Actinobolin. I. Structure of Actinobolamine[1]

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Abstract: The structure elucidation of actinobolamine, the basic fragment derived from the acid hydrolysis of the intact antibiotic, is described. The N-acetate of the free base was shown to possess a tertiary amide unit, $-HC(CHOHCH_3)N(Ac)CH<$, a ketone carbonyl, flanked on either side by a methylene group, which entered into facile hemiketal formation with the hydroxyl group of the hydroxyethyl side chain, an additional secondary alcohol unit, *i.e.*, $>$CHOH, and a $\gg$CH unit. Inspection of the chemical and spectral (infrared, nmr, and mass) properties of actinobolamine and its derivatives reveals expression **1** for the actinobolamine molecule.

The preliminary characterization of the antibiotic actinobolin was first described by Haskell and Bartz.[3] The compound, isolated from an antibiotic beer cultured by an actinomycete designated *Streptomycetes griseoviridus* var. *atrofaciens*, possesses broad spectrum antimicrobial activity and has demonstrated some value as a chemotherapeutic agent in certain types of neoplastic diseases.[4] Because of these properties, its unusually low toxicity,[3] and current interest in its mode of action,[5] an examination of the unique arrangement of atoms in space that characterizes actinobolin appeared timely.

While not large in comparison to some biosynthetic substances, the actinobolin molecule, $C_{13}H_{20}N_2O_6$,[6] was still too substantial in size and complexity to reveal its molecular array merely upon inspection of its simple chemical and spectral properties. Disruption of the molecule into smaller, more readily characterized fragments was indicated. Vigorous acid hydrolysis of the crystalline sulfate salt of the intact antibiotic with refluxing 4 *N* sulfuric acid gives rise to a fragment containing nine of the original 13 carbon atoms. The chemistry and structure elucidation of this optically active, amorphous, but homogeneous free base, designated actinobolamine (**1**), comprises the subject of this report.

The presence of N–H and/or O–H and an unconjugated, undistorted carbonyl group in the actinobolamine molecule is indicated by its infrared absorption spectrum ($\nu_{C=O}$ 1715 cm^{-1}). Upon treatment with ethanolic acetic anhydride, conditions designed to acetylate amine nitrogen but suppress acetylation of hydroxyl oxygen, the amorphous free base is converted to a crystalline N-acetate, N-acetylactinobolamine, $C_{11}H_{17}NO_4$,[6b] thus establishing the molecular formula $C_9H_{15}NO_3$ for the free base, actinobolamine. This crystalline N-acetate provides the point of departure for the structural studies.

The partial structure of N-acetylactinobolamine (**2**), shown at the left in Scheme I as a mobile equilibrium between a hydroxy ketone and its cyclic hemiketal, is derived on the basis of the following evidence. Acylation of the N-acetate **2** with *p*-nitrobenzoyl chloride gives rise to a diester **3** whose infrared spectrum indicates the absence of bands characteristic of N–H and O–H; therefore the presence of a tertiary amide and *two* and *only* two hydroxyl groups is disclosed. The infrared absorption spectrum of the N-acetate **2** supports the assignment of a tertiary amide ($\nu_{C=O}$ 1615 cm^{-1}), but, in contrast to the free base **1**, the band at 1715 cm^{-1} is absent. It is this observation that suggests the presence of ketone carbonyl (evidence described later rules out an aldehyde group) in N-acetylactinobolamine (**2**) that is masked as a cyclic hemiketal. Such an assignment is confirmed in a number of ways: (1) sodium borohydride reduction to the triol **4**; (2) formation of the cyclic dithioketal **5**, a derivative of the carbonyl-containing component of the equilibrium, **2a**; and (3) formation of the cyclic methyl ketal **6**, a derivative of the hemiketal-containing component of the equilibrium, **2b**. Both the dithioketal **5** and cyclic methyl ketal **6** can be reconverted to N-acetylactinobolamine (**2**), thus precluding the occurence of any skeletal rearrangement during their formation. Diborane reduction of N-acetylactinobolamine methyl ketal (**6**) followed by acid hydrolysis provides a route to N-ethylactinobolamine (**8**), the infrared spectrum of which displays ketone carbonyl absorption ($\nu_{C=O}$ 1720 cm^{-1}), again, in contrast to the N-acetyl compound **2**.

