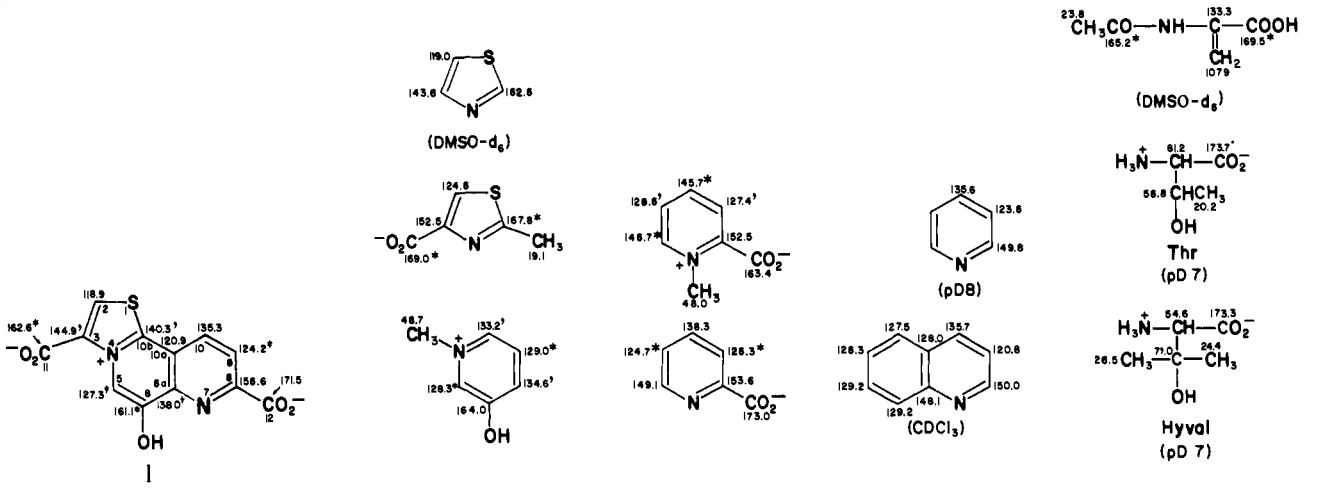
^{13}C NMR Spectrum of Berninamycinic Acid. The assignment of the ^{13}C NMR spectrum of berninamycinic acid (ammonium salt, D_2O, pH 9, internal dioxane as standard) is shown in Scheme VI. The assignments are based upon authentic model compounds, also shown in Scheme VI, whose ^{13}C NMR spectra were run under the same conditions, except as noted. The off-resonance decoupled spectrum of ammonium berninamycinate shows four doublets at 135.3, 127.3, 124.2, and 118.9 ppm, which must be assigned to the four hydrogen-bearing carbons, C-10, C-5, C-9, and C-2 (Scheme VI). Picolinic acid's C-3 (found at 126.3 ppm) and C-4 (at 138.3 ppm) provide good models for C-9 and C-10, respectively, of berninamycinic acid. Only slight changes are expected from the presence of the fused ring, since C-2 and C-4 of quinoline[21] appear at almost exactly the same position as like carbons of pyridine (Scheme VI), while C-3 is shifted slightly (~3 ppm) upfield in quinoline. Thus, C-10 of berninamycinic acid can only be the signal at 135.3 ppm and C-9 is either at 124.2 or 127.3 ppm, more likely the former. 3-Hydroxy-1-methylpyridinium chloride's C-2 (129.0 ppm) provides a good model for C-5 in berninamycinic acid, which thus must appear at either 127.3 or 124.2 ppm, more likely the former. The remaining proton-bearing carbon of berninamycinic acid (C-2) must be the signal found at 118.9 ppm, at the same position as the corresponding carbon (C-5, at 119.0 ppm) of thiazole but somewhat upfield from C-5 (at 124.8 ppm) of 2-methylthiazole-4-carboxylic acid (Scheme VI).

The remaining eight carbons of berninamycinic acid are nonprotonated. The three resonances at lowest field (171.5, 162.6, and 161.1 ppm) are those for C-12, C-11, and C-6, which are oxygen-bearing. The carboxylate carbons in picolinic acid (173.0 ppm) and *N*-methylpicolinic acid (165.4 ppm) are like C-12 and C-11, respectively, in ammonium berninamycinate; thus, C-12 is the resonance at lowest field. Quaternarization of the ring nitrogen in picolinic acid results in a large (−7 ppm) upfield shift of the carboxylate carbon. 3-Hydroxy-1-methylpyridinium chloride provides a good model (in C-3, at 164.0 ppm) for C-6 of ammonium berninamycinate. However, the observed resonances for C-6 and C-11 (161.1 and 162.6 ppm) in ammonium berninamycinate are too close to allow definitive assignment from the two models (165.4 and 164.0 ppm).

Of the remaining five signals for nonprotonated carbons (120.9, 138.0, 140.3, 144.9, and 156.6 ppm), that at highest field (120.9 ppm) can be assigned as C-10a by analogy to the model quinoline (Scheme VI),[21] in which C-4a appears at 128.0 ppm (whereas no other carbons are expected at higher field than 136 ppm), and that at lowest field (156.6 ppm) can be assigned as C-8 by analogy to picolinic acid, in which C-2 appears at 153.6 ppm.

Three carbons (C-3, C-6a, and C-10b) remain and these can be only tentatively assigned, since the models are imperfect.

Scheme VI. ^{13}C NMR Assignments of Ammonium Berninamycinate and Model Compounds (Determined in D_2O at pD 9 except as Noted). Quinoline Values Are from Ref 21



I

The resonance at 138.0 ppm is probably due to C-6a, since the shift for C-8a in 8-hydroxyquinoline can be calculated as 135.5 ppm, 12.6 ppm upfield[22,23] from C-8a of quinoline (Scheme VI). In the models studied C-3 of berninamycinic acid is best approximated by C-5 of 2-methylthiazole-4-carboxylic acid (at 152.5 ppm) and C-10b of berninamycinic acid by C-2 of thiazole (also at 152.5 ppm). These signals should both be shifted upfield (and C-6a downfield) by the quaternization of N-4, but they cannot be definitively assigned.

^{13}C NMR Spectrum of Berninamycin A. The ^{13}C NMR spectrum of berninamycin A (Table II) cannot be uniquely assigned in terms of subunits f, g, and i, since so many similar sp^2 carbons are present in the antibiotic. However, assignments can be made of all the sp^3 and some of the sp^2 carbons in berninamycin A, and the remaining carbons can be assigned in groups.

The sp^3 carbons in the threonine and β-hydroxyvaline residues (subunits g and Hyval) were assigned by comparison to the ^{13}C NMR spectra of the free amino acids (Scheme VI) and by the off-resonance decoupled spectrum of berninamycin A, where C-3 of Hyval is the only singlet carbon in the region 55–75 ppm.

