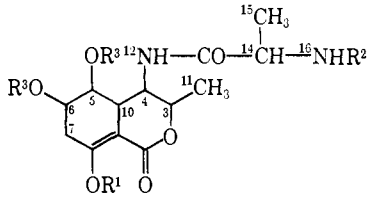
The Structure and Chemistry of Actinobolin[1]

Frederick J. Antosz,[2] Denny B. Nelson, Delbert L. Herald, Jr., and Morton E. Munk

Contribution from the Department of Chemistry, Arizona State University, Tempe, Arizona 85281. *Received December 31, 1969*

Abstract: The structure, chemistry, and absolute configuration of actinobolin (**1**), an antibiotic possessing a broad spectrum of biological activity, is described. When the chemical and spectral properties of N-acetylactinobolin (**2**) led to a partial structure expressed as a series of four fragments, which together accounted for *all* atoms in the molecule, computer program CMBN was employed to generate all structures consistent with available evidence. All six structures outputted possessed a β-ketolactone system. Spin decoupling studies on **2**, the periodate oxidation of a sodium borohydride reduction product of **2**, and formation of an isopropylidene derivative of **2** narrowed the number to two structures. An nmr study of the totally acetylated 5-pyrazolone derived from **2** upon treatment with hydrazine allowed the assignment of expression **2** as the structure of N-acetylactinobolin. The isolation of L-alanyl-L-threonine from the mild permanganate oxidation of actinobolin established the configuration at C-3, C-4, and C-14 as *R*, *R*, and *S*, respectively. The absolute configuration at C-5, C-6, and C-10 could be assigned as *R*, *R*, and *R*, respectively, by a study of the 100-MHz nmr spectra of **2** and its methyl enol ether **5**.

The antibiotic actinobolin ($C_{13}H_{20}N_2O_6$), which possesses a broad spectrum of biological activity, was first isolated by Haskell and Bartz[3] as the crystalline sulfate salt. The products arising from the acid hydrolysis of actinobolin[4] and from the base hydrolysis of N-acetylactinobolin[5] have been described. The results of these degradations coupled with other chemical and spectral data led to the assignment of expression **1** as the structure of the intact antibiotic.[5b] We now wish to report further chemical evidence in support of structure **1** and describe the work leading to the absolute configuration of actinobolin.



1, $R^1 = R^2 = R^3 = H$

2, $R^1 = R^3 = H$; $R^2 = CO\text{–}{}^{18}CH_3$

3, $R^1 = R^3 = H$; $R^2 = COCH_2CH_3$

4, $R^1 = R^2 = R^3 = COCH_3$

5, $R^1 = {}^{19}CH_3$; $R^2 = CO\text{–}{}^{18}CH_3$; $R^3 = H$

6, $R^1 = H$; $R^2 = COCH_3$; $R^3R^3 = C(CH_3)_2$

Structure of Actinobolin

In arriving at the structure of actinobolin, a computer program,[5a,6] recently developed in our laboratory, was employed as an aid in evaluating the structural implications of experimental data. Program CMBN was designed to generate all possible structures for a molecule by combining the structural units derived from a study of chemical and spectral properties in all possible ways consistent with the evidence at hand. The utility of program CMBN has been demonstrated in the structure elucidation of N-acetylalanylactinobicyclone (**7**),[5a,6b] one of the products of mild base hydrolysis of N-acetylactinobolin (**2**).

The structural units used as input data for program CMBN are shown in Figure 1. The presence of two secondary hydroxyl groups (fragment A) was suggested by the nmr spectrum of N-acetylactinobolin in dimethyl sulfoxide solution which exhibited two, one-proton doublets at δ 4.77 and 5.05 (J = 4 Hz), each of which immediately disappeared upon the addition of deuterium oxide.[7] A third signal, a one-proton singlet, at δ 13.15 also disappeared immediately upon the addition of deuterium oxide and was tentatively assigned to an enolic proton. The isolation of the tri-O-acetate **4** upon treatment of N-acetylactinobolin with acetic anhydride containing perchloric acid was consistent with the presence of three acetylatable hydroxyl groups, while the formation of the enol ether **5** upon treatment of N-acetylactinobolin with diazomethane provided support for the presence of an enolic hydroxyl group.

Evidence for the presence of fragment B in N-acetylactinobolin was derived from the structure of N-acetylalanylactinobolone (**8**),[5b,8] the second product of base hydrolysis of N-acetylactinobolin. The mildness of the conditions employed in the conversion to **8** and the small change in molecular formula ($C_{15}H_{22}N_2O_7 + H_2O \rightarrow C_{14}H_{24}N_2O_6 + CO_2$) suggested that no deep-seated rearrangement had occurred, hence the 1,3 relationship between the ketone carbonyl and the side chain shown in fragment B. The positioning of the hydroxyl groups specifically at positions 4 and 5

(1) (a) Support of this work by the National Institutes of Health through Research Grant No. AI-04720 is gratefully acknowledged. (b) This paper is based in large part on the Ph.D. Dissertation of F. J. Antosz, Arizona State University, Tempe, Arizona, 1969. (c) A portion of the work described was presented before the Division of Organic Chemistry at the 156th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 1968, ORGN 022.

(2) (a) NASA Trainee, 1965–1968; (b) Arizona State University Graduate Fellow, 1968–1969.

(3) T. H. Haskell and Q. R. Bartz, *Antibiot. Ann., 1958–1959*, 505 (1959).

(4) M. E. Munk, C. S. Sodano, R. L. McLean, and T. H. Haskell, *J. Amer. Chem. Soc.*, **89**, 4158 (1967).

(5) (a) D. B. Nelson, M. E. Munk, and K. B. Gash, 156th National Meeting of the American Chemical Society, Atlantic City, New Jersey, Sept 1968, MEDI 040; (b) M. E. Munk, D. B. Nelson, F. J. Antosz, D. L. Herald, Jr., and T. H. Haskell, *J. Amer. Chem. Soc.*, **90**, 1087 (1968).

(6) (a) D. L. Herald, Jr., Ph.D. Dissertation, Arizona State University, Tempe, Arizona, 1969; (b) D. B. Nelson, M. E. Munk, K. B. Gash, and D. L. Herald, Jr., *J. Org. Chem.*, **34**, 3800 (1969).

(7) O. L. Chapman and R. W. King, *J. Amer. Chem. Soc.*, **86**, 1256 (1964).

(8) D. B. Nelson and M. E. Munk, *J. Org. Chem.*, in press.

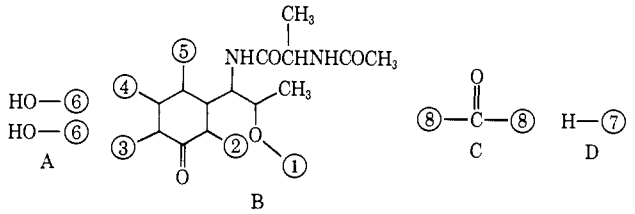
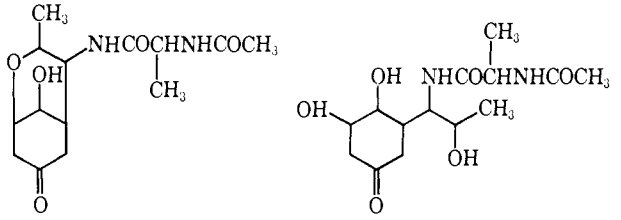


Figure 1. Partial structure of N-acetylactinobolin (**2**).

as suggested by N-acetylalanylactinobolone was not considered secure, and, therefore, to obtain the broadest spectrum of plausible structures for evaluation, C–O bonds at these sites were not assumed. More secure was the placement of the oxygen in the side chain, as shown by the presence of a similar structural unit in the product of *both* acid hydrolysis, actinobolamine,[4] and basic hydrolysis, **8**. Further evidence supporting the nature of the side chain included the isolation of the dipeptide L-alanyl-L-threonine[9] upon mild permanganate oxidation of N-acetylactinobolin and the appearance of strong peaks in the mass spectrum of N-acetylactinobolin at *m/e* 131 $NH_2C(=OH^+)CH(CH_3)NHCOCH_3$, 114 $O^+{\equiv}CC(CH_3)CHNHCOCH_3$, and 86 ($CH_3CH{=}NH^+COCH_3$), and a minor peak at *m/e* 170 $CH_3\dot{C}HCH{=}NH^+COCH(CH_3)NHCOCH_3$. Three of the four peaks underwent a shift of 14 mass units in the mass spectrum of N-propionylactinobolin (**3**); the peak corresponding to *m/e* 170 did not appear.



The ultraviolet absorption spectrum of N-acetylactinobolin exhibited a single maximum at 261 mμ in water solution which underwent a bathochromic shift to 288 mμ upon the addition of sodium hydroxide. This behavior, which is characteristic of β-keto esters[10] and β-diketones,[11] suggested the presence of a second carbonyl group, fragment C. Evidence for the 1,3 relationship of carbonyl groups includes: (1) the loss of 1 mol of carbon dioxide during the mild basic hydrolysis of N-acetylactinobolin;[8] (2) the appearance of the one-proton singlet at δ 13.15 in the nmr spectrum of N-acetylactinobolin; (3) the isolation of the enol acetate **4** and the enol ether **5**; and (4) a pK_a of 8.8 for N-acetylactinobolin.

Taken together, fragments A, B, and C account for all of the atoms present in N-acetylactinobolin, except for one hydrogen atom. This necessitated the inclusion of fragment D in the list of structural units.

(9) Although the sequence of the dipeptide was not rigorously proven, presumptive evidence for its assignment was obtained from the acid hydrolysis of the 2,4-dinitrophenyl (DNP) derivative of **1** which resulted in the isolation of DNP-alanine. Thus the free amino group of actinobolin is that of the alanyl moiety suggesting that the free amino group of the dipeptide is also that of alanine.

(10) (a) R. A. Morton and W. C. V. Rosney, *J. Chem. Soc.*, 706 (1926); (b) S. J. Rhoads, J. C. Gilbert, A. W. Decora, T. R. Garland, R. J. Spangler, and M. J. Urbigkit, *Tetrahedron*, **19**, 625 (1963).

(11) (a) A. I. Scott, "Interpretation of the Ultraviolet Spectra of Natural Products," The Macmillan Co., New York, N. Y., 1964, p 69–70; (b) B. Eistert and W. Reiss, *Chem. Ber.*, **87**, 108 (1954).