Base-catalyzed deuterium exchange studies, monitored by nmr, disclosed the nature of the environment about the carbonyl group. Addition of sodium deuterioxide to an nmr sample tube containing N-acetylactinobolamine (**2**) in deuterium oxide effects the loss of four protons from an original total of seven protons in the poorly resolved δ 1.6–2.4[7] region. After 1 hr, only the sharp, three-proton singlet (δ 2.15), assigned to the N-acetyl methyl protons, remains in that region.

(1) (a) Support of this work by the National Institutes of Health through Research Grant AI-04720 is gratefully acknowledged. (b) This paper is based in large part on the Ph.D. Dissertation of C. S. Sodano, Arizona State University, 1967. (c) A portion of the work described was presented before the Organic Division at the 152nd National Meeting of the American Chemical Society, New York, N. Y., Sept 1966, p 72S.

(2) Research Laboratories, Parke, Davis and Co., Ann Arbor, Mich.

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

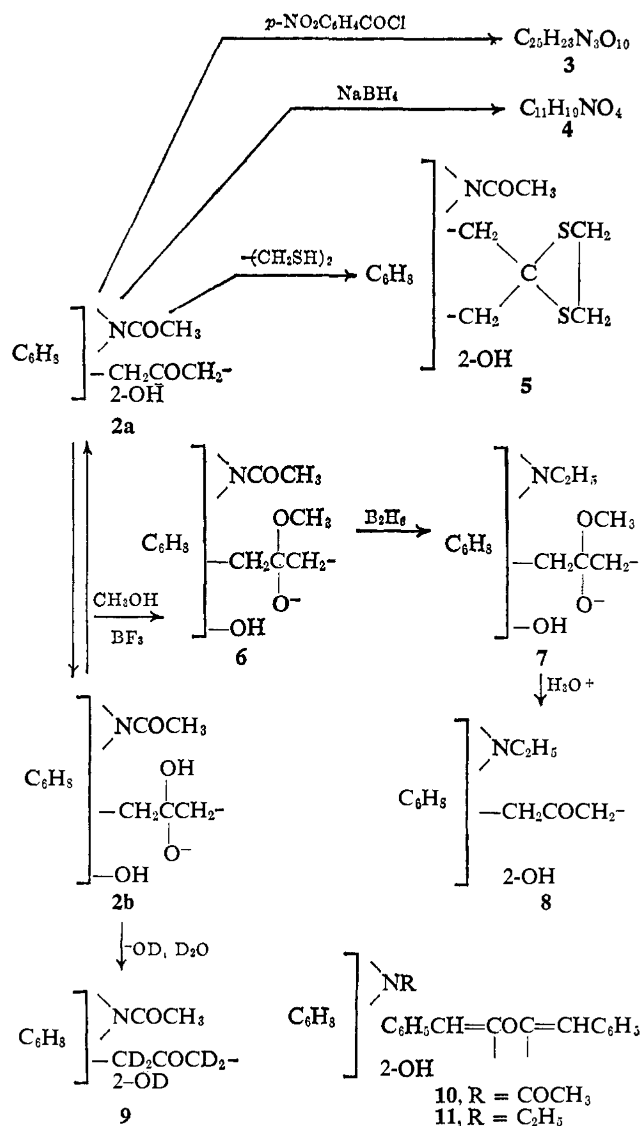
(4) (a) K. Suguira and H. C. Reilly, *ibid.*, 522 (1959); (b) J. H. Burchenal, *ibid.*, *1959–1960*, 954 (1960); (c) K. Suguira, *ibid.*, p 924; (d) M. N. Teller, R. Wolff, and S. F. Wagshul, *Cancer Res.*, **24**, 114 (1964); (e) M. N. Teller, *Trans. N. Y. Acad. Sci.*, 24, 158 (1961); (f) P. C. Merker, M. Bowie, and P. Ando, *Cancer Res.*, 22, 352 (1962); (g) F. M. Schabel, Jr., T. P. Johnston, G. McCaleb, J. A. Montgomery, W. R. Laster, and H. E. Skipper, *Cancer Res.*, **23**, 725 (1963); (h) J. H. Burchenal, H. F. Oettgen, M. Lyman, and J. Purple, *Acta Unio Intern. Contra Cancrum*, 20, 256 (1964).

(5) (a) D. Smithers, *Proc. Am. Assoc. Cancer Res.*, 7, 66 (1966); (b) R. F. Pittillo, F. M. Schabel, Jr., and B. G. Quinnelly, *Antibiot. Chemotherapy*, **11**, 501 (1961).

(6) (a) Reported by Haskell and Bartz.[3] (b) Confirmed mass spectrometrically.