The methyl resonances at 11.4 and 11.5 ppm in the ^{13}C NMR spectrum of berninamycin A (Me_2SO-d_6) are quite similar to those of the aromatic methyl carbon (11.7 ppm) in **14** and can be assigned as the C-5α carbons in the oxazole residues of f and g. The remaining sp^3 carbon is the C-2γ methyl carbon in g, and it is assigned to the methyl resonance at 13.6 ppm (Me_2SO-d_6). The ^{13}C NMR spectrum of α-acetamidoacrylic acid (*N*-acetyldehydroalanine) contains a signal at 107.9 ppm (Scheme VI); thus, the chemical shifts of the β-carbons in the dehydroalanine residues of f, g, and Deala and the C-2β carbon in the oxazole of subunit f (Scheme V) should be approximately 108 ppm, i.e., the six resonances between 102 and 109 ppm (multiplets in the off-resonance spectrum). If one assumes that the Deala residues will be those closest together, oxazole A C-2β is assigned as the signal at 108.4 ppm.

Five protonated carbons remain to be assigned in the ^{13}C NMR spectrum (Me_2SO-d_6), at 122.8, 126.2, 128.9, 133.6, and 139.0 ppm (all doublets in the off-resonance spectrum). These must correspond to C-2β in g and to C-2, C-5, C-9, and C-10 in i. An imperfect model for the C-2β carbon in g is provided by a similar carbon (found at 113.0 ppm) in tryptophan dehydrobutyrine diketopiperazine,[7] which must appear at lower field in i. The protonated carbons in ammonium berninamycinate (Scheme VI), which were noted above to appear at 118.9, 124.2 127.3, and 135.3 ppm, also provide imperfect models, since the spectrum of ammonium berninamycinate was obtained in deuterium oxide at pD 9 (rather than Me_2SO-d_6), its C-6 carbon bears an oxygen (rather than a nitrogen), and C-12 is a carboxylate (rather than a carboxamide). Nevertheless, the signal at 139.0 ppm can be tentatively assigned to C-10 of i, the signals at 133.6 and 128.9 ppm to C-5 and C-9 of i, and the signals at 122.8 and 126.2 ppm to C-2 of i and C-2β of g.

The remaining resonances in the ^{13}C NMR spectrum of berninamycin A are from nonprotonated carbons and are not sufficiently unique to allow specific assignment, although they can be assigned in groups. The C-10a carbon in i is expected at highest field and the five dehydroalanine C-2 carbons (in f, g, and Deala) at next highest field since C-10a of ammonium berninamycinate appears at 120.9 ppm and C-2 of *N*-acetyldehydroalanine at 133.3 ppm (Scheme VI). Thus, C-10a is assigned the signal at 126.6 ppm and the dehydroalanine C-2 carbons have five closely grouped resonances in the region 128.4–130.3 ppm (C_5D_5N). There are 11 singlet resonances in the region 134.9–155.5 ppm (C_5D_5N). The C-4 carbons in f and g are expected in the region 130–135 ppm since that carbon appears at 130.8 ppm in **14**. Thus, they must be two of the three at highest field. The C-2α positions in f and g are similar to C-2 in α-acetamidoacrylic acid (133.3 ppm) but differ slightly due to the methyl substituent in g and should appear at slightly lower field since they bear aromatic substituents. Thus, they are assigned to one of the three highest field carbons and one of the two near 141 ppm. The chemical shifts for C-3, C-6a, and C-10b in ammonium berninamycinate (Scheme VI) are 140.3, 138.0, and 144.9 ppm, respectively; C-3 should not change, C-6a and C-10b might be shifted downfield somewhat in i. Thus, C-3 is assigned to one of the signals near 141 ppm, C-6a to the signal near 147 ppm, and C-10b to one of those between 150 and 154 ppm.

The C-5 carbons in the oxazoles in f and g are nearly identical with C-5 in **14** (155.9 ppm) and are assigned to the signals at 155.0 and 155.5 ppm. The C-6 and C-8 carbons in i are expected in the region 150–156 ppm and are assigned to the resonances at 150.9 and 153.6 ppm (C_5D_5N). The C-8 carbon in ammonium berninamycinate appears at 156.6 ppm. The C-6 carbon in ammonium berninamycinate appears at 161.1 or 162.6 ppm; however, C-6 bears an oxygen in ammonium berninamycinate but a nitrogen in i. Thus, C-6 in i is expected at considerably higher field than the C-6 carbon in ammonium berninamycinate.

Of the 11 remaining resonances the five closest together, those between 160 and 163 ppm (C_5D_5N), should be the dehydroalanine carbonyl carbons. The two C-2 carbons in the oxazoles in f and g are expected to be near one another and at approximately 160 ppm since C-2 in **14** occurs at 162.4 ppm; they can be assigned as the signals at 157.1 and 158.2 ppm. The

carboxylate carbon (C-11) of i should appear very near its signal in ammonium berninamycinate (162.6 ppm) and is assigned at 163.6 ppm. The saturated threoninamide and β-hydroxyvalinamide carbonyl carbons should be at lowest field (169.8 and 170.7 ppm). The remaining three carbonyl carbons (oxazolecarboxamides and berninamycinamide) are assigned the signals at 164.0, 165.3, and 166.6 ppm.

Experimental Section

Melting points, determined on a Kofler hot stage, and boiling points are uncorrected. Infrared (IR) spectra were recorded on a Perkin-Elmer 521 or a Beckman IR-12 spectrophotometer and ultraviolet (UV) spectra on a Beckman DB or a Beckman Acta II spectrophotometer. Optical rotations were measured on a Zeiss polarimeter. Proton magnetic resonance (^{1}H NMR) spectra were determined on Varian A-60, HA-100, and HR-220 spectrometers. Chemical shifts are reported in parts per million from Me_4Si as internal standard. Carbon magnetic resonance (^{13}C NMR) spectra were obtained on either a Varian XL-100 or a JEOL FX-60 spectrometer with dioxane (67.4 ppm) as internal standard for samples obtained in deuterium oxide and Me_4Si as internal standard for all other solvents. Low-resolution mass spectra were obtained on a Varian MAT mass spectrometer, Model CH-5DF. Gas chromatography–mass spectrometry (GC–MS) samples were analyzed on a Varian MAT mass spectrometer, Model CH-7. High-resolution (HRMS) mass spectra were obtained on a Varian MAT mass spectrometer, Model 731. Field desorption (FDMS) mass spectra were obtained by the techniques employed previously.[24]

Thin-layer chromatography was carried out on Eastman chromatogram sheets or silica gel plates (5 × 20 cm) which were either prepared from silica gel GF_{254} (Brinkmann) or obtained commercially (Brinkmann or Analtech). Preparative TLC separations were made on plates (20 × 20 cm) prepared with a 1-mm layer of silica gel PF_{254} (Brinkmann) or on commercial 2-mm plates (Brinkmann or Analtech). Compounds were detected by fluorescence quenching or the use of an appropriate spray reagent.