In arriving at the computer-generated structures, certain restrictions were placed on the input data. The computer was instructed to reject any structure which contained a double bond, an aldehyde, a peroxide, or an acid—*i.e.*, arrays containing any one of the following number pairs were rejected: 3–4, 4–5, 7–8, 1–6, or 6–8 functional groups clearly inconsistent with the chemical and physical properties. The input data also required the rejection of any array containing the number pair 1–7, because this would have resulted in the generation of structures containing three secondary hydroxyl groups. Arrays containing the number pair 6–7 were also rejected. This could have resulted in the formation of a molecule of water and, therefore, multimolecule answers. Finally, because all available evidence indicated that N-acetylactinobolin possessed a 1,3-dicarbonyl linkage, the computer was programmed to output only those structures containing either number pair 2–8 or 3–8.

The six structures generated by the computer appeared as the number-pair arrays shown in Table I.

Table I. Computer Output of Possible Structures for N-Acetylactinobolin

Structure no.	No.-pair array				
11	1–8	2–6	3–8	4–6	5–7
12	1–8	2–6	3–8	4–7	5–6
13	1–8	2–7	3–8	4–6	5–6
14	1–8	2–8	3–6	4–6	5–7
15	1–8	2–8	3–6	4–7	5–6
2	1–8	2–8	3–7	4–6	5–6

Each is a β-ketolactone. Although not specifically instructed, the computer generated no β-diketones. Thus the structural information *already described* (and expressed as computer input), which does not explicitly restrict the β-diketone linkage, is sufficient to exclude such structures. For confirmation, N-acetylactinobolin was treated with hydrazine. The isolation and characterization of the amorphous 5-pyrazolone **9**[12] as its crystalline hexaacetate **10** militated against a β-diketone, which would have given rise to a pyrazole.[13]

The presence of a lactone was further substantiated by the isolation of the hydroxylactone **16** upon reduction of N-acetylactinobolin with sodium borohydride. The infrared spectrum of **16** exhibited bands characteristic of a δ-lactone[14] at 1730 and 1210 cm^{-1}, and **16** gave the expected hydroxamic acid test.[15,16] Another derivative containing the lactone ring was obtained upon hydrogenation of the enol ether **5** over platinum oxide. The resulting dihydro enol ether **17** displayed characteristic bands in its infrared spectrum at 1730, 1235, and 1070 cm^{-1}.[14]

(12) R. H. Wiley and P. Wiley in "Pyrazolones, Pyrazolidones, and Derivatives," A. Weissberger, Ed., Interscience Publishers, New York, N. Y., 1964, Chapter 2.

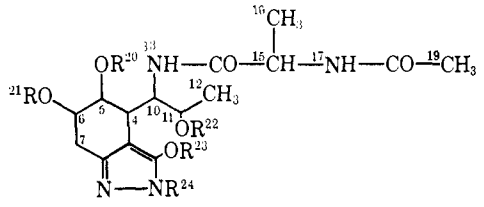
(13) R. Fusco in "Pyrazoles, Pyrazolines, Pyrazolidenes, Indazoles, and Condensed Rings," R. H. Wiley, Ed., Interscience Publishers, New York, N. Y., 1967, Part I.

(14) K. Nakanishi, "Infrared Absorption Spectroscopy," Holden-Day, Inc., San Francisco, Calif., 1962, Chapter 1.

(15) M. Abdel-Akher and F. Smith, *J. Amer. Chem. Soc.*, 73, 5859 (1951).

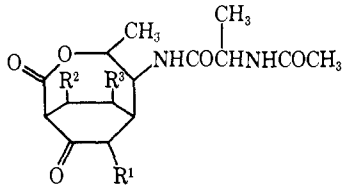
(16) Under identical conditions, 7 and 8 gave a positive test only after 3 hr, thus precluding the amide groups as the source of the immediate positive test.[17]

(17) F. Bergman, *Anal. Chem.*, 24, 898 (1952).



9, R = H

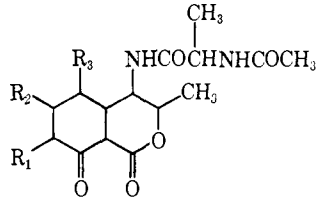
10, R = $COCH_3$



11, $R^1 = R^2 = OH; R^3 = H$

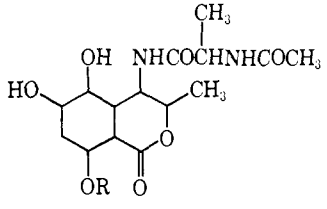
12, $R^1 = R^3 = OH; R^2 = H$

13, $R^1 = H; R^2 = R^3 = OH$



14, $R_1 = R_2 = OH; R_3 = H$

15, $R_1 = R_3 = OH; R_2 = H$



16, R = H

17, R = CH_3

Three experiments narrowed the list of possible structures for N-acetylactinobolin to two candidates: (1) the periodate oxidation of hydroxylactone **16** resulted in the consumption of *only 1* mol of periodate; (2) treatment of N-acetylactinobolin with acetone in the presence of *p*-toluenesulfonic acid resulted in the isolaton and characterization of the isopropylidene derivative **6**; and (3) the protons on the carbon atoms bearing the hydroxyl groups in N-acetylactinobolin were shown by nmr decoupling studies to be spin–spin coupled.[18] These data require adjacent hydroxyl groups, neither of which is α to the ketone carbonyl. Of the six possible structures, only **2** and **13** meet this requirement.

An examination of structures **2** and **13** revealed that they differed only in the position at which the lactone carbonyl was joined to the cyclohexanone ring. A thorough study of the nmr spectrum of the totally acetylated pyrazolone **10** provided information which allowed a distinction to be made. Specifically, irradiation of the multiple signal at δ 5.25 which includes H-6, resulted in the collapse of the H-7 doublet at δ 3.42 to a singlet (Table II). Furthermore, the C-4 methine proton (δ 3.07) was shown to be spin–spin coupled to two low-field signals by irradiation at δ 4.60 and 5.25. No coupling could be observed between the methylene protons and this methine proton, thereby ruling out structure **13** which would give rise to a 5-pyrazolone requiring spin coupling between the methylene and methine protons. These data establish expression **2** as the unique structure of N-acetylactinobolin.

Table II. Spin Decoupling Studies on Compound **10**[a,b]

Signal irradiated (δ, multiplicity, J in Hz)	Signal obsd (δ)	Multiplicity change
H-15 (4.63, quintet, 7)	H-16 (1.30)	d → s
H-11 (5.25, m)	H-12 (1.15)	d → s
H-10 (5.25, m)	H-4 (3.07)	d of d → d J = 10 Hz
H-5 (4.60, t, 10)	H-4 (3.07)	d of d → s (broad)
H-6 (5.25, m)	H-7 (3.42)	d → s

[a] Field-swept decoupling at 60 MHz. [b] In $CDCl_3$ solution at 15% *vs.* internal TMS.

The structure of N-acetylactinobolin suggests the feasibility of aromatization of the carbocyclic ring. This goal was realized by the sequence of reactions pictured in Scheme I. Treatment of the enol ether **5** with acetic anhydride and pyridine resulted in the isolation of di-O-acetate **18**. On passage over an alumina column **18** was converted to **19**,[19] but all attempts to eliminate the second mole of acetic acid failed. However, **19** was successfully aromatized by treatment with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) to yield **20**. Although a satisfactory elemental analysis of this compound was not possible due to the ease of acetate cleavage, the spectral properties (see Experimental Section) of **20** verified that it was a dihydroisocoumarin. The isolation of **20** requires a 1,2 relationship of lactone carbonyl and side chain on the carbocyclic ring of N-acetylactinobolin and thus militates against structure **13**. Furthermore, the magnitude of the coupling constant, $J_{6,7}$, indicated that the two aromatic protons of **20** were *ortho* to each other,[21] thus establishing the loss of the hydroxyl group at C-6 during the sequence of steps leading to aromatization.

Treatment of **20** with 0.1 *N* sodium methoxide for 15 min resulted in the isolation of the free phenol **21** which was methylated with diazomethane to yield **22**. When **20** was treated with 0.1 *N* sodium methoxide for 30 hr, two unexpected products were formed; methyl N-acetyl-L-alanate and the phthalimidine **24** whose structure was assigned on the basis of the following

(18) In dimethyl sulfoxide solution at 60 MHz, the signals for the protons on carbon bearing the hydroxyl groups appear as a triplet of doublets centered at δ 3.10 and a multiplet at 3.33–3.92. Upon the addition of deuterium oxide, the signal at δ 3.10 collapses to a triplet while the multiplet collapses to a triplet of doublets centered at 3.70 thereby confirming the above assignments. These same signals in deuterium oxide solution at 100 MHz appear as a triplet at δ 3.70 and a triplet of doublets at 4.34, respectively. Upon irradiation of the signal at δ 4.34, the triplet at 3.70 collapses to a doublet. When the signal at δ 3.70 was irradiated, coupling with the proton at 4.34 could be demonstrated although exact multiplicity changes could not be determined.

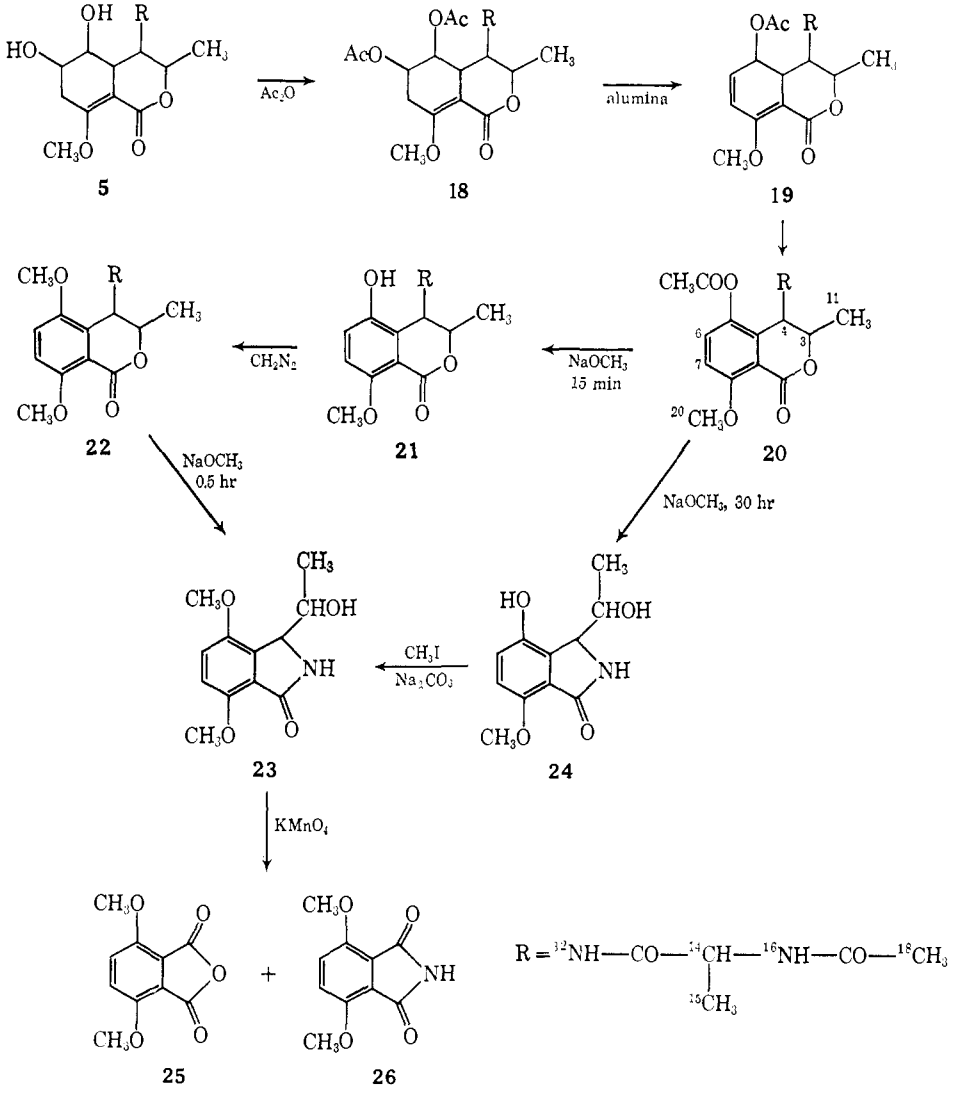
(19) The isolation of upon olefins treating alkyl acetates with alumina has been reported.[20]

(20) R. Taylor, *Chem. Ind. (London)*, 1684 (1962).