(7) Parts per million downfield of 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt as an internal standard.

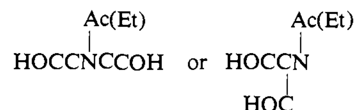
Scheme I



The N-acetate **2** can be recovered unchanged, except of course for deuterium incorporation (compound **9**), following treatment with sodium deuterioxide–deuterium oxide. In contrast, similar treatment of N-acetyldihydroactinobolamine (**4**), the control compound for this study, produces no change in its nmr spectrum. Further, a crystalline dibenzylidene derivative **10** can be prepared upon treatment of actinobolamine with benzaldehyde followed by acetylation. These observations are consistent with a structure possessing a ketone carbonyl function which is flanked on either side by a methylene group and militate against the presence of an aldehyde group in the molecule. The absence of a signal in the nmr spectra of actinobolamine and its derivatives characteristic of the aldehydic proton (δ 9.4–10.0 ppm) lends support to the latter conclusion. In addition, N-ethylactinobolamine (**8**) demonstrates identical base-catalyzed deuterium-exchange behavior and, in contrast to N-acetylactinobolamine, forms a dibenzylidene derivative **11** directly.

The nmr spectrum (D_2O) of N-acetylactinobolamine (**2**) displays a group of poorly resolved, overlapping signals in the δ 3.7–4.6[7] region whose integrated intensity corresponds to four protons; thus the presence of four protons on carbon bearing amide nitrogen and hydroxyl oxygen is suggested. Confirmation of this assignment and an elaboration of the nature of the carbon atoms involved were achieved as a result of the oxidation studies and nmr work described next.

Periodate oxidation studies proved useful in relating the positions of the amino and hydroxyl functions to one another on the carbon skeleton. N-Acetylactinobolamine (**2**) and N-acetyldihydroactinobolamine (**4**) fail to consume periodate; N-ethylactinobolamine (**8**) consumes 2 moles. These observations provide presumptive evidence for the absence of either a vicinal diol or an α-hydroxy ketone grouping and suggest the presence of an oxygen (carbonyl or hydroxyl) on each of the two carbon atoms contiguous to carbon bearing nitrogen, *e.g.*



N-Acetylactinobolamine (**2**) possesses a number of functional groups, *e.g.*, hydroxyl groups, that provide sites for oxidative cleavage. Indeed, mild permanganate oxidation of the N-acetate **2** gives rise to, among other products, a mixture of a number of amino acids from which L-threonine and D-aspartic acid are isolated. The formation of L-threonine is consistent with the presence of a 1-hydroxyethyl side chain attached to carbon bearing nitrogen, *i.e.*, $-HC(CHOHCH_3)N(Ac)-$, a finding in accord with the results of periodate oxidation. The expected three-proton methyl doublet is found in the nmr spectrum of N-acetylactinobolamine (**2**) centered at δ 1.12[7] (D_2O). The presence of a proton on carbon bearing nitrogen is suggested, but not required, by the production of L-threonine. Its presence is confirmed by the appearance of a one-proton quintet ($J \cong 6.5$ cps) centered at δ 4.70[7] (DMSO-d_6) in the nmr spectrum of N-acetylactinobolamine ethanedithiol ketal (**5**), a compound in which the hydroxyethyl side chain must be free. This signal is assigned to the proton on carbon bearing the hydroxyl group; its observed multiplicity requires the presence of one proton on adjacent carbon in addition to the three of the adjacent methyl group. The one-proton quintet undergoes the expected paramagnetic shift (to δ 6.25;[7] $\Delta\delta$ 1.55[8]) upon conversion of the thioketal **5** to its *di-p*-nitrobenzoate ester. A second *one-proton* signal in the nmr spectrum of the thioketal **5**, whose chemical shift is difficult to assess because the signal overlaps the absorption of the protons on carbon bearing sulfur, also experiences a paramagnetic shift ($\Delta\delta \sim 1.8$[8]) upon conversion to the diester and appears as a one-proton triplet ($J = 5$ cps) at δ 5.30.[7] *Thus the second hydroxyl group must also be secondary in character.*[9]