Berninamycin A. A Craig type countercurrent distribution apparatus[6] (H. O. Post Scientific Co.) having 400 tubes, each containing 10 ml of upper and 10 ml of lower phase, was employed. The solvent system chloroform–cyclohexane–0.1 N aqueous sodium chloride–methanol (27:13:10:30) was used after 24 h of equilibration. Crude berninamycin[5] (15 g) was added to 220 ml of lower phase and the mixture was stirred 1 h Upper phase (220 ml) was added, stirring was continued for an additional 1 h, the mixture was rapidly filtered through glass wool, and the layers were separated. Saturated lower phase (10 ml) and saturated upper phase (10 ml) were added to tubes 1–19, and 10 ml of fresh lower phase was added to tubes 20–400 Distribution used 17 inversions and 5-min settling time. The UV (250 nm, methanol) profile for suitable dilutions of the lower phase in each tube was determined and, after 800 transfers, the following fractions were pooled: fraction I, tubes 1–160; fraction II, 166–230; fraction III, 231–280; fraction IV, 281–340; and fraction V, 350–400 plus 6 l. of effluent upper phase collected at tube 400. Fractions I and V were found by TLC (silica gel; methanol–chloroform, 1:19 and 1:9) to remain at the origin.

Fraction III was evaporated to dryness at reduced pressure, dissolved in 200 ml of methanol–methylene chloride (1:19), dried with magnesium sulfate, filtered, and concentrated to a minimal volume (~20 ml). The concentrate was added dropwise with stirring to 750 ml of ether–petroleum ether (1:1). After standing 1 h at room temperature, the precipitate was filtered and dried 24 h at 55 °C (0.01 Torr), affording 8.55 g of berninamycin A: mp >290 °C dec; UV (EtOH) end absorption 280–200 nm (ϵ_{200} >15 000); IR (KBr) 3370, 2980, 1665 (br), 1510, 1200, and 885 cm^{-1}; ^{1}H and ^{13}C NMR, see Tables I and II. Anal. Calcd for $C_{51}H_{52}N_{14}O_{17}S$ ($C_{51}H_{50}N_{14}O_{16}S\cdot H_2O$): C, 52.57; H, 4.50; N, 16.83; S, 2.75; mol wt, 1164. Calcd for $C_{51}H_{50}N_{14}O_{16}S$: C, 53.40; H, 4.39; N, 17.10; S, 2.80; mol wt, 1146. Found: C, 52.67; H, 4.69; N, 16.78; S, 2.80 (average of two analyses); mol wt, 1147 (M + H, FDMS 20 mA).

Fraction IV was worked up in the same manner and yielded 2.40 g of berninamycin A with properties identical with those of fraction III. The total yield of berninamycin A was thus 10.85 g.

Berninamycin B. Fraction II was treated in the same manner as above and yielded 0.65 g of berninamycin B: melting point, UV, and IR like those of berninamycin A; ^{1}H NMR (C_5D_5N) 0.93 (3 H, d, J = 8 Hz), 1.02 (3 H, br s), 1.11 (6 H, br s), 1.36 (6 H, m), 1.73 (3 H, br s), 1.91 (3 H, d, J = 7 Hz), 2.52 (3 H, s), 2.64 (3 H, s), 3.98 (1 H, m), 4.32 (1 H, m), 4.95 (1 H, m), 5.05 (1 H, m), 5.14 (1 H, br s), 5.39 (1 H, br s), 5.73 (1 H, s), 5.77 (1 H, s), 5.84 (1 H, s), 5.95 (1 H, s), 6.14 (1 H, s), 6.25 (1 H, s), 6.34 (1 H, s), 6.48 (9 H, s), 6.80 (1 H, s), 6.89 (1 H, s), 6.93 (1 H, s), 7.00 (1 H, s), 8.07 (1 H, d, J = 8 Hz), 8.34 (1 H, d, J = 8 Hz), 8.59 (1 H, s), 8.89 (1 H, s), 9.11 (1 H, s), 9.18 (1 H, s), 9.73 (1 H, s), 9.77 (1 H, s), 9.91 (2 H, s), 10.00 (1 H, s), 10.39 (1 H, s), and 11.00 ppm (1 H, s); ^{13}C NMR (C_5D_5N) 172.2, 169.7, 166.7, 165.6, 164.0, 163.6, 162.8, 162.1, 161.0, 160.5, 160.3, 157.9, 157.2, 155.5, 155.1, 153.7, 150.1, 149.5, 147.3, 141.6, 141.3, 140.7, 135.8, 135.7, 135.2, 134.2, 130.3, 129.7, 129.1, 128.2, 126.7, 122.7, 121.1, 104.5, 104.2, 104.0, 103.4, 102.6, 83.8, 68.2, 62.1, 58.9, 30.4, 19.9, 19.3, 19.2, 14.5, 11.5, and 11.3 ppm; ^{13}C NMR (Me_2SO-d_6) 171.2, 168.6, 165.1, 163.8, 163.3, 163.0, 162.6, 161.5, 159.8, 159.5, 159.4, 158.0, 156.6, 155.2, 154.2, 153.5, 149.4, 149.1, 146.2, 140.3, 140.2, 139.1, 135.0, 134.6, 133.8, 133.3, 130.2, 129.2, 129.1, 128.7, 126.6, 123.0, 121.1, 106.0 (br), 77.3, 68.4, 60.5, 58.2, 29.8, 20.3, 18.9, 18.8, 13.7, 11.4, and 11.3 ppm. Anal. Calcd for $C_{59}H_{74}N_{14}O_{22}S$: C, 51.97; H, 5.47; N, 14.38; S, 2.35. Found: C, 51.95; H, 5.41; N, 14.28; S, 2.35.

Separation of Berninamycins A and B by High-Pressure Liquid Chromatography (HPLC). A Waters Associates, Model ALC/GPC-502, HPLC system equipped with a differential UV detector, 6000 psi pump, and a U6K injector was employed. Separation of a mixture of 1 mg each of berninamycins A and B in 1 ml of tetrahydrofuran (2-μl injection) was achieved on a C_{18}-Porasil B column (⅛ in. × 2 ft, Waters Associates) with tetrahydrofuran–water (7:13, 1 ml/min): retention times, 8.5 and 12.5 min, respectively.

Hydrolysis of Berninamycin A. (a) Isolation and Characterization of Berninamycinic Acid. A sealed tube containing 1.0 g of berninamycin A in 15 ml of 6 N hydrochloric acid under nitrogen was heated at 110 °C for 18 h, cooled, opened, and washed out with water. The solvent was removed at reduced pressure below 40 °C. The residue was triturated with 10 ml of 1 N acetic acid, the insoluble precipitate was removed by filtration, and the filtrate was evaporated to dryness to give the amino acid fraction. The insoluble precipitate (crude berninamycinic acid[2]) was dissolved in 5 ml of 0.1 N sodium hydroxide, and the solution was acidified to pH 1 with concentrated hydrochloric acid and let stand at room temperature overnight. The tan precipitate was collected by suction filtration, washed with acetone, and dried at 110 °C (0.02 Torr), affording 143 mg of berninamycinic acid (**1**): mp 210 °C; pK_a = 5.8; UV max (0.01 N HCl) 228 nm (ε 13 500) and 272 (13 000); UV max (0.01 N NaOH) 232 nm (ε 9500) and 294 (13 000); IR (KBr) 3400, 1725, 1625, 1460, 1375, and 1250 cm^{-1}; ^{1}H and ^{13}C NMR, see text.