(21) For aromatic compounds, the coupling between various protons is usually $J_{ortho} \sim 7.0$–9.2 Hz, $J_{meta} \sim 1.1$–3.1 Hz, and $J_{para} \sim 0.0$–0.7 Hz.[22]

(22) J. W. Emsley, J. Feeney, and L. H. Sutcliffe, "High Resolution Nuclear Magnetic Resonance Spectroscopy," Vol. 2, Pergamon Press, Long Island City, N. Y., 1966, pp 770–773.

Scheme I. Aromatization and Subsequent Oxidation of Enol Ether **5**



data. The infrared spectrum of **24** exhibited an absorption band at 1680 cm^{-1}, but lacked absorption characteristic of the lactone carbonyl (~1715 cm^{-1}) and the secondary amide group (~1540 cm^{-1}, amide II), thus suggesting cleavage at the alanyl carbonyl and concomitant formation of a γ-lactam. The nmr spectrum of lactam **24**, which displayed *one* methyl doublet at δ 0.84 (J = 6.5 Hz) and a one-proton singlet at δ 8.17 assigned to the proton on the lactam nitrogen, and lacked a signal for the N-acetate methyl group, confirmed the loss of alanine and the proposed structure. Treatment of **24** with methyl iodide and potassium carbonate in acetone solution or with diazomethane led to the methyl ether **23**. Compound **23** was also formed from **22** by treatment with 0.1 N sodium methoxide for 0.5 hr.[23]

Although permanganate oxidation of the lactone **22** did not yield any identifiable products, the γ-lactam gave rise to a mixture of the known 3,6-dimethoxyphthalic anhydride (**25**) and 3,6-dimethoxyphthalimide (**26**). The sequence described in Scheme I confirms the 1,4 relationship of ketone and hydroxyl group in the carbocyclic ring, verifies the point of attachment of the lactone carbonyl to the carbocyclic ring, and is consistent with the presence of the second hydroxyl group at position 6 of N-acetylactinobolin. Expression **2** for N-acetylactinobolin is thus secured.

Absolute Configuration of Actinobolin

The focal point of the examination of configuration was the 100-MHz nmr spectra of N-acetylactinobolin (**2**) and its methyl enol ether derivative **5**. A detailed interpretation of these nmr spectra, coupled with a chemical degradation, not only provided strong confirmation for the structure proposed, but, in addition, made possible the assignment of the absolute configuration of actinobolin and suggested a preferred conformation for N-acetylactinobolin in solution.

The similarities between the nmr spectra of N-acetylactinobolin (**2**) and its enol ether **5** in deuterium oxide solution (Table III) suggested that the enol form of **2** is heavily favored in solution. This suggestion was strengthened by the one-proton singlet at δ 13.15 observed in the nmr spectrum of **2** in dimethyl sulfoxide

(23) The phthalimidine can be pictured as resulting from initial opening of the lactone ring by methoxide ion followed by an N → O acyl migration.[24] Because of favorable geometry γ-lactam formation occurs. Transesterification then yields the phthalimidine **24** and methyl N-acetylalanate.

(24) L. A. Cohen and B. Witkop in "Molecular Rearrangements," Vol. II, P. de Mayo, Ed., Interscience Publishers, New York, N. Y., 1963, Chapter 15.

Table III. Nmr Spectra of Compounds 2 and 5[a]

Proton (no.)[b]	Chemical shift[c] (multiplicity) 2	5	Pertinent coupling constants,[d] compd 2[e]
H-11 (3)	1.75 (d)	1.70(d)	$J_{3,11} = 6.5$
H-15 (3)	1.83 (d)	1.82 (d)	$J_{14,15} = 7$
H-18 (3)	2.45 (s)	2.44 (s)	
H-7_{ax}[f] (1)	2.85 (q of d)	2.87 (q of d)	$J_{7ax,7eq} = 18.5$; $J_{6,7ax} = 10$; $J_{7ax,10} = 2.5$
H-10 (1)	3.29 (d of t)[g,h]	3.29 (d of t)[h]	$J_{5,10} = 9.5$; $J_{4,10} = 3$ ($J_{7ax,10}$)
H-7_{eq}[f] (1)	3.35 (d of d)[g,h]	3.60 (d of d)[h]	$J_{6,7eq} = 6.5$ ($J_{7ax,7eq}$)
H-5 (1)	3.70 (t)	3.66 (t)	$J_{5,6} = 9.5$ ($J_{5,10}$)
H-6 (1)	4.34 (t of d)	4.30 (t of d)[i]	($J_{6,7ax}$; $J_{6,7eq}$; $J_{5,6}$)
H-14 (1)	4.77 (q)	4.76 (q)	($J_{14,15}$)
H-4 (1)	4.98 (d of d)	4.96 (d of d)	$J_{3,4} = 2$ ($J_{4,10}$)
H-3(1)	5.23 (q of d)[j]	5.16 (q of d)[j]	($J_{3,11}$; $J_{3,4}$)
H-19 (3)		4.32 (s)	

[a] Recorded at 100 MHz on a Varian Model HA-100 nmr spectrometer in D_2O solution at a concentration of 10%. H on nitrogen and oxygen exchanged for D. [b] Number of protons. [c] In δ units (parts per million) downfield of external TMS (capillary). [d] J values reported in hertz. [e] With the exception of $J_{6,7ax} = 9.5$, $J_{7ax,10} = 3$, and $J_{4,10} = 2.5$, the other J values for compound 5 are identical with those of 2. [f] ax = axial-like; eq = equatorial-like. [g] Signal partially obscured by overlap of signals of H-7_{eq} and H-10. [h] Broadening of peaks due to long-range homoallylic coupling between H-10 and H-7_{eq}; $J < 1$ Hz. [i] Signal partially obscured by overlap with OCH_3 (H-19) signal. [j] Signal partially obscured by HDO peak..

solution which immediately disappeared upon the addition of deuterium oxide.

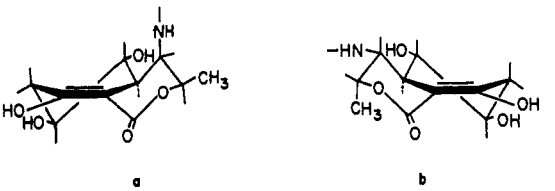
The mild permanganate oxidation of 2, which resulted in the isolation of L-alanine, L-threonine, and the dipeptide L-alanyl-L-threonine,[9] provided information concerning three of the six asymmetric centers present in actinobolin. The absolute configuration at C-3, C-4, and C-14 of 1 is thereby established as *R*, *R*, and *S*, respectively.

An examination of Dreiding stereomodels of the basic hexahydroisocoumarin skeleton of enolic actinobolin clearly suggests the double chair conformation shown in both a and b of Figure 2 to be most probable.[25] This conformation minimizes both angle strain and nonbonded interaction.

In the most favorable conformation, the configuration at C-3 and C-4, as required by their relationship to L-threonine, imposes an axial–equatorial relationship of substituents in both a and b of Figure 2. The small coupling constant of 2 Hz between H-3 and H-4 is consistent with this arrangement. As expected, H-3, being attached to carbon bearing ester oxygen, appears at lowest field.

The six-peak, one-proton signal centered at δ 4.34 and attributed to H-6 suggests spin coupling to three other protons. The large J values (9.5–10.0 Hz) of two of these interactions requires an H-5–H-6 axial–axial relationship. The appearance of the H-5 signal as a triplet ($J = 9.5$ Hz) is consistent with the assignment and the axial orientation of H-10 in Figure 2. Thus the nmr data require equatorial hydroxyl groups at C-5 and C-6, an arrangement in accord with both configuration a and b in Figure 2. It is the nature of the H-10 and H-4 signals that permits a choice to be made between the absolute configurations shown.

The H-10 signal appears as a doublet of triplets with two of the three proton–proton interactions displaying coupling constants of 3 Hz or less. The pattern confirms the H-10–H-5 axial–axial relationship but implicates an H-10–H-4 axial–equatorial relationship that is only compatible with configuration a of Figure 2.



a b

Figure 2. Possible configurations of actinobolin.

The long-range homoallylic coupling[26] with the C-7 axial proton is more clearly visible in the H-7_{ax} signal centered at δ 2.85 which, as expected, appears upfield of the C-7 equatorial proton signal. Homoallylic coupling between H-10 and H-7_{eq} is also evident, but only as slight broadening of the peaks of the H-7_{eq} signal, *i.e.*, $J_{7eq,10} = <1$ Hz.[27] The consequence of this coupling is more clearly seen as peak broadening in the signals for H-7_{eq} and H-10 of the enol ether 5. The axial–equatorial H-10–H-4 arrangement is also reflected in the signal for H-4, a doublet of doublets ($J_{4,10} = 3$ Hz, $J_{3,4} = 2$ Hz).