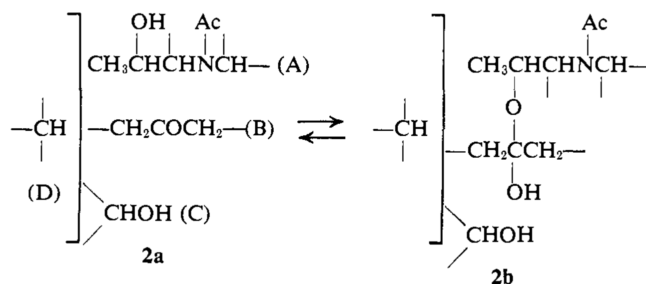
Of the suggested total of four protons on carbon bearing oxygen and nitrogen, the presence of two protons on carbon bearing hydroxyl oxygen has just been established; the presence of two protons on carbon bearing nitrogen is therefore inferred. The latter de-

(8) L. M. Jackman, "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry," Pergamon Press Inc., New York, N. Y., 1959, p 55, reports that a paramagnetic shift of about 1.33 ppm is observed as a consequence of benzoylation of secondary alcohols.

(9) Attempts to classify the hydroxyl groups according to the nmr method of O. L. Chapman and R. W. King, *J. Am. Chem. Soc.*, **86**, 1256 (1964), met with incomplete success in our hands. See J. G. Traynham and G. A. Knesel, *ibid.*, **87**, 4220 (1965).

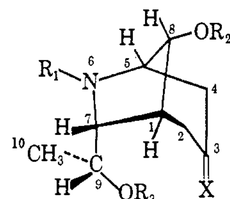
duction received support from the observation that as a result of the conversion of N-ethylactinobolamine methyl ketal (7) to its *p*-toluenesulfonic acid salt *two* one-proton signals experience a paramagnetic shift of at least 0.5 ppm.[10] These signals, assigned to methine protons on carbon bearing nitrogen, appear at δ 3.64[7] (multiplet) and 3.82[7] (doublet, J = 7 cps) (D_2O) in the salt, *downfield* of the methoxy methyl singlet (δ 3.24[7]); in the free base **7**, the position of these signals is difficult to assess because of overlapping with other signals, but they are *upfield* of the methoxy methyl singlet.

The structural implications of the data presented thus far are summarized in the partial structure shown below for N-acetylactinobolamine (**2**). The involve-

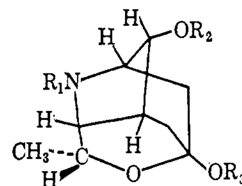


ment of the hydroxyl group of the 1-hydroxyethyl side chain in hemiketal formation is clearly established on the basis of the nmr studies described later. The ease of the hemiketal and methyl ketal formation requires that this hydroxyl group be favorably disposed in space with respect to the ketone carbonyl. The molecular formula of actinobolamine, $C_9H_{15}NO_3$, requires the presence of three double bonds or their equivalent (the ketone carbonyl group accounts for one). Three observations militate against the presence of carbon–carbon multiple bond linkages: N-acetylactinobolamine does not consume ozone under conditions expected to cleave even a tetrasubstituted olefin, *i.e.*, no reaction was observed at $-30°$ using a 3% ozone gas stream in ethanol solution;[11] it fails to consume hydrogen in glacial acetic acid in the presence of a platinum catalyst; there are no vinyl proton signals in the nmr spectra of actinobolamine and its derivatives. Thus the incorporation of two rings in the actinobolamine molecule is indicated.

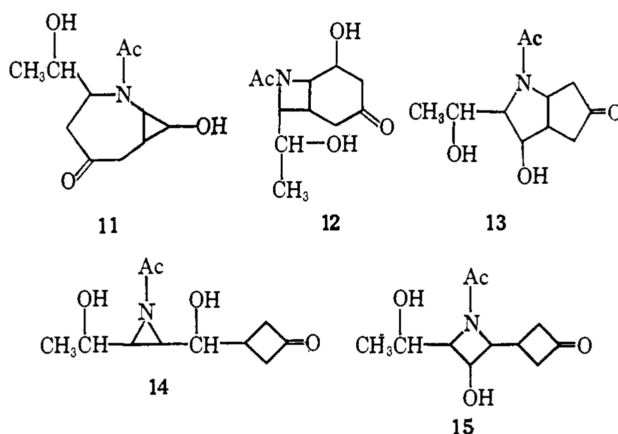
At this point the search for the structure of actinobolamine was facilitated by an examination of *all* structures consistent with the partial structure shown. In what we believe to be an interesting and advantageous application of computer techniques, the four fragments (A–D) of partial structure **2a** were fed to a computer programmed to generate *all* possible ways of joining the fragments together (*i.e.*, "structures") consistent with the following requirements: (1) fragment C must join directly to A (required by periodate oxidation studies) and (2) "structures" with multiple bond linkages are excluded.[12] An examination of the six structures (**2**, **11–15**) generated by the computer reveals only one expression, namely **2**, consistent with the chemical and spectral properties of actinobolamine. Briefly, structures **13**, **14**, and **15** are excluded, in part, on the basis of the carbonyl stretching frequency of 1715 cm^{-1} for actinobolamine and 1720 cm^{-1} for its N-ethyl derivative, values consistent with a cyclohexanone carbonyl, but not a cyclobutanone or cyclopentanone carbonyl;[13] structure **11** because of its anticipated instability to the strongly acidic conditions employed in the preparation of actinobolamine and its derivatives;[14]