Crude berninamycinic acid (**1**, 200 mg) was dissolved in a minimal volume of 1 N sodium hydroxide and let stand in a refrigerator overnight. The resulting **sodium berninamycinate** was filtered off and recrystallized from water–ethanol, affording 150 mg of orange plates, which were recrystallized four times from water–ethanol to yield a product giving constant microanalyses: mp 300 °C dec; IR (Nujol) 3120, 1650, 1560, and 1380 cm^{-1}. Anal. Calcd for $C_{12}H_5N_2NaO_5S$: C, 56.16; H, 1.61; N, 8.97; Na, 7.37; S, 10.27. Calcd for $C_{12}H_5N_2NaO_5S\cdot 0.2NaOH$: C, 45.00; H, 1.64; N, 8.75; Na, 8.62; S, 10.01. Found: C, 44.89; H, 1.88; N, 8.73; Na, 8.60; S, 9.97.

Crude **1** (1.5 g) was dissolved in 20 ml of 7 N ammonium hydroxide and diluted with 100 ml of water. The solution was lyophilized to yield 1.6 g of crude **ammonium berninamycinate:** mp 270 °C dec; FDMS (17 mA) m/e 246 (M − NH_3 − CO_2) and 202 (246 − CO_2).

(b) Identification of Amino Acids. The amino acid fraction from (**a**) was dissolved in 10 ml of 1 N hydrochloric acid and layered on top of a Dowex 50W-X4 column (4.5 × 75 cm), which was prepared and washed with 1 N hydrochloric acid. The column was eluted with 1 N hydrochloric acid and 20-ml fractions were analyzed with ninhydrin solution[25] at 570 nm and pooled as follows: fraction I, tubes 111–122; II, 128–137; III, 145–178; and IV, 225–264. The fractions were lyophilized and saved.

Each amino acid fraction was dissolved in 1 ml of 0.5 N acetic acid, layered on top of a Dowex 1-X8 (acetate) column (2.5 × 20 cm), and eluted with 0.5 N acetic acid. The 3-ml fractions containing the single ninhydrin-positive band from each column were pooled and lyophilized to the free amino acid. Fraction **I** yielded 110 mg of L-threonine, which was crystallized from water–ethanol to give 75 mg of needles: mp 246 °C dec (lit.[26] 255 °C dec); $[\alpha]^{24}D$ −15.5° (c 0.8, 6 N HCl) [lit.[26] $[\alpha]^{24}D$ −15.0° (c 1.0, 5 N HCl)]. The IR spectrum (KBr) was iden-

tical with that of an authentic sample of L-threonine.

Fraction II gave 90 mg of L-β-hydroxyvaline, which was crystallized from water–acetone, affording needles (65 mg): mp 190 °C dec (lit.[7] 196–197 °C dec); $[\alpha]^{24}D$ +12.0° (*c* 0.2, 6 N HCl) [lit.[7] $[\alpha]^{24}D$ +13.5° (*c* 2.0, 5 N HCl)]. The IR spectrum (KBr) was identical with a literature spectrum[7] of L-β-hydroxyvaline.

Fraction III gave 144 mg of a sticky residue whose TLC (Eastman cellulose; 1-butanol–acetone–diethylamine–water, 30:30:6:15) indicated only one compound, with the R_f of ammonium acetate. At pH 11 the compound gave the characteristic ammonia odor.

Fraction IV gave ca. 60 mg of a black, hygroscopic residue which gave a very weak ninhydrin test. The residue had no peaks visible in its ^1H NMR spectrum and amino acid analysis indicated only a trace of glycine. Attempts at further characterization were fruitless.

Amino acid analysis of a second sample of berninamycin A (10 mg), which was similarly hydrolyzed, indicated β-hydroxyvaline, threonine, glycine, and ammonia in the molar ratio 1.0:1.0:0.1:8–9.

(c) Isolation of Pyruvic Acid. Berninamycin A (1.0 g) was hydrolyzed exactly as in the preceding section, then the tube was cooled to liquid nitrogen temperature, opened, inverted into a beaker containing a solution of 1.3 g of 2,4-dinitrophenylhydrazine in 60 ml of ethanol, and allowed to thaw. After the mixture had been stirred 1 h, 60 ml of water was added and the resulting light brown precipitate (crude berninamycinic acid, **1**) was filtered off. The filtrate was extracted with methylene chloride (5 × 60 ml) and the combined lower layers were back extracted with 60 ml of water. The water layers were combined and saved.

The combined methylene chloride layers were extracted twice with 100 ml of 0.2 N sodium bicarbonate, dried over magnesium sulfate, and evaporated to yield excess 2,4-dinitrophenylhydrazine (260 mg) plus a trace of acetone 2,4-dinitrophenylhydrazone (10 mg), identified by TLC and mass spectral comparison with authentic samples. The sodium bicarbonate phase was concentrated to 100 ml, acidified to pH 1 with concentrated hydrochloric acid, and let stand 2 h in the refrigerator. The resulting precipitate was filtered off and dried in vacuo for 24 h, yielding 1.04 g of pyruvic acid 2,4-dinitrophenylhydrazone, mp 215–217 °C (lit.[27] 217–218 °C). Examination by IR, ^1H NMR, and mass spectrometry indicated that the sample was pure. Two additional experiments afforded 0.99 and 1.05 g. The average yield was thus 1.03 g (3.85 mequiv) per 1.0 g of berninamycin A.

2-Carbomethoxy-6-(1-carbomethoxy-2-methylthioethenyl)-8-methoxy-5-oxo-5,6-dihydro-1,6-naphthyridine (2). A slurry of 100 mg of **1**, 100 mg of silver oxide, and 2 ml of methyl iodide in 50 ml of ethanol-free chloroform (prepared by passing reagent chloroform down a short column of activated basic alumina) was stirred in the dark under a drying tube. Additional aliquots of silver oxide and methyl iodide (100 mg and 2 ml, respectively) were added daily for 5 days, the precipitate was filtered off, and the filtrate was concentrated to an oily residue (~144 mg), which was chromatographed by preparative TLC (silica gel, 1 mm; methanol–chloroform, 1:49). A very broad fraction (R_f 0.1–0.6) was isolated and rechromatographed to give two bands, which were removed and eluted with chloroform. The more polar band was rechromatographed to give four bands, labeled fractions A, B, C, and D in order of decreasing polarity.