The spatial relationships deduced from the nmr spectrum of N-acetylactinobolin are best accommodated by the arrangement of atoms expressed as a of Figure 2. A close examination of the stereomodel of Figure 2b, however, revealed that if the lactone ring is converted to the more *mobile*, but energetically *less favorable* (eclipsing of substituents), and, therefore,

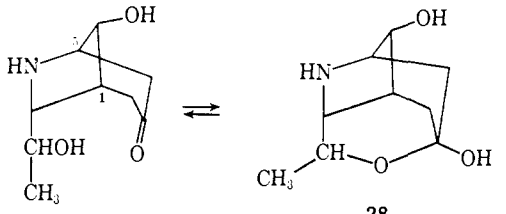
(25) (a) The basic hexahydroisocoumarin skeletons of a and b of Figure 2 (*i.e.*, no substituents) are enantiomerically related, differing in configuration at C-10. (b) The ring system in its most favorable conformation is relatively rigid. A double ring inversion (*e.g.*, as with *cis*-decalin) is not possible. In fact, chair–chair inversion of the lactone ring alone, which converts the C-5–C-10 bond to an axial-like position, leads to great strain in the system (the lactone ring can, however, assume a boat-like conformation as discussed later). The slightly deformed half-chair of the carbocyclic ring can undergo conformational inversion; however, as a consequence, eclipsing of substituents at C-5 and C-10 occurs that cannot be relieved by any favorable change in the shape of the lactone ring.

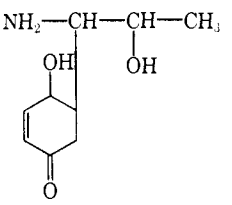
(26) N. S. Bhacca and D. H. Williams, "Applications of NMR Spectroscopy in Organic Chemistry," Holden-Day, Inc., San Francisco, Calif., 1964, pp 49–61.

(27) On a Jeolco 100-MHz nmr instrument, a measurable value for $J_{7e,10}$ of 0.75 Hz was obtained in the case of N-acetylactinobolin.

less likely, boat conformation, the nmr data could be accommodated. Specifically, the H-10–H-4 dihedral angle can be reduced from 180 to 120° without seriously altering other spatial relationships.

A review of the stereochemistry of actinobolamine (**27**)[4] led to decisive evidence in support of configuration a of Figure 2. Although no direct evidence is available, it is likely that under conditions of strong acidic hydrolysis actinobolamine arises *via* an intramolecular conjugate addition in the intermediate α,β-unsaturated ketone **29**, and is the energy well of the series of hydrolysis products.[28] The chemical nature of the C-10 carbon atom of actinobolin and the pathway by which **29** and **27** are formed suggest that the original configuration of C-10 is retained in both of these derivatives. The formation of L-threonine upon oxidation of actinobolamine confirms the retention of the configuration at centers C-3 and C-4 of actinobolin.[4] The independently established *R* configuration at C-1 of





29

actinobolamine[4,30] requires an *R* configuration at C-10 of actinobolin. Of the two configurations proposed in Figure 2, only a possesses an *R* center at C-10. The absolute configuration of actinobolin is thereby established as *R*, *R*, *R*, *R*, *R*, and *S* at C-3, C-4, C-5, C-6, C-10, and C-14, respectively.

Experimental Section

All melting points are corrected (unless otherwise noted) and were determined on a Thomas-Hoover capillary melting point apparatus. Infrared spectra were obtained on a Perkin-Elmer 237B Infracord as potassium bromide pellets and ultraviolet spectra on a Cary Model 14 spectrophotometer. Nuclear magnetic resonance spectra were determined in an appropriate solvent on a Varian Associates A-60 or HA-100 spectrometer; tetramethylsilane was used as either an external or as internal standard, and chemical shift values are reported in δ units. Field-sweep decoupling experiments at 60 MHz utilized a Varian Associates Model V-6058A spin decoupler. Rotations were determined on a Rudolf Model 80 polarimeter at the sodium D line. Mass spectra were obtained on an Atlas CH-4B mass spectrometer with a heated direct inlet system at an ionizing current of 19 μA and an ionizing energy of 70 eV. Thin-layer chromatographic (tlc) plates were prepared with Bio-Sil A silicic acid (10–30 μ) with 5% binder (purchased from Bio-Rad Laboratories) and with Merck aluminum oxide G (purchased from Brinkman Instruments, Inc.). Mallinckrodt ChromAR 500 sheets (silicic acid) were used for preparative tlc. The solvent systems for tlc and the methods for visualization are listed where used. Column chromatography separations were performed with Bio-Sil A silicic acid (100–200 mesh) (purchased from Bio-Rad Laboratories) and with neutral Woelm aluminum oxide (purchased from Alupharm Chemicals). Microanalyses were performed by Midwest Microlab, Inc., Indianapolis, Ind.

Preparation of N-Acetylactinobolin (2). A modification of the procedure of Haskell and Bartz[3] was followed. A solution of 9.00 g (12.2 mmol) of actinobolin sulfate dihydrate in 150 ml of water was passed over a column containing 130 ml of Bio-Rad AG 21-K ion-exchange resin (hydroxide form). The column was eluted with water until the eluent was neutral. This was followed by elution with 400 ml of 10% acetic acid (v/v). The acetic acid eluent was freeze dried. The amorphous residue remaining after freeze-drying was dissolved in 60 ml of ethanol and 30 ml of acetic anhydride, and the solution was magnetically stirred at room temperature for 24 hr. The slightly pink solid was filtered to yield 3.14 g of crude **2**. The filtrate was taken to dryness *in vacuo*, and the residue was crystallized from ethanol to yield an additional 1.68 g of **2**. The solid material was combined and recrystallized from ethanol to give 4.80 g (56%) of the crystalline N-acetylactinobolin (**2**): mp 254–255° dec; $[\alpha]^{26}D$ +30.0° (*c* 3.8, H_2O); $\lambda_{max}^{H_2O}$ 261 mμ (ϵ 9570), $\lambda_{max}^{0.1\ N\ HCl}$ 262 mμ (ϵ 9370), $\lambda_{max}^{0.1\ N\ NaOH}$ 288 mμ (ϵ 14,300); ν_{max} 1685 (enol lactone C=O), 1635 (amide C=O), 1610 (enol C=C), and 1555 cm^{-1} (amide II); nmr (D_2O) see Table III, (DMSO-d_6) δ 1.18 (3 H, d, J = 6.5, H-11), 1.21 (3 H, d, J = 7, H-15), 1.83 (3 H, s, H-18), 1.90–2.84 (3 H, m, H-7$_{ax}$, H-7$_{eq}$, H-10), 3.10 (1 H, t of d, J = 9 and 4, H-5), 3.33–3.92 (1 H, m, H-6), 4.17–4.72 (3 H, m, H-3, H-4, H-14), 4.77 (1 H, d, J = 4, CHO*H*), 5.05 (1 H, d, J = 4.5, CHO*H*), 7.76 (1 H, d, J = 9.5, H-12), 8.02 (1 H, d, J = 7, H-16), 13.15 (1 H, s, C=C—O*H*); mass spectrum *m/e* (relative intensity) 342 (11), 324 (11), 306 (2), 283 (4), 256 (6), 212 (93), 211 (36), 194 (81), 176 (49), 171 (82), 170 (73), 165 (67), 152 (47), 131 (94), 114 (100), 87 (67), 86 (67), 72 (54), 57 (93).

Preparation of N-Propionylactinobolin (3). A solution of 2.76 g (9.20 mmol) of actinobolin-free amine in 10 ml of ethanol and 6 ml of propionic anhydride was stirred at room temperature for 10 hr. Removal of solvent under high vacuum left a film which was dissolved in 25 ml of water and passed through a column containing 20 ml of Amberlite IR 120 ion-exchange resin (acid form). The water eluent was freeze dried and the resulting solid was crystallized from ethanol to give 1.13 g (34%) of N-propionylactinobolin (**3**), mp 200–201° dec. Recrystallization from ethanol gave an analytical sample: mp 200–201° dec; $[\alpha]^{25}D$ +29.9° (*c* 2.36, H_2O); $\lambda_{max}^{H_2O}$ 263 mμ (ϵ 9500), $\lambda_{max}^{0.1\ N\ NaOH}$ 287 mμ (ϵ 14,700); ν_{max} 3435, 3355, and 3325 (hydroxyl OH and amide NH), 1685 (enol lactone C=O), 1650–1625 (amide C=O), 1600 (enol C=C), 1535 (amide II), and 1215 cm^{-1} (lactone C–O); mass spectrum *m/e* (relative intensity) M + 1 = 357 (7), 356 (2), 338 (6), 320 (1), 297 (1), 256 (2), 145 (98), 128 (100), 101 (10), 100 (67), 57 (72).

Anal. Calcd for $C_{16}H_{24}N_2O_7$: C, 53.92; H, 6.79; mol wt, 356. Found: C, 54.04; H, 6.99; mol wt 356 (mass spectrum, M + 1 = 357).

Preparation of N-Acetyltri-O-acetylactinobolin (4). A solution of 700 mg (2.10 mmol) of **2** in 10 ml of acetic anhydride at 0° was degassed with nitrogen for 10 min. To the vigorously stirred, degassed solution was added 10 drops of 70% perchloric acid. The reaction was slowly allowed to warm up to room temperature while stirring was continued for 1.5 hr. The reaction mixture was extracted with 20 ml of methylene chloride. The organic layer was back-washed with water (two 10-ml portions) and the combined water solutions were freeze dried to yield 510 mg of unreacted starting material. The organic layer was then washed with 10 ml of a 10% sodium bicarbonate solution followed by 10 ml of water. After drying over magnesium sulfate the methylene chloride solution was filtered, reduced in volume under vacuum, and taken to dryness under high vacuum. The residue was chromatographed over 50 g of alumina (activity IV, acetic acid washed). Elution with a mixture of benzene–methanol (99:1, v/v) yielded 258 mg of a white powder which was crystallized from benzene to give 169 mg of crystalline **4**, mp 173–175°. Recrystallization from methylene chloride–methylcyclohexane yielded 162 mg (30% based on starting material consumed): mp 175–175.5°; $[\alpha]^{25}D$ +29.4° (*c* 3.8, CH_3OH); λ_{max}^{EtOH} 227 mμ (ϵ 8480); ν_{max} 1760 and 1750 (acetate C=O), 1690 (lactone C=O), 1670 (amide C=O), and 1535 cm^{-1} (amide II); nmr ($CDCl_3$), δ 1.92 (3 H, s, NHCOC*H*$_3$), 1.98 (3

(28) The pathways relating the various hydrolysis products of actinobolin are detailed in a manuscript accepted for publication.[8] An α,β-unsaturated ketone similar to **26** has been proposed as an intermediate in the formation of 6-benzyl-3-oxo-6-azabicyclo[3.2.1]octane.[29]

(29) W. J. Gensler, C. D. Gatsonis, and Q. A. Ahmed, *J. Org. Chem.*, **33**, 2968 (1968).

(30) In arriving at the absolute configuration of actinobolamine (**27**) it was shown that an *S* configuration at C-1 requires an *exo*-1-hydroxyethyl group. The ease of formation of the hemiketal **28** and its derivatives militates against such an assignment.