1a, $R_1 = R_2 = R_3 = H$; $X = O$
2a, $R_1 = Ac$; $R_2 = R_3 = H$; $X = O$
4, $R_1 = Ac$; $R_2 = R_3 = H$; $X = H, OH$
5, $R_1 = Ac$; $R_2 = R_3 = H$; $X = (SCH_2)_2$
8, $R_1 = C_2H_5$; $R_2 = R_3 = H$; $X = O$



1b, $R_1 = R_2 = R_3 = H$
2b, $R_1 = Ac$; $R_2 = R_3 = H$
6; $R_1 = Ac$; $R_2 = H$; $R_3 = CH_3$
7, $R_1 = C_2H_5$; $R_2 = H$; $R_3 = CH_3$



and structure **12** because of the resistance of N-acetylactinobolamine to dehydration[15] and its nmr spectrum in basic deuterium oxide (see below).

The facile hemiketal and ketal formation requires the *endo* configuration for the 1-hydroxyethyl side chain at C-7. Molecular models clearly demonstrate the strain-free quality of the cyclic derivative **2b**. The isolation of L-threonine—arising from carbon atoms 10, 9, 7, and 1—and D-aspartic acid—arising from carbon

(10) P. W. K. Woo, H. W. Dion, L. Durham, and H. S. Mosher, *Tetrahedron Letters*, 735 (1962), report that the C-3 proton signal of desosamine is shifted downfield about 0.7 ppm upon conversion to its hydrochloride salt.

(11) R. Criegee, *Ann.*, **583**, 6 (1953).

(12) The program, written by Mr. Kenneth Gash, will be described in a forthcoming publication.

(13) See K. Nakanishi, "Infrared Absorption Spectroscopy," Holden-Day, Inc., San Francisco, Calif., 1962, p 42.

(14) The susceptibility of cyclopropanols to ring opening under acidic and basic conditions has been described: C. H. DePuy, and F. W. Bruin, *J. Am. Chem. Soc.*, **88**, 3347 (1966).

(15) (a) For example, in hot dimethyl sulfoxide, according to the method of V. J. Traynelis, W. L. Hergenrother, J. R. Livingston, and J. A. Valicenti, *J. Org. Chem.*, 27, 2377 (1962). (b) Studied by Mr. Denny B. Nelson.

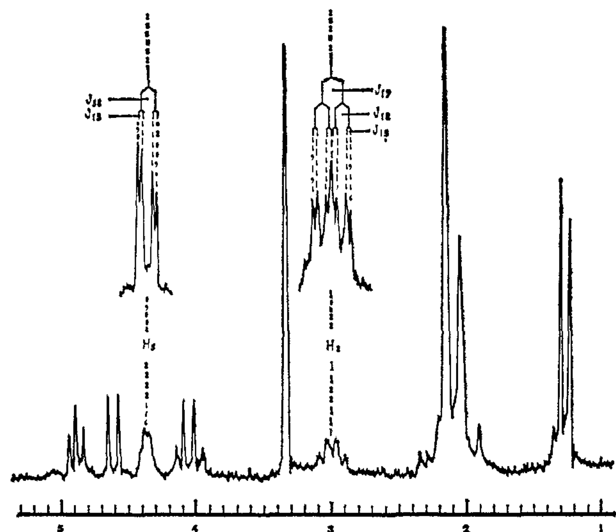


Figure 1. Nmr spectrum of the *p*-nitrobenzoate ester of N-acetylactinobolamine methyl ketal.