Fraction D, the major component, afforded an oil (20 mg) upon drying: UV max (EtOH) 275 nm (ε 15 000); IR ($CHCl_3$) 3000, 1725 (br), 1670, 1630, 1580, 1440 (br), 1285, and 1145 cm^{-1}; ^1H NMR ($CDCl_3$) 2.53 (3 H, s), 3.80 (3 H, s), 3.94 (3 H, s), 4.07 (3 H, s), 6.61 (1 H, s), 7.92 (1 H, s), 8.25 (1 H, d, *J* = 8 Hz), and 8.86 ppm (1 H, d, *J* = 8 Hz); mass spectrum (70 eV) *m/e* (rel intensity) 364 (3), 331 (14), 317 (100), and 303 (3). Anal. Calcd for $C_{16}H_{16}N_2O_6S$: mol wt, 364.0729. Found: mol wt, 364.0734 (HRMS).

Hydrolysis of Sodium Borohydride Reduced Berninamycin A. (a) Complete Reduction. A solution of 1.024 g of berninamycin A and 0.5 g of sodium borohydride in 45 ml of tetrahydrofuran–ethanol (9:1) stood at room temperature for 25 h, then was adjusted to pH 3, let stand 13 h at room temperature, and was finally evaporated to dryness. The residue was dissolved in 50 ml of water and extracted three times with 50 ml of chloroform. The chloroform extracts were worked up in the usual manner to yield 890 mg of crude reduced product.

A sample of the crude product (400 mg) in 10 ml of 6 N hydrochloric acid was hydrolyzed as above to give a mixture of amino acids separated by ion-exchange chromatography as before. The four amino acids obtained were identified as threonine, β-hydroxyvaline, alanine, and butyrine by TLC and IR comparison with authentic samples of these amino acids.

A smaller sample (50 mg) of the crude reduced product was hydrolyzed in a similar manner, and the residue after opening the tube and evaporating the solvent was derivatized for quantitative gas chromatography after the method of Gehrke.[10] The derivatized mixture was separated on a gas chromatographic column (3% OV17 on Gaschrom Q, 6 ft). The five peaks obtained were identified by comparison to authentic samples of derivatized threonine, β-hydroxyvaline, alanine, and butyrine, and the peak assignments were confirmed by GC–MS. β-Hydroxyvaline yields two peaks which correspond to the mono- and bistrifluoroacetylated products. Integration of the GC trace of the sample obtained from hydrolysis of the crude reduced product and correction for the relative molar responses of the amino acids (1.00:1.17:0.85:0.97, Thr–Hyval–Ala–But) indicated a molar ratio of threonine–β-hydroxyvaline–alanine–butyrine (1.0:0.8:6.0:1.1).

(b) Partial Reduction. When a solution of 1.0 g of berninamycin A and 0.5 g of sodium borohydride in 50 ml of dioxane–ethanol (9:1) stood at room temperature for 10 h, an orange precipitate appeared. The mixture was acidified to pH 3, let stand 4 h at room temperature, and evaporated to dryness. The residue was hydrolyzed and treated with 2,4-dinitrophenylhydrazine in the same manner as described above for the isolation of pyruvic acid 2,4-dinitrophenylhydrazone. When the methylene chloride layer was evaporated to dryness, TLC analysis (silica gel; methanol–chloroform, 1:1) indicated three polar compounds. The residue was added to 20 ml of 3 N methanolic hydrogen chloride and the mixture was stirred at room temperature 18 h, then evaporated, and purified by preparative TLC (silica gel; benzene) to afford three fractions. The two less polar fractions contained 42 and 54 mg, respectively, of the 2,4-dinitrophenylhydrazones of methyl pyruvate and methyl α-ketobutyrate, identified by comparison of their mass spectra with those of authentic samples.

The most polar fraction contained 18 mg of a mixture of **4** and **5**, which could not be separated by the TLC systems tested. The mixture had mp 176–178 °C: IR ($CHCl_3$) 3410, 3045, 1750, 1680, 1620, 1525 (br), and 1345 cm^{-1}; ^1H NMR (pyridine-d_5) 1.23 (~1.5 H, t, *J* = 7 Hz), 1.82 (3 H, d, *J* = 7 Hz), 2.57 (~1.5 H, s), 2.79 (3 H, s), 2.95 (1 H, q, *J* = 7 Hz), 3.72 (3 H, s), 4.89 (1 H, q, *J* = 7 Hz), 8.23 (1 H, d of d, *J* = 3 and 9 Hz), 8.48 (1 H, d, *J* = 9 Hz), and 9.11 ppm (1 H, d, *J* = 3 Hz); mass spectrum (70 eV) *m/e* (rel intensity) 448.1330 (89, $C_{18}H_{20}N_6O_8$), 434.1233 (62, $C_{17}H_{18}N_6O_8$), 389.1192 (100, $C_{16}H_{17}N_6O_6$), 375.1057 (73, $C_{15}H_{15}N_6O_6$), 346.0802 (15, $C_{14}H_{12}N_5O_6$), 332.0631 (10, $C_{13}H_{10}N_5O_6$), 183 (37), 149 (34), 71 (27), 64 (43), and 43 (21). Anal. Calcd for $C_{17}H_{18}N_6O_8$: mol wt, 434.1185. Found: mol wt, 434.1233 (HRMS). Anal. Calcd for $C_{18}H_{20}N_6O_8$: mol wt, 448.1341. Found: mol wt, 448.1330 (HRMS).

Methanolysis of Berninamycin A. (a) 36-h Reaction. A solution of 2.0 g of berninamycin A in 35 ml of 3 N methanolic hydrogen chloride was heated in a pressure bottle for 36 h at 95 °C. After the solution had been evaporated to dryness, the residue was dissolved in 60 ml of water and extracted six times with 10 ml of chloroform. The chloroform layers were combined, dried over magnesium sulfate, and evaporated to yield an oily residue, which was purified on a silica gel column (1.5 × 20 cm) eluted with methanol–chloroform (1:49). One major fraction was detected by TLC (Eastman silica gel; methanol–chloroform, 1:49) of 3-ml fractions and identical tubes were pooled. The residue upon evaporation of the solvent was crystallized from chloroform–cyclohexane, affording 35 mg of **2-acetyl-4-carbomethoxy-5-methyloxazole (6):** mp 74–75 °C; UV max (MeOH) 204 nm (ε 7600) and 265 (9200); IR (CCl_4) 3025, 1750, 1730, 1610, 1545, 1450, 1400, 1370, 1355, 1130, and 1100 cm^{-1}; ^1H NMR, see text; mass spectrum (70 eV) *m/e* (rel intensity) 183 (12), 151 (10), 123 (3), 110 (7), 109 (7), and 43 (100). Anal. Calcd for $C_8H_9NO_4$: C, 52.45; H, 4.91; N, 7.65; mol wt, 183.0531. Found: C, 52.71; H, 5.05; N, 7.60; mol wt, 183.0531 (HRMS).

Crude **6** (before crystallization) contained a trace amount of homologue, 4-carbomethoxy-5-methyl-2-propionyloxazole (**18**), which was not isolated in pure form but was detected by mass spectrometry. Anal. Calcd for $C_9H_{11}NO_4$: mol wt, 197.0688. Found: mol wt, 197.0689 (HRMS).