H, s, OCOCH_3), 2.02 (3 H, s, OCOCH_3), 2.18 (3 H, s, C=C—OCOCH_3).

Anal. Calcd for $C_{21}H_{28}N_2O_{10}$: C, 53.84; H, 6.02; N, 5.98; O, 34.16. Found: C, 53.98; H, 6.35; N, 5.99; O, 34.39.

Preparation of N-Acetylactinobolin Methyl Enol Ether (5). To a magnetically stirred ethereal solution of diazomethane[31] (~700 mg) in a 200-ml round-bottomed flask which was fitted with a Dry Ice condenser and drying tube was added a solution of 1.03 g (3.01 mmol) of **2** (freeze dried from water before using to enhance solubility) in 60 ml of ethanol. The mixture was stirred at room temperature for 3 hr, after which time the excess diazomethane was destroyed by the dropwise addition of acetic acid. The solvents were removed at reduced pressure, and the residue was crystallized from ethanol–methylcyclohexane to yield 683 mg (61%) of crystalline enol ether, mp 160–163°. Recrystallization from 95% ethanol raised the melting point to 161–163°; $[\alpha]^{26}D$ +29.2° (*c* 2.5, H_2O); $\lambda_{max}^{H_2O}$ 264 mμ (ε 10,950); ν_{max} 3565 and 3510 (OH), 3340 (NH), 1685 (enol lactone C=O), 1635 (amide C=O), 1585 (enol C=C), and 1550 cm^{-1} (amide II); nmr (D_2O) see Table III; mass spectrum *m/e* (relative intensity) 356 (4), 338 (2), 320 (1), 297 (35), 226 (37), 179 (100), 169 (98), 152 (25), 139 (26), 131 (15), 114 (46), 87 (14) 86 (38), 72 (8), 57 (51).

Anal. Calcd for $C_{16}H_{24}N_2O_7 \cdot H_2O$: C, 51.33; H, 7.00, N, 7.48; O, 34.19; mol wt, 356. Found: C, 51.54; H, 7.03; N, 7.50; O, 34.23; mol wt, 356 (mass spectrum).

Treatment of N-Acetylactinobolin with Hydrazine. Preparation of Pyrazolone (9). A solution of **2** (687 mg, 2.00 mmol), hydrazine (0.2 ml, 6.00 mmol), and 20 ml of methanol was immersed in an ice bath. After stirring for 12 hr, the solvent was removed *in vacuo* and the residue was chromatographed over 20 g of silicic acid, eluting with ethanol. Fractions containing the desired compound were combined to yield 721 mg of an amorphous, cream-colored solid. All attempts at crystallization failed. An analytical sample was prepared by precipitating the material twice from methanol–acetone solution, mp decomposes above 200°: λ_{max}^{EtOH} 246 (ε 2780), 228 mμ (ε 2380), $\lambda_{max}^{H_2O}$ 243 mμ (ε 6550), $\lambda_{max}^{0.1\ N\ HCl}$ 229 mμ (ε 4860), $\lambda_{max}^{0.1\ N\ NaOH}$ 236 mμ (ε 5330); nmr (D_2O) δ 1.20 (3 H, d, *J* = 6.5, H-12), 1.33 (3 H, d, *J* = 7, H-16), 1.92 (3 H, s, H-18), 2.46 (1 H, d of d, *J* = 15.5 and 9, H-7_{ax}), 2.72 (1 H, d of d, *J* = 9 and 2, H-4), 2.97 (1 H, d of d, *J* = 5 and 15.5, H-7_{eq}), 3.47 (1 H, t, *J* = 9, H-5), 3.84 (1 H, t of d, *J* = 9 and 5, H-6), 4.15 (1 H, q, *J* = 7, H-15), 4.17 (1 H, q of d, *J* = 4 and 6.5, H-11), 4.43 (1 H, d of d, *J* = 2 and 4, H-10); mass spectrum *m/e* (relative intensity) 356 (<1), 338 (28), 320 (7), 251 (13), 225 (33), 208 (56) 193 (27), 190 (27), 164 (21), 151 (24), 131 (98), 114 (100), 87 (72), 86 (79), 72 (29), 57 (72).

Anal. Calcd for $C_{15}H_{24}N_4O_6$: C, 50.55; H, 6.79; N, 15.73; O, 26.94; mol wt, 356. Found: C, 50.25; H, 7.47; N, 15.30; O, 27.04; mol wt, 356 (mass spectrum).

Acetylation of the N-Acetylactinobolin–Hydrazine Adduct. Preparation of (10). A solution of 320 mg (0.898 mmol) of **9**, 3 ml of pyridine and 2 ml of acetic anhydride was stirred at room temperature for 34 hr. The solvents were removed under high vacuum, and the residue was chromatographed over 25 g of silicic acid, eluting with ethyl acetate. The fractions homogeneous to tlc [silicic acid; ethyl acetate–ethanol (2:1, v/v); sulfuric acid char] were combined and crystallized from carbon tetrachloride to yield 264 mg (52%) of the white, crystalline hexaacetate derivative **10**, mp 128.5–131°. An analytical sample was recrystallized from benzene: mp 129.5–131°; $[\alpha]^{26}D$ −72.3° (*c* 3.18, CH_3OH); λ_{max}^{EtOH} 242 mμ (ε 10,800); ν_{max} 1775 (enol acetate C=O) 1740 (normal acetate C=O), 1650 (amide C=O), and 1530 cm^{-1} (amide II); nmr ($CDCl_3$) 1.15 (3 H, d, *J* = 6, H-12), 1.30 (3 H, d, *J* = 7, H-16), 2.02 (3 H, s, COCH_3), 2.12 (3 H, s, COCH_3), 2.12 (3 H, s, COCH_3), 2.15 (3 H, s, COCH_3), 2.52 (3 H, s, COCH_3), 2.62 (3 H, s, COCH_3), 3.07 (1 H, d of d, *J* = 10 and 2–3, H-4), 3.42 (2 H, d, *J* = 4, H-7), 4.60 (1 H, t, *J* = 10, H-5), 4.63 (1 H, quintet, *J* = 7, H-15), 5.25 (3 H, m, H-6, H-10, H-11), 6.16 (1 H, d, *J* = 7, H-17), 7.05 (1 H, d, *J* = 10, H-13); mass spectrum *m/e* (relative intensity) 566 (7), 524 (1), 404 (2), 396 (4), 336 (1), 316 (3), 306 (6), 264 (19), 229 (88), 204 (13), 193 (16), 176 (60), 169 (61), 151 (23), 134 (28), 117 (33), 116 (100), 114 (50), 87 (10), 86 (40), 74 (38), 57 (26).

Anal. Calcd for $C_{25}H_{34}N_4O_{11}$: C, 53.00; H, 6.05; N, 9.89; O, 31.06; mol wt, 566. Found: C, 53.21; H, 6.03; N, 9.72; O, 31.28; mol wt, 566 (mass spectrum).

Sodium Borohydride Reduction of 2. Isolation of Hydroxylactone (16). To a stirred solution of 1.00 g (2.92 mmol) of **2** in 50 ml of water was added dropwise over 0.5 hr a solution of 527 mg (13.8 mmol) of sodium borohydride in 25 ml of water. The mixture was stirred at room temperature for 11 hr after which the excess sodium borohydride was destroyed by the dropwise addition of acetic acid until the solution was neutral. The resulting solution was passed over a column containing 30 ml of Bio-Rad AG 50-WX8 ion-exchange resin (acid form). Elution with water and freeze drying the water eluent yielded a white, fluffy residue which was dissolved in 10 ml of methanol, and the methanol was removed *in vacuo*. The procedure with methanol was repeated two more times to yield 839 mg of a white, amorphous solid. This solid was chromatographed (dry loaded) over 70 g of silicic acid and eluted with mixtures of ethyl acetate–ethanol. The collected fractions beginning with those eluted with ethyl acetate–ethanol (4:1, v/v) were divided into three groups based on their tlc results [silicic acid; ethyl acetate–ethanol (2:1, v/v); sulfuric acid char]. The first group of fractions (62 mg) contained pure hydroxylactone **16**. These fractions were combined and crystallized from ethanol to yield 40 mg of white, crystalline **16**, mp 245–247° dec. The second group of fractions (225 mg) was a mixture of two components. Column chromatography as above of these mixed fractions yielded an additional 10 mg of crystalline **16**. The last groups of fractions which contained the bulk of the material appeared to be a mixture of isomers of totally reduced **2** and was not investigated further.

The total yield of **16** was 50 mg. Recrystallization from ethanol yielded an analytical sample: mp 247–248° dec; ν_{max} 1730 (lactone C=O), 1675 and 1640 (amide C=O), and 1530 and 1515 cm^{-1} (amide II); mass spectrum *m/e* (relative intensity) 344 (<1), 326 (8), 308 (2), 293 (12), 267 (65), 114 (61), 87 (100), 86 (88), 85 (45), 72 (43), 57 (54).

Anal. Calcd for $C_{15}H_{24}N_2O_7$: C, 52.32; H, 7.02; N, 8.14; O, 32.52; mol wt, 344. Found: C, 52.43; H, 7.15; N, 7.95, O, 32.71; mol wt, 344 (mass spectrum).

Periodate Oxidation of N-Acetylactinobolin (2) and the Hydroxylactone 16. A solution of 12 mg of compound and 10.0 ml of 0.0449 *M* sodium periodate was kept in the dark at 5°. At measured time intervals 1-ml aliquots were withdrawn, and the periodate uptake was determined by the standard procedure.[34] A blank was analyzed along with each unknown sample. Table IV summarizes the results.

Table IV. Periodate Oxidation of Compounds **2** and **16**

	Mol of periodate reduced	
Time, hr	**2**	**16**
1	3.64	0.94
2	3.26	1.03
3	3.52	
3.5		1.05
4.5	3.29	
9.5	3.46	
10		1.06
24	5.08	
75	5.35	

Detection of the Presence of a Lactone Moiety in 16. The procedure of Abdel-Ahker and Smith[15] was followed. Pure samples of N-acetylactinobolin (**2**), N-acetylalanylactinobolone (**8**), N-acetylalanylactinobicyclone (**7**), and hydroxylactone **16** were applied to two pieces of filter paper. One of the pieces of filter paper was sprayed with a 2% ferric chloride solution containing 1% hydrochloric acid; only **2** gave a positive test (a purple color). The other pieces of filter paper was sprayed first with a freshly prepared basic hydroxylamine solution—made by mixing equal portions of 1 *M* hydroxylamine hydrochloride in methanol and 1.1 *N* potassium hydroxide in methanol just before using. After standing for 10 min, the paper was sprayed with the ferric chloride solution; only

(31) Generated from "Diazald"[32] according to the procedure of T. J. deBoer and H. J. Becker.[33]

(32) Purchased from Aldrich Chemical Co., Milwaukee, Wis.

(33) (a) T. J. de Boer and H. J. Becker, *Recl. Trav. Chim. Pays-Bas*, **73**, 229 (1954); (b) Technical Information Sheet, D2800-O, Aldrich Chemical Co., Milwaukee, Wis., Jan 1967.

(34) R. D. Guthrie, *Methods Carbohyd. Chem.*, **1**, 435 (1962).

2 and **16** gave immediate positive hydroxamic acid tests (rust-colored spots). After approximately 3 hr, rust-colored spots began to appear for **7** and **8**.

Hydrogenation of N-Acetylactinobolin Methyl Enol Ether (5). A solution of 300 mg (0.0805 mmol) of **5** in 20 ml of ethanol was vigorously stirred in a hydrogen atmosphere in the presence of 90 mg of platinum oxide catalyst at room temperature for 48 hr. The mixture was then filtered through Celite, and the filtrate was taken to dryness *in vacuo* to yield 308 mg of an amorphous solid. The solid was chromatographed (dry loaded) over 20 g of silicic acid. Elution with ethyl acetate–ethanol (88:12, v/v) yielded 256 mg of crude dihydro enol ether **17**. Crystallization from ethanol–methylcyclohexane gave 141 mg (49%), mp 201–202°. Recrystallization yielded an analytical sample: mp 205–206.5°; ν_{max} 1740 and 1715 (lactone C=O), 1650 (amide C=O), 1540 (amide II), and 1235 and 1070 cm^{-1} (ester CO and ether CO, respectively); mass spectrum *m/e* (relative intensity) 358 (<1), 340 (<1), 326 (2), 308 (<1), 282 (6), 267 (100), 114 (43), 87 (54), 86 (47), 85 (33), 72 (22), 57 (31).

Anal. Calcd for $C_{16}H_{26}N_2O_7$: C, 53.62; H, 7.31; N, 7.82; O, 31.25; mol wt, 358. Found: C, 53.41; H, 7.45; N, 7.90; O, 31.53; mol wt, 358 (mass spectrum).

Preparation of the Isopropylidene of N-Acetylactinobolin (6). In a 500-ml round-bottomed flask fitted with a Soxhlet condenser containing 3A Molecular Sieves in the thimble was placed 1.19 g (3.48 mmol) of **2**, 250 ml of acetone, and 60 mg of *p*-toluenesulfonic acid monohydrate. The solution was refluxed for 22 hr, cooled, and passed onto a column containing 20 ml of Amberlite IR-45 weak anion-exchange resin. The eluent was reduced in volume *in vacuo* and taken to dryness under high vacuum. The resultant solid was triturated with cold acetone and filtered to give 657 mg of **2** identified by tlc [silicic acid; ethyl acetate–ethanol (2:1, v/v); sulfuric acid char]. The filtrate was loaded into a column containing 20 g of silicic acid and eluted with acetone. The first 125 ml of acetone contained 557 mg of the isopropylidene derivative **6**. Further elution gave an additional 13 mg of **2** to bring the yield of recovered **2** to 670 mg (1.96 mmol). The crude yield of **6** based on starting material utilized was 95%. Recrystallization from acetone followed by vacuum drying at 78° gave an analytical sample: mp 238–240° dec; $[\alpha]^{26}D$ +26.3° (*c* 3.6, CH_3OH); λ_{max}^{EtOH} 262 (ε 9000), $\lambda_{max}^{EtOH,OH^-}$ 287 mμ (ε 17,400); ν_{max} 3340, 3300–3250, and 3055 (amide NH), 1687 (enol lactone C=O), 1660–1625 (amide C=O), 1590 (enol C=C), 1525 (amide II), and 1390 and 1380 cm^{-1} (*gem*-dimethyl); nmr (acetone-d_6) δ 1.27 (3 H, d, *J* = 6.5, H-11), 1.33 (3 H, d, *J* = 7, H-15), 1.38 (6 H, s, $C(CH_3)_2$), 1.88 (3 H, s, H-18), 2.47 (1 H, q of d, *J* = 17.5, 10 and 2.5, H-7$_{ax}$), 2.90 (1 H, d of d, *J* = 17.5 and 6, H-7$_{eq}$), 3.07 (1 H, m, H-10), 3.45 (1 H, t, *J* = 9, H-5), 3.81 (1 H, t of d, *J* = 7, 9, and 10, H-6), 4.35 (1 H, quintet, *J* = 7, H-14), 4.5 (1 H, m, H-4), 4.77 (1 H, q of d, *J* = 2 and 6.5, H-3), 7.25–7.65 (2 H, m, H-12 and H-16), 13.70 (1 H, s, C=C—O*H*); mass spectrum *m/e* (relative intensity) 382 (3), 367 (27), 324 (30), 307 (8), 296 (4), 252 (6), 238 (12), 194 (51), 171 (100), 170 (64), 131 (35), 87 (65), 86 (73), 72 (25), 57 (77).

Anal. Calcd for $C_{18}H_{26}N_2O_7$: C, 56.53; H, 6.85; O, 29.29; mol wt, 382. Found: C, 56.71; H, 6.99; O, 29.13; mol wt, 382 (mass spectrum).

Acetylation of N-Acetylactinobolin Methyl Enol Ether. A solution of 215 mg (0.575 mmol) of **5**, 2 ml of pyridine, and 2 ml of acetic anhydride was stirred at room temperature for 34 hr. The solvents were removed under high vacuum, and the residue was dissolved in ethanol and decolorized with charcoal. Crystallization from ethyl acetate–methylcyclohexane yielded 190 mg (75%) of the cream-colored di-O-acetate derivative **18**, mp 143–145°. Recrystallization raised the melting point to 146–148°; ν_{max} 1745 (acetate C=O), 1690–1650 (C=O), 1585 (enol C=C), 1530 (amide II), and 1245–1210 cm^{-1} (acetate CO).

Preparation of 3-Methyl-4-(N-acetylalanyl)amino-5-O-acetyl-8-methoxy-3,4,6,7-tetrahydroisocoumarin (19). A solution of 300 mg (0.802 mmol) of **5**, 2 ml of pyridine, and 2 ml of acetic anhydride was stirred at room temperature for 24 hr. The solvents were removed under high vacuum, and the residue was chromatographed over 30 g of alumina (activity III). Elution with ethyl acetate–ethanol (98:2, v/v) gave a yellow powder which could be crystallized from 95% ethanol to yield 226 mg (76%) of **19**, mp 210–211° dec. Recrystallization from 95% ethanol yielded an analytical sample: mp 212–212.5° dec; $[\alpha]^{25}D$ +245° (*c* 3.45, CH_3OH); λ_{max}^{EtOH} 320 (ε 5800), 235 mμ (ε 3200); ν_{max} 3570 and 3495 (water OH), 3285 and 3055 (amide NH), 1750 (acetate C=O), 1690–1625 (C=O), 1540 (amide II), and 1225 cm^{-1} (acetate CO); nmr (DMSO-d_6) δ 1.02 (6 H, d, *J* = 6.5–7, H-11 and H-15), 1.73 (3 H, s, H-18), 1.92 (3 H, s, OCOC*H*$_3$), 3.30 (1 H, d of d, *J* = 5 and 17, H-10), 3.75 (3 H, s, OC*H*$_3$), 4.40–4.67 (3 H, m, H-3, H-4, and H-14), 5.20 (1 H, d, *J* = 17, H-5), 6.46 (2 H, s, H-6 and H-7), 7.87 (1 H, d, *J* = 7, H-16), 8.02 (1 H, d, *J* = 10, H-12); mass spectrum *m/e* (relative intensity) M + 1 = 381 (11), 363 (5), 234 (79), 223 (18), 208 (78), 207 (55), 193 (79), 192 (100), 191 (73), 190 (54), 189 (54), 177 (56), 170 (76), 163 (78), 151 (57), 148 (48), 135 (65), 114 (51), 87 (36), 86 (43), 77 (28), 74 (44), 72 (29), 65 (15), 57 (74).

Anal. Calcd for $C_{18}H_{24}N_2O_7 \cdot {}^1/_2H_2O$: C, 55.82; H, 6.47; N, 7.19; O, 30.82. Found: C, 55.61 (55.57); H, 6.53 (6.71); N, 7.19 (7.05); O, 30.54 (30.82).

Preparation of 3-Methyl-4-(N-acetylalanyl)amino-5-O-acetyl-8-methoxy-3,4-dihydroisocoumarin (20). To a 20-ml round-bottomed flask equipped with a condenser and drying tube was added 117 mg (0.300 mmol) of **19**, 85 mg (0.375 mmol) of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, and 5 ml of dioxane. After refluxing for 22 hr the mixture was cooled, and the solvent was removed *in vacuo*. The residue was chromatographed over 7.5 g of alumina (activity III). Elution was carried out by collecting 20-ml fractions of ethyl acetate–ethanol (1:1, v/v) as fast as possible to minimize hydrolysis of the aromatic acetate. Removal of solvents *in vacuo* yielded 111 mg of a white solid which was crystallized from acetone to give 77 mg (68%) of crystalline **20**, mp 241–243°. An elemental analysis could not be obtained due to the ease of hydrolysis of the aromatic acetate: λ_{max}^{EtOH} 305 (ε 4700), 246 (ε 7400), 212 mμ (ε 28,000); nmr (DMSO-d_6) δ 0.99 (3 H, d, *J* = 7, H-15), 1.10 (3 H, d, *J* = 6.5, H-11), 1.73 (3 H, s, H-18), 2.10 (3 H, s, H-19), 3.75 (3 H, s, H-20), 4.14 (1 H, quintet, *J* = 7, H-14), 4.56 (1 H, q of d, *J* = 2.5 and 6.5, H-3), 5.05 (1 H, d of d, *J* = 2.5 and 9.5, H-4), 7.13 and 7.35 (2 H, AB q, *J* = 9, H-6, H-7), 7.90 (1 H, d, *J* = 7, H-16), 8.45 (1 H, d, *J* = 9.5, H-12); spin-decoupling studies (signal irradiated, signal observed, multiplicity change) H-14, H-15, d → s; H-3, H-11, d → s; H-11, H-3, q of d → d (*J* = 2.5 Hz); H-12, H-4, d of d → d (*J* = 2.5 Hz); H-14, H-16, d → s; H-4, H-12, d → s.