The aqueous phase was lyophilized and the residue was acetylated with 10 ml of acetic anhydride–pyridine (1:9). After 12 h, the solvent was removed at reduced pressure and the residue was separated by preparative TLC (silica gel; methanol–chloroform, 1:19). Two bands were detected by fluorescence quenching along with considerable material which remained at the origin. The compounds isolated from

the two bands (14 and 17 mg) were identified as the methyl esters of *N,O*-diacetylthreonine and *N*-acetyl-β-hydroxyvaline, respectively, by comparison of their mass spectra with those of authentic similarly derivatized samples.

(b) 3-h Reaction. A solution of 1.0 g of berninamycin A in 70 ml of 3 N methanolic hydrogen chloride was heated at reflux for 3 h and then evaporated to dryness. The residue was dissolved in 60 ml of hot water and extracted four times with 30 ml of ethyl acetate. The ethyl acetate extracts were worked up in the usual manner to yield 400 mg of an orange oil, which was dissolved in 20 ml of ethyl acetate, added to 1 g of silica gel, and evaporated to dryness. The adsorbed sample was layered on top of a silica gel column (1.5 × 30 cm) and eluted with methanol–chloroform (1:49); 10-ml fractions were collected. Four fractions were identified by TLC (Eastman silica gel; methanol–chloroform, 1:49) and identical tubes were pooled.

Fraction I was crystallized from chloroform–cyclohexane, affording 42 mg of **2-acetyl-4-carboxamido-5-methyloxazole (14)**: mp 186–186.5 °C; UV max (MeOH) 217 nm (ε 9500) and 269 (9100); IR ($CHCl_3$) 3530, 3420, 1720, 1680, 1605, 1525, 1450, 1430, and 1360 cm^{-1}; ^1H NMR ($CDCl_3$) two sharp singlets at 2.66 and 2.76 ppm; ^{13}C NMR (Me_2SO-d_6) 185.1, 162.5, 162.4, 155.9, 130.8, 26.2, and 11.7 ppm; mass spectrum (70 eV) *m/e* (rel intensity) 168 (39), 151 (28), 125 (3), 124 (4), and 43 (100). Anal. Calcd for $C_7H_8N_2O_3$: C, 50.00; H, 4.76; N, 16.66; mol wt, 168.0535. Found: C, 49.98; H, 4.84; N, 16.54; mol wt, 168.0533 (HRMS).

Crude **14** (before crystallization) contained homologue 2-propionyl-4-carboxamido-5-methyloxazole (**15**), which was not isolated in pure form but was detected by ^1H NMR and mass spectrometry: ^1H NMR (C_5D_5N) 1.15 (t, J = 7.5 Hz, $-CH_2CH_3$), 2.55 (s), 2.69 (s), and 3.00 ppm (q, J = 7.5 Hz, $-CH_2CH_3$). Anal. Calcd for $C_8H_{10}N_2O_3$: mol wt, 182.0692. Found: mol wt, 182.0691 (HRMS).

The aqueous layer was evaporated to dryness and the residue was acetylated with 5 ml of acetic anhydride–acetic acid (1:1) for 18 h. The solvent was removed at reduced pressure and the residue was separated by preparative TLC (silica gel; methanol–chloroform, 1:19). Three major bands were detected by fluorescence quenching. The first two bands (5 and 9 mg, respectively) were identified as the methyl esters of *N,O*-diacetylthreonine and *N*-acetyl-β-hydroxyvaline by comparison of their mass spectra with those of authentic samples.

The third band afforded 40 mg of the major component, **4-carbomethoxy-5-methyl-2-(1-threoninamido-1-propenyl)oxazole diacetate (17)**: mp 215 °C; IR ($CHCl_3$) 1720 (br), 1495, 1440, and 1365 cm^{-1}; ^1H NMR, see text; mass spectrum (70 eV) *m/e* (rel intensity) 381.1538 (14, $C_{17}H_{23}N_3O_7$), 321.1334 (24, $C_{15}H_{19}N_3O_5$), 223.0968 (90, $C_{10}H_{13}N_3O_3$), 196.0849 (100, $C_9H_{12}N_2O_3$), 191 (58), 164 (28), 136 (49), 99.0320 (38, $C_4H_5NO_2$), 73 (38), and 43 (55). Anal. Calcd for $C_{17}H_{23}N_3O_7$: mol wt, 381.1530. Found: mol wt, 381.1538 (HRMS).

Conversion of 6 to 14. A solution of 10 mg of **6** in 1 ml of concentrated ammonium hydroxide was heated at 55 °C for 15 min. The yellow solution was cooled and extracted six times with 1 ml of chloroform, and the extracts were combined, washed with 2 ml of 1 N hydrochloric acid, dried, and evaporated to yield a white solid. After recrystallization from acetone–cyclohexane, the product (9 mg, mp 184–186 °C) exhibited the same IR, ^1H NMR, and mass spectra as **14**.

***N*-(α-Benzyloxypropionyl)-*O-tert*-butyl-DL-threonine Methyl Ester (9).** *O-tert*-Butyl-DL-threonine methyl ester[14] (**7**, 10 g) was added at 0 °C to a stirred solution of 15 ml of triethylamine in 50 ml of ethanol followed by 10.4 g of α-benzyloxypropionyl chloride[15] (**8**). The solution was allowed to warm to room temperature, stirred overnight, concentrated, and diluted with 60 ml of water. The solution was extracted three times with 50 ml of ether and the ether extracts were washed with 50 ml of 0.1 N hydrochloric acid, dried, and evaporated to yield a crude oil. The oil was distilled to give 15.2 g (78%) of **9**: bp 173–175 °C (0.2 Torr); IR (thin film) 3440, 2980, 1775, 1695, and 1520 cm^{-1}; ^1H NMR ($CDCl_3$) 1.08 (9 H, s), 1.40 (3 H, d, J = 7 Hz), 3.68 (3 H, s), 4.20 (3 H, m), 4.56 (2 H, s), and 7.28 ppm (5 H, s). Anal. Calcd for $C_{19}H_{29}NO_5$: C, 64.95; H, 8.26; N, 3.99. Found: C, 64.91; H, 8.23; N, 3.86.

***N*-(α-Benzyloxypropionyl)-DL-threonine Methyl Ester (10).** Compound **9** (10.5 g) was added to 50 ml of trifluoroacetic acid at 0 °C, and the solution was allowed to stand 1 h at 0 °C and an additional 1 h at 15 °C.[16] Excess trifluoroacetic acid was removed at reduced pressure and the residue was dissolved in benzene and lyophilized. The residual oil was dissolved in 50 ml of chloroform and extracted twice with 50 ml of water. The chloroform layers were worked up in the usual manner, affording a yellow oil, which was purified by column chromatography (silica gel; 2 × 50 cm; chloroform). The major band yielded a colorless oil which solidified upon standing. Crystallization from chloroform–hexane yielded **10** (7.55 g, 83%): mp 74–75 °C; IR ($CHCl_3$) 3420, 3025, 1760, 1695, and 1525 cm^{-1}; ^1H NMR ($CDCl_3$) 1.19 (3 H, d, J = 7 Hz), 1.44 (3 H, d, J = 7 Hz), 2.45 (1 H, s), 3.76 (3 H, s), 4.02 (1 H, d, J = 7 Hz), 4.40 (2 H, m), 4.60 (2 H, s), and 7.33 ppm (5 H, s). Anal. Calcd for $C_{15}H_{21}NO_5$: C, 61.01; H, 7.12; N, 4.74. Found: C, 59.94; H, 6.95; N, 4.61.