Preparation of 3-Methyl-4-(N-acetylalanyl)amino-5-hydroxy-8-methoxy-3,4-dihydroisocoumarin (21). A solution of 178 mg (0.471 mmol) of **20** and 5.5 ml of 0.1 *N* sodium methoxide in methanol was stirred at room temperature 15 min. The yellow reaction mixture was passed over a column containing 17 ml of Amberlite IR 120 exchange resin (acid form, methanol washed) and eluted with 150 ml of methanol. Removal of solvent *in vacuo* yielded 159 mg of a white solid which was crystallized from methanol to give 90 mg (57%) of crystalline **21**: mp 282–284° dec (uncorrected); λ_{max}^{EtOH} 337 (ε 5650), 240 (ε 5000), 218 (ε 18,800), 203 mμ (ε 21,400); ν_{max} 1715 (lactone C=O), 1680 and 1645 (amide C=O), 1590, and 1500 cm^{-1}; mass spectrum *m/e* (relative intensity) 336 (100), 318 (4), 294 (1), 250 (31), 224 (35), 223 (76), 222 (46), 208 (70), 207 (35), 206 (93), 205 (31), 204 (18), 179 (42), 177 (35), 164 (30), 114 (27), 91 (5), 87 (20), 86 (22), 77 (7), 72 (12), 65 (7).

Anal. Calcd for $C_{16}H_{20}N_2O_6$: C, 57.13; H, 5.99; N, 8.33; O, 28.54; mol wt, 336. Found: C, 57.39; H, 6.22; N, 8.37. O, 28.69; mol wt, 336 (mass spectrum).

Preparation of 3-Methyl-4-(N-acetylalanyl)amino-5,8-dimethoxy-3,4-dihydroisocoumarin (22). To a solution of 100 mg (0.298 mmol) of **21** in 20 ml of methanol was added an ethereal solution of diazomethane[31] (20 ml, 140 mg). The reaction flask was fitted with a Dry Ice condenser and drying tube. After stirring for 1 hr, the yellow color of diazomethane was gone; however, tlc (silicic acid; ethyl acetate–ethanol (2:1, v/v); sulfuric acid char] indicated a large amount of starting material was still present. An additional 140 mg of diazomethane was added. After an additional 1.5 hr, the solution was again colorless, and tlc indicated only a small amount of starting material. The solvents were removed *in vacuo* and the residue was chromatographed (dry loaded) over 10 g of silicic acid. Elution began with ethyl acetate and proceeded to mixtures of ethyl acetate–ethanol. The desired material came off in the fractions eluted with ethyl acetate–ethanol (95:5, v/v). These fractions were combined and crystallized from ethyl acetate to yield 67 mg (64%) of crystalline **22**, mp 248–249.5° An analytical sample was recrystallized from ethyl acetate followed by acetone to a constant melting point of 248.5–250;[9] $[\alpha]^{25}D$ −190° (*c* 3.37, 95% EtOH); λ_{max}^{EtOH} 333 (ε 5560), 237 (ε 7000), 218 (ε 21,500), and 203 mμ (ε 24,600); ν_{max} 3300 (amide NH), 1720 (lactone C=O), and 1635 cm^{-1} (amide C=O); nmr (DMSO-d_6) δ 1.03 (3 H, d, *J* = 7, H-15), 1.13 (3 H, d, *J* = 6.5, H-11), 1.77 (3 H, s, NHCOC*H*$_3$), 3.67 (3 H, s, OC*H*$_3$), 3.73 (3 H, s, OC*H*$_3$), 4.20 (1 H, quintet, *J* = 7. H-14), 4.53 (1 H, q of d, *J* = 2.5 and 6.5, H-3), 5.13 (1 H, d of d, *J* = 2.5 and 9, H-4), 7.06 and 7.24 (2 H, AB q, *J* = 9. H-6 and H-7), 7.92 (1 H, d, *J* = 7, H-16), 8.17 (1 H, d, *J* = 9, H-12); mass spectrum *m/e* (relative intensity) 350 (24), 291 (7), 264 (39), 237 (64),

222 (97), 221 (76), 220 (42), 219 (100), 208 (76), 207 (81), 193 (89), 178 (77), 91 (7), 87 (34), 86 (32), 77 (11), 72 (23), 65 (6).

Anal. Calcd for $C_{17}H_{22}N_2O_6$: C, 58.27; H, 6.34; N, 8.00; O, 27.40; mol wt, 350. Found: C, 58.42; H, 6.60; N, 8.26; O, 27.62; mol wt, 350 (mass spectrum).

Preparation of 3-(1′-Hydroxyethyl)-4-hydroxy-7-methoxyphthalimidine (24). A solution of 707 mg (1.87 mmol) of **20** in 50 ml of 0.1 *N* sodium methoxide was stirred at room temperature for 30 hr. The yellow solution was then passed over a column containing 20 ml of Amberlite IR 120 ion-exchange resin (acid form, methanol washed) and eluted with 50-ml fractions of methanol. The first two fractions, after removal of solvent, yielded 616 mg of an oil. These two fractions were combined and crystallized from ethyl acetate to give 364 mg of crystalline **24**, mp 210–211°.

The filtrate was combined with the material obtained from fractions 3–6 and chromatographed over 14 g of silicic acid. Eluting with ethyl acetate yielded 250 mg (92%) of a clear oil which proved to be L-methyl N-acetylalanate **29**: $[\alpha]^{25}D$ −23.1° (*c* 4.42, H_2O) [lit.[35] $[\alpha]^{23}D$ −91.7 (*c* 2, H_2O)]; $n^{26}D$ 1.4504 [lit.[36] $n^{20}D$ 1.4510]; ν_{max}^{neat} 3280 and 3070 (amide NH), 1745 (ester C=O), 1655 (amide C=O), 1540 (amide II), and 1220 cm^{-1} (ester C—O); nmr ($CDCl_3$) δ 1.42 (3 H, d, J = 7, $CHCH_3$), 2.05 (3 H, s, $NHCOCH_3$), 3.78 (3 H, s, $COOCH_3$), 4.62 (1 H, quintet, J = 7, $NHCHCH_3$), 6.41–6.84 (1 H, broad hump, NH).

Continued elution with ethyl acetate–ethanol (97:3, v/v) yielded an additional 12 mg of **24** bringing the total yield of **24** to 376 mg (90%). An analytical sample was obtained upon recrystallization from ethyl acetate: mp 214.5–216.5°; $[\alpha]^{25}D$ +64.9° (*c* 2.01, EtOH); λ_{max}^{EtOH} 317 (ε 5300), 237 (ε 6200), 215 (ε 22,200), and 202 mμ (ε 15,800); ν_{max} 1680 (lactam C=O), 1612 and 1505 cm^{-1}; nmr (DMSO-d_6) δ 0.84 (3 H, d, J = 6.5, $CHCH_3$), 3.00–3.58 (1 H, broad s, OH), 3.68 (3 H, s, OCH_3), 4.22 (1 H, q of d, J = 3 and 6.5, $CHCH_3$), 4.45 (1 H, d, J = 3, NHCHCH), 6.81 and 6.87 (2 H, AB q, J = 9.5, aromatic), 7.17–8.00 (1 H, broad s, aromatic OH), 8.17 (1 H, s, NH); mass spectrum *m/e* (relative intensity) 223 (33), 179 (98), 178 (100), 164 (30), 148 (20), 107 (10).

Anal. Calcd for $C_{11}H_{13}NO_4$: C, 59.18; H, 5.87; N, 6.28; O, 28.67; mol wt, 223. Found: C 59.15; H, 5.67; N, 6.32; O, 28.68; mol wt, 223 (mass specrum).

Preparation of 3-(1′-Hydroxyethyl)-4,7-dimethoxyphthalimidine (23). Method A. To a solution of 376 mg (1.69 mmol) of **24** in 120 ml of acetone was added 500 mg of potassium carbonate and 6 ml of methyl iodide. The reaction flask was wrapped with aluminum foil to exclude light, and the mixture was vigorously stirred for 49 hr at room temperature. After filtering, the slightly yellow filtrate was taken to dryness *in vacuo* and the residue was crystallized from ethanol–ethyl acetate to yield 221 mg of a cream-colored solid, mp 188–190°. Recrystallization from ethanol–ethyl acetate yielded 192 mg of white, crystalline **23**, mp 199–201°.

The mother liquors were combined and chromatographed (dry loaded) over 15 g of silicic acid. Elution wth ethyl acetate–ethanol (96:4, v/v) yielded 115 mg of crude **23**. Crystallization from ethyl acetate yielded 69 mg of **23**, mp 199–201°, which brought the total yield of **23** to 261 mg (65%).

An analytical sample was obtained by recrystallization to constant melting point from ethyl acetate: mp 200–201.5°; $[\alpha]^{25}D$ −115° (*c* 1.74, CH_3OH); λ_{max}^{EtOH} 313 (ε 5330), 234 (ε 7200), 214 (ε 25,900), 212 (ε 25,500), and 203 mμ (ε 20,200); ν_{max} 2830 (aromatic OCH_3) and 1690 cm^{-1} (lactam C=O); nmr (CF_3CO_2H) δ 0.95 (3 H, d, J = 6.5, $CHCH_3$), 3.53 (6 H, s, OCH_3), 4.46 (1 H, q of d, J = 2 and 6.5, $CHCH_3$), 4.60 (1 H, d, J = 2, NHCHCH), 6.68 and 6.92 (2 H, AB q, J = 9, aromatic), 8.17–8.93 (1 H, broad s, NH); mass spectrum *m/e* (relative intensity) 237 (10), 207 (2), 193 (79), 192 (100), 178 (18), 177 (10), 174 (12), 164 (13), 163 (11), 162 (16), 132 (3).

Anal. Calcd for $C_{12}H_{15}NO_4$: C, 60.75; H, 6.37; N, 5.90; O, 26.97; mol wt, 237. Found: C, 60.51; H, 6.38; N, 5.75; O, 27.20; mol wt, 237 (mass spectrum)

Method B. To a solution of 112 mg (0.502 mmol) of **24** in 40 ml of ethanol was added 250 mg of diazomethane[31] in 25 ml of ether. The reaction flask was fitted with a Dry Ice condenser with a drying tube, and the mixture was stirred at room temperature for 2 hr. The excess diazomethane was destroyed by the dropwise addition of acetic acid. The solvents were removed *in vacuo* and the residue was crystallized from acetone–ethyl acetate to give 100 mg of crude **23**, mp 191–194°. Recrystallization from ethyl acetate yielded 75 mg (63%) of **23**, mp 197–199°. The material prepared in this manner was identical with that prepared by method A.

Methanolysis of 22. A solution of 5.8 mg of **22** in 1 ml of 0.1 *N* sodium methoxide was stirred at room temperature. Periodically the reaction was examined *via* tlc [alumina; ethyl acetate–ethanol (2:1, v/v); sulfuric acid char]. After 0.5 hr, the major product was **23** (R_f 0.45) with a small trace of **22** still present. With time, **23** appeared to be converted to a new compound (R_f 0.23). After 24 hr, tlc indicated a mixture of **23** and the unknown compound. Further reaction with sodium methoxide had no effect.