Methyl α-(α-Benzyloxypropionamido)acetoacetate (11). Jones reagent[28] was added dropwise to a solution of 0.916 g of **10** in 50 ml of acetone at 50 °C. Addition was continued until the color was no longer discharged. Water (100 ml) was added and the solution was extracted three times with 50 ml of ether. The ether layers were worked up in the usual manner to afford a light brown oil, which was purified on a short column of silica gel (5 g) eluted with chloroform. The product appeared in one band, which was collected and evaporated to yield 0.732 g (90%) of **11**. Anal. Calcd for $C_{15}H_{19}NO_5$: C, 61.43; H, 6.48; N, 4.78. Found: C, 61.39; H, 6.72; N, 4.51.

2-(1-Benzyloxyethyl)-4-carbomethoxy-5-methyloxazole (12). A solution of 0.480 g of **11** in 5 ml of thionyl chloride was allowed to stand at 0 °C for 24 h and then was evaporated to an oil, which was dissolved in 10 ml of chloroform and immediately poured into a solution of 40 ml of 10% aqueous sodium carbonate. The mixture was vigorously stirred 1 h, and the chloroform layer was separated and worked up in the usual manner to afford 0.42 g (62%) of crude **12.** Anal. Calcd for $C_{15}H_{17}NO_4$: C, 65.44; H, 17.14; N, 5.09. Found: C, 65.14; H, 17.05; N, 4.71.

4-Carbomethoxy-2-(1-hydroxyethyl)-5-methyloxazole (13). Hydrogen gas was gently bubbled for 18 h through a mixture of 53 mg of crude **12**, 0.1 ml of concentrated hydrochloric acid, and 50 mg of 10% palladium on carbon in 10 ml of methanol. The mixture was filtered and the filtrate was evaporated to yield an oily residue which was purified by preparative TLC (silica gel; methanol–chloroform, 1:49). The major band (R_f 0.45) was isolated and the compound was eluted with chloroform, which upon evaporation afforded 22 mg (57%) of crude **13**. Anal. Calcd for $C_8H_{11}NO_4$: C, 51.89; H, 5.99; N, 7.56. Found: C, 51.75; H, 6.09; N, 7.20.

2-Acetyl-4-carbomethoxy-5-methyloxazole (6). Jones reagent[28] (~0.2 ml) was added dropwise with stirring to a solution of 20 mg of crude **13** in 10 ml of acetone at 50 °C and the solution was let stand 15 min. A second aliquot of the reagent (0.2 ml) was added and the solution was let stand an additional 20 min. Water (50 ml) was added and the solution was extracted three times with 20 ml of chloroform. The chloroform layers were worked up in the usual manner and the residue was sublimed [50 ° (0.2 Torr)] to afford colorless needles of **6** (6.6 mg, 33%), mp 75-76 °C. The UV, IR, ^1H NMR, and mass spectra of the authentic synthetic material are identical with those obtained for the methanolysis product **6**.

Sodium Borohydride Reduction of Crude 2-Acetyl-4-carboxamido-5-methyloxazole (14). A sample of crude **14** (40 mg) obtained as a methanolysis product of berninamycin A was prepared for ^1H NMR analysis (C_5D_5N). The spectrum showed a major component (2.55 ppm, 3 H, s, $-COCH_3$; 2.69 ppm, 3 H, s, $ArCH_3$) and a minor component (1.15 ppm, t, J = 7.5 Hz, $-CH_2CH_3$; 3.00 ppm, q, J = 7.5 Hz, $-CH_2CH_3$). The sample was added to 10 ml of ether, giving 38 mg of recovered **14**. Sodium borohydride (20 mg) was added to the recovered **14** dissolved in 10 ml of ethanol, and the solution was stirred 1 h and then evaporated to dryness. The residue was dissolved in 10 ml of 0.1 N hydrochloric acid, the aqueous solution was extracted seven times with 15 ml of chloroform, and the chloroform layers were worked up in the usual manner to afford 40 mg of crude **16**, which was purified by preparative TLC (silica gel; methanol–methylene chloride, 1:19). The major band was removed and eluted with chloroform. Upon evaporation of the solvent, 21 mg of a mixture of 2-(1-hydroxyethyl)-4-carboxamido-5-methyloxazole (**16a**) and 2-(1-hydroxypropyl)-4-carboxamido-5-methyloxazole (**16b**) was obtained: the ^1H NMR spectrum showed a major component (**16a**, 1.75 ppm, 3 H, d, J = 7 Hz, $ArCHOHCH_3$; 2.64 ppm, 3 H, s, $ArCH_3$; 5.18 ppm, 1 H, q, J = 7 Hz, $ArCHOHCH_3$) and a minor component (**16b**, 1.07 ppm, t, J = 7 Hz, $ArCHOHCH_2CH_3$; 2.12 ppm, m, $ArCHOHCH_2CH_3$). The homologue of **14** was thus identified as 4-carboxamido-5-methyl-2-propionyloxazole (**15**).

Methanolysis of 17. A sample of 5 mg of **17** in 2 ml of 3 N metha-

nolic hydrogen chloride was heated in a sealed tube (under nitrogen) at 95 °C for 36 h. After evaporation of the solvent, the residue was dissolved in 1 ml of acetic anhydride–pyridine (1:1) and let stand 18 h. The mixture was added to 25 ml of 0.1 N hydrochloric acid and the aqueous solution was extracted twice with 25 ml of chloroform. After evaporation of the combined chloroform layers, the residue was purified by preparative TLC (silica gel; methanol–methylene chloride, 1:49) to afford two compounds identified as 4-carbomethoxy-5-methyl-2-propionyloxazole (**18**) and *N,O*-diacetylthreonine methyl ester by comparison of their mass spectra with those of authentic samples.

Hydrochloric Acid–Glacial Acetic Acid Degradation of Berninamycin A. Hydrochloric acid (1 N) in glacial acetic acid[17] was prepared by weighing the solution while hydrogen chloride gas bubbled through. A solution of 1.000 g of berninamycin A in 100 ml of 1 N hydrochloric acid in glacial acetic acid was flushed with nitrogen, placed in an oil bath at 110 °C for 10 min, then immediately frozen, and lyophilized to give 1.020 g of pale white product. Analysis by TLC (silica gel; methanol–chloroform, 1:9 and 1:19) indicated eight new compounds, which were more polar than berninamycin A. A portion of the product (520 mg) was purified by preparative TLC (silica gel, six 2-mm plates; methanol–chloroform, 1:9). Four bands were isolated and worked up in the usual manner to give, in order of increasing polarity, fractions I (7 mg), II (34 mg), III (147 mg), and IV (264 mg). Fraction I contained a single compound but was not further investigated due to its small quantity. The other three fractions were found to be mixtures of compounds by TLC (silica gel; methanol–methylene chloride, 1:9). Preparative TLC (silica gel, 2-mm; methanol–chloroform, 1:19) divided fraction II into two bands, fractions IIa (2 mg) and IIb (31 mg). The latter was found by ^{1}H NMR, UV, and mass spectrometry to be identical with the methanolysis product **14**, mp 186–187 °C.

Fraction III yielded three bands on preparative TLC (silica gel, two 2-mm plates; methanol–chloroform, 1:9; developed twice): fractions IIIa (2 mg), IIIb (43 mg), and IIIc (3 mg). Fraction IIIb was dissolved in 1 ml of methanol, precipitated with cyclohexane, and dried in vacuo to give 34 mg of **4-carboxamido-5-methyl-2-[1-(*N*-pyruvylthreoninamido)-1-propenyl]oxazole (19)**: mp 158–165 °C dec; UV max (EtOH) 230 nm to end absorption ($\epsilon \sim 15\,000$); IR ($CHCl_3$) 3025, 1700 (br), 1510, and 1425 cm^{-1}; ^{1}H NMR ($CDCl_3$ + 1% CD_3OD) 1.38 (3 H, d, J = 8 Hz), 1.78 (3 H, d, J = 7 Hz), 2.48 (3 H, s), 2.58 (3 H, s), and 8.25 (4 H, br s); mass spectrum (70 eV) *m/e* (rel intensity) 352 (2), 309 (11), 295 (5), 294 (33), 194 (7), 183 (15), 169 (14), 168 (100), 152 (21), 151 (4), 141 (13), and 140 (5). Anal. Calcd for $C_{15}H_{20}N_4O_6$: mol wt, 352.1383. Found: mol wt, 352.1396 (HRMS).

Preparative TLC (silica gel, two 2-mm plates; methanol–chloroform, 1:9; developed twice) of fraction IV afforded fractions IVa (6 mg) and IVb (131 mg). The latter material, mp 168–174 °C dec, did not give interpretable spectral data. It appeared to be similar to fraction IIIb (**19**), although probably larger since its mass spectrum contained peaks, due to decomposition, up to *m/e* 622. The latter ion had the composition $C_{27}H_{26}N_8O_8S$ (calcd, 622.1589; found, 622.1594). Attempts to purify further fraction IVb were unsuccessful, since it apparently decomposed rapidly, yielding only material remaining at the origin by TLC.

The remaining crude acetolysis product lyophilizate (400 mg) was similarly purified after having been frozen for 2 days; however, it had mainly decomposed and only yielded 15 mg of **14.**

Methanolysis of 19. A sample of **19** (10 mg) was methanolyzed in the same manner as described for **17** to give diacetylthreonine methyl ester and **18,** again identified by mass spectrometry.

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References and Notes

(1) (a) Presented in part at the 170th National Meeting of the American Chemical Society, Chicago, Ill., August 1975; cf. Abstract No. ORGN 20; (b) taken mainly from the Ph.D. Thesis of J. M. Liesch, University of Illinois, Urbana, 1975; (c) a minor portion of this material has appeared in a preliminary communication.[2]
(2) J. M. Liesch, J. A. McMillan, R. C. Pandey, I. C. Paul, K. L. Rinehart, Jr., and F. Reusser, *J. Am. Chem. Soc.*, **98**, 299 (1976).
(3) University of Illinois Fellow, 1971–1973; Mobil Foundation Fellow, 1973–1974; Uniroyal Fellow, 1974–1975.
(4) F. Reusser, *Biochemistry*, **8**, 3303 (1969).
(5) M. E. Bergy, J. H. Coats, and F. Reusser, U.S. Patent 3 689 639 (Jan 23, 1969); *Chem. Abstr.*, **77**, 150582v (1972).
(6) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *Anal. Chem.*, **23**, 1236 (1951).
(7) G. W. Edwards and M. L. Minthorn, Jr., *Can. J. Biochem.*, **46**, 1227 (1968).
(8) M. Bodanszky, J. A. Scozzie, and I. Muramatsu, *J. Antibiot.*, **23**, 9 (1970).
(9) K. Kakinuma and K. L. Rinehart, Jr., *J. Antibiot.*, **27**, 733 (1974).
(10) C. W. Gehrke, W. M. Lanikin, D. L. Stalling, and F. Shahrokhi, *Biochem. Biophys. Res. Commun.*, **19**, 328 (1965).
(11) B. Anderson, D. Hodgkin, and M. A. Viswamitra, *Nature (London)*, **225**, 233 (1970).
(12) L. A. Paquette in "Principles of Modern Heterocyclic Chemistry", W. A. Benjamin, Boston, Mass., 1968, p 191.
(13) J. P. Greenstein and M. Winitz in "Chemistry of the Amino Acids", Vol. 2, Wiley, New York, N.Y., 1961, p 853.
(14) E. Schröder, *Justus Liebigs Ann. Chem.*, **670**, 127 (1963).
(15) S. Shapiro and L. Freedman, U.S. Patent 2 967 886 (Jan 10, 1961).
(16) H. C. Beyerman and J. S. Bontekoe, *Recl. Trav. Chim. Pays-Bas*, **81**, 696 (1962).
(17) E. Gross and H. H. Kiltz, *Biochem. Biophys. Res. Commun.*, **50**, 559 (1973).
(18) L. M. Jackman and S. Sternhell, "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry", 2d ed, Pergamon Press, Braunschweig, Germany, 1969.
(19) The pK_a of an authentic sample of picolinic acid is 5.3 and that of 2-carboxy-1-methylpyridinium chloride is less than 2.
(20) S. Pataki, Ed., "The Chemistry of the Amino Group", Interscience, New York, N.Y., 1967.
(21) L. F. Johnson and W. C. Jankowski, "Carbon-13 NMR Spectra", Wiley-Interscience, New York, N.Y., 1972.
(22) J. B. Stothers, "Carbon-13 NMR Spectroscopy", Academic Press, New York, N.Y., 1972.
(23) G. C. Levy and G. L. Nelson, "Carbon-13 Nuclear Magnetic Resonance for Organic Chemists" Wiley-Interscience, New York, N.Y., 1972.
(24) K. L. Rinehart, Jr., J. C. Cook, Jr., K. H. Maurer, and U. Rapp, *J. Antibiot.*, **27**, 1 (1974).
(25) J. M. Clark in "Experimental Biochemistry", W. H. Freeman, San Francisco, Calif., 1964, p 217.
(26) H. A. Sober, Ed., "The Handbook of Biochemistry", 2d ed, Chemical Rubber Publishing Co., Cleveland, Ohio, 1970.
(27) S. Liebermann, D. Fukushima, and K. Dobriner, *J. Biol. Chem.*, **182**, 299 (1956).
(28) K. Bowden, I. M. Heilbron, E. R. Jones, and B. C. Weedon, *J. Chem. Soc.*, 39 (1946).