Permanganate Oxidation of 3-(1′-Hydroxyethyl)-4,7-dimethoxyphthalimidine (23). To a stirred solution of 262 mg (1.10 mmol) of **23** in 75 ml of water was added 540 mg of potassium permanganate in small portions over 1.5 hr. After the addition was complete, another 25 ml of water was added, and the purple reaction mixture was stirred at room temperature. After 9 hr, small needles were visible. At this time, the reaction mixture was heated on a steam bath for 10 min and allowed to cool to room temperature. This procedure was repeated after 10 hr. After 11 hr, the excess potassium permanganate and the manganese dioxide were destroyed by the addition of hydrochloric acid and sodium bisulfite. The resulting yellow solution was filtered to yield 65 mg of a yellow solid. The filtrate was extracted with ethyl acetate (three 50-ml portions). The combined organic extracts were dried over magnesium sulfate, filtered, and evaporated to dryness *in vacuo* to yield 64 mg of yellow solid. The two solids were identical *via* tlc [silicic acid; chloroform; sulfuric acid char] and were combined.

The combined solids were chromatographed (dry loaded) over 10 g of silicic acid and eluted with 20-ml fractions of chloroform. Fractions 2–4 came off as a blue fluorescent band which yielded 33 mg of a yellow solid upon evaporation of solvent. The solid material was combined and crystallized from acetone to give 25 mg of 3,6-dimethoxyphthalic anhydride (**25**) as bright yellow needles: mp 261–262° [lit.[37] mp 261–262°]; λ_{max}^{EtOH} 375 (ε 3350), 250 (ε 5490), 238 (ε 16,000), and 210 mμ (ε 18,100); ν_{max} 1830 and 1770 cm^{-1} (anhydride C=O); nmr (DMSO-d_6) δ 3.88 (6 H, s, OCH_3) 7.55, (2 H, s, aromatic); mass spectrum *m/e* (relative intensity) M = 208 (100), 193 (7), 190 (36), 179 (45), 178 (38), 163 (60), 162 (68), 161 (57), 150 (45), 134 (45), 133 (43), 106 (24), 105 (23), 78 (41), 76 (40).

Upon continued elution with chloroform, fractions 7–13 came off as yellow band and were combined. Evaporation of solvent *in vacuo* yielded 77 mg of a yellow solid which was crystallized from acetone to give 46 mg of 3,6-dimethoxyphthalimide (**26**): mp 251.5–253.5° [lit.[38] mp *ca.* 200°]; λ_{max}^{EtOH} 372 (ε 6440), 227 (ε 24,200), 221 mμ (ε 25,800); ν_{max} 3175 (imide NH), 1775, 1725, and 1705 cm^{-1} (phthalimide C=O); nmr (DMSO-d_6) δ 3.73 (6 H, s, OCH_3), 7.23 (2 H, s, aromatic), 10.83 (1 H, s, NH); the signal at δ 10.83 disappeared immediately upon the addition of D_2O; mass spectrum *m/e* (relative intensity) 207 (30), 206 (7), 192 (20), 190 (18), 189 (26), 188 (52), 179 (39), 178 (92), 175 (29), 174 (31), 163 (21), 162 (57), 161 (69), 160 (100), 135 (46), 134 (33), 133 (51), 132 (65), 131 (37), 108 (20), 107 (25), 106 (46), 91 (8), 78 (24), 77 (29), 76 (31), 75 (24).

Anal. Calcd for $C_{10}H_9NO_4$: C, 57.97; H, 4.38; N, 6.76; O, 30.89; mol wt, 207. Found: C, 57.75; H, 4.49; N, 6.83; O, 30.96; mol wt, 207 (mass spectrum).

Permanganate Oxidation of N-Acetylactinobolin (2).[39] A solution of 344 mg (1.00 mmol) of **2** in 20 ml of water was heated at 80° and a 0.5 *M* potassium permanganate solution was added with stirring until it took 2–3 min to decolorize. The total amount added was 16 ml. The cooled solution was filtered through Celite and rinsed with water. To the slightly alkaline filtrate was added solid Dowex 50W X 8 ion-exchange resin (acid form) and rinsed with water. The acidic eluent was concentrated *in vacuo* and the residual oil dissolved in 10 ml of 3 *N* sulfuric acid and heated on a steam bath for 5 hr. The hot solution was then neutralized with warm barium hydroxide, and the barium sulfate was removed by filtration through Celite. The filtrate and washings were concentrated *in vacuo* to a volume of 20 ml, and the resulting solution (pH 5.8) was passed over a column containing 8 ml of ZeoRex ion-exchange resin (acid form) and rinsed with water. The column was then eluted with 0.15 *M* ammonium hydroxide, and the ninhydrin-positive fractions

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were examined by paper electrophoresis and chromatography. One band corresponding to neutral amino acids was obtained by electrophoresis in 0.5 *M* pyridine acetate (pH 5.0) and two to three zones by paper chromatography in *t*-butyl alcohol, acetic acid, water (2:1:1, v/v/v), R_f 0.48 (threonine), 0.56 (alanine), and 0.64 (unknown). Six fractions (*ca.* 8 ml) were collected from the column. The first four contained only the threonine–alanine zones, and these were combined and concentrated to dryness *in vacuo* affording 124 mg of solid. The last two fractions afforded 44 mg of solid upon evaporation (alanine and unknown).

A. Isolation of Alanine and Threonine. The 124-mg sample was dissolved in 1 ml of water and applied to a Whatman 3M large paper sheet. The sheet was developed overnight with *sec*-butyl alcohol–4% aqueous ammonia (5:1, v/v), dried, and redeveloped again with the same solvent. Guide strips were cut, and the zones corresponding to alanine and threonine were cut horizontally and eluted with water. The fraction corresponding to threonine (51 mg) was passed over a small Dowex 50W X 8 ion-exchange resin (acid form) column, eluted with 0.2 *N* ammonium hydroxide, and the eluent concentrated to dryness. The residue on trituration with ethanol and acetone afforded pure L-threonine, $[\alpha]^{29}D$ −28.6 (*c* 1.0, H_2O) [lit.[40] $[\alpha]^{26}D$ −28.3° (*c* 1.1, H_2O)].

The alanine fraction (47 mg) was treated in an identical manner and afforded pure L-alanine, $[\alpha]^{29}D$ +15° (*c* 2.0, 1 *N* HCl) [lit.[41] $[\alpha]^{23}D$ +14.4 (*c* 6.5, 1.0 *N* HCl)].

B. Isolation of the Dipeptide Alanylthreonine (R_f = 0.64 Zone). The 44 mg from the final two tubes were dissolved in water and applied to two Whatman 3M paper sheets and developed overnight with *t*-butyl alcohol–acetic acid–water (2:1:1, v/v/v). Guide strips were cut and the faster zone was eluted with water and concentrated to dryness affording 10 mg of chromatographically homogeneous product (R_f 0.64). The product was purified on Dowex 50WX8 ion-exchange resin as above and afforded 8 mg of hydroscopic solid, pK_a = 3.6 and 8.0. Its approximate molecular weight was 240. (The calculated molecular weight of alanylthreonine is 289.) The product on further hydrolysis (3 *N* sulfuric acid) showed two zones corresponding to threonine and alanine (R_f 0.49 and 0.57, respectively) by paper chromatography in the *t*-butyl alcohol system.

Preparation of 2,4-Dinitrophenylactinobolin. The procedure of Cowgill and Pardee[42] was followed. To a solution of 220 mg (0.734 mmol) of **1** in 15 ml of a saturated sodium bicarbonate solution was added 160 mg (0.860 mmol) of 2,4-dinitrofluorobenzene in 15 ml of ethanol. The mixture was vigorously stirred for 1.5 hr at room temperature. The ethanol was removed *in vacuo*, and the yellow suspension was dissolved by the addition of 50 ml of water. The aqueous yellow solution was extracted with three 50-ml portions of ethyl acetate. The combined extracts were dried over magnesium sulfate, filtered, and taken to dryness *in vacuo* to yield 250 mg of a yellow residue. The yellow residue was precipitated from ethanol–methylcyclohexane to yield 170 mg of a yellow solid which was composed of two components *via* tlc [silicic acid; chloroform–benzyl alcohol–acetic acid (20:30:3, v/v/v)] R_f 0.10 (minor) and R_f 0.33 (major). A portion of this material (107 mg) was recrystallized from acetone–petroleum ether (30–60°) to yield 42 mg of 2,4-dinitrophenylactinobolin (R_f 0.33): mp 253–256° dec (uncorrected); ν_{max} 3375, 3320, 3110, and 3080 (amide NH and hydroxyl OH), 1690 (enol lactone C=O), 1655 (amide C=O), 1425 and 1335 (nitro NO), and 1230 cm^{-1} (lactone CO). Recrystallization from ethanol gave an analytical sample 260–261° dec (uncorrected).

Anal. Calcd for $C_{19}H_{22}N_4O_{10}$: C, 48.93; H, 4.75; N, 12.01. Found: C, 48.71; H, 4.89; N, 11.91.

Acid Hydrolysis of 2,4-Dinitrophenylactinobolin. A solution of 34 mg (0.073 mmol) of 2,4-dinitrophenylactinobolin in 10 ml of 4 *N* sulfuric acid was refluxed for 11 hr, cooled, diluted with 20 ml of water, and extracted with three 25-ml portions of ether (all of the yellow color goes into the ether layer). The combined extracts were dried over magnesium sulfate, filtered, and taken to dryness *in vacuo* to give 21 mg of a yellow oil. Crystallization from ethanol–water yielded 10 mg (54%) of 2,4-dinitrophenylalanine, mp 175–177° [lit.[43] mp 177°]. A mixture melting point with an authentic sample was undepressed, and the infrared spectrum was identical with that of the authentic sample.

Acknowledgment. Without the generous supply of antinobolin provided by Parke, Davis and Co., and their enthusiastic cooperation throughout the course of this work, its successful completion could not have been achieved. The authors are especially pleased to recognize the sustained interest and many important contributions of Dr. T. H. Haskell of Parke, Davis and Co.

Helpful discussion with Professor F. A. L. Anet of the University of California, Los Angeles, Calif., about the nmr spectra and with Professor Peter Brown of Arizona State University about the mass spectra is gratefully acknowledged. We are also grateful to Miss Katie Reimer (Arizona State University) for the 60-MHz nmr spectra, Dr. Victor Thalacker (University of Arizona) and Mr. William C. Jankowski (Varian Associates) for the 100-MHz nmr spectra, and Mr. Edwin Bebee (Arizona State University) for the mass spectra. Purchase of the Atlas CH4B mass spectrometer was made possible through Grant No. GB-4939 from the National Science Foundation.

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