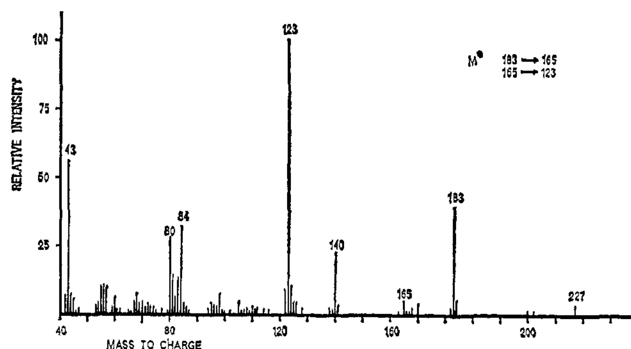


Figure 2. Mass spectrum of N-acetylactinobolamine.

atoms 9, 7, 1, and 2[16]—are compatible with structure **2** and permit the assignment of absolute configuration as shown. The assignment of configuration of the hydroxyl group at C-8, *i.e.*, *syn* to the three-atom bridge, is based on the nmr data described next.

In the nmr spectrum of the *p*-nitrobenzoate ester of N-acetylactinobolamine methyl ketal (**6**) (Figure 1) the H_8 proton appears as a triplet centered at δ 4.90 (J = 5 cps).[17] The observed coupling constant is consistent with the value calculated from the Karplus equation[18] using dihedral angles derived from a molecular model of **6** (Table I). With the hydroxyl group at C-8 *anti* to the three-atom bridge a singlet would be expected for the H_8 proton since models indicate that dihedral angles H_1–H_8 and H_5–H_8 would then approach 90°.[19]

Table I

Spin-coupled protons	Measured H–C–C–H angle, deg	*J*, cps Calcd	Obsd
H_1–H_5	40 ± 2	4.6	5.0
H_5–H_8	40 ± 2	4.6	5.0
H_7–H_9	78 ± 2	0	0
H_1–H_7	20 ± 2	7.2	7.5
H_1–H_5	Long range	1–2[a]	1.5

[a] Experimentally observed; see footnote 22.

Evidence is found in the same spectrum to verify the involvement of the hydroxyl group of the hydroxyethyl side chain in ketal formation. The proton at C-9 appears as a quartet (centered at δ 4.07; J = 6.5 cps) rather than a quintet as observed in derivatives where the hydroxyethyl side chain is free, *e.g.*, in the thioketal **5** and N-ethylactinobolamine (**8**). This reduction in multiplicity is consistent with the development of a rigid H_7–H_9 dihedral angle of 78 ± 2° in the ketal. Further, when the methyl ketal **6** is converted to its *p*-nitrobenzoate ester, it is the triplet assigned to H_8 that experiences the paramagnetic shift (from δ 3.8–4.0[20] to δ 4.90). *Therefore the hydroxyl group at C-8 must be free in the methyl ketal* **6**.

The observed multiplicity and coupling constant for the signal assigned to H_7 (δ 4.61, doublet, J = 7.5 cps) are consistent with that expected on the basis of the measured H_1–H_7 and H_7–H_9 dihedral angles. The multiplicities of the bridgehead protons, H_1 (δ 3.00) and H_5 (δ 4.37), while ambiguous in the nmr spectrum of the *p*-nitrobenzoate of the methyl ketal **6**, become more lucid when the protons on carbon adjacent to the carbonyl in N-acetylactinobolamine (**2**) and N-ethylactinobolamine (**8**) are exchanged for deuterium.[21] The H_1 signal of 2,2,4,4-d_4-**2** and the H_5 signal of 2,2,-4,4-d_4-**8** are shown in the inserts of Figure 1. The observed doublet of doublets for H_5 and the symmetrical septet for H_1 are comparable to patterns expected on the basis of coupling constants derived from measured dihedral angles. Pertinent dihedral angles and calculated and observed coupling constants for the *p*-nitrobenzoate ester of the methyl ketal **6** are summarized in Table I. The H_1–H_5 long-range coupling constant of 1.5 cps is comparable (Table I) to that observed in similar rigid systems that possess the required "M" arrangement of four σ bonds.[22]

Finally, the mass spectrum of N-acetylactinobolamine (**2**), shown in Figure 2, is also consistent with the structure proposed. The electron-induced fragmentation is best interpreted in terms of the collapse of the molecular ions derived from both components of the equilibrium mixture, the hydroxy ketone **2a** and its cyclic hemiketal **2b**.[23] In both cases electron deficiency at nitrogen

(16) Aspartic acid can also arise from carbon atoms 9, 7, 1, and 8 or 3, 4, 5, and 8; in each case structure **2** gives rise to the D configuration.

(17) (a) Nmr spectrum (100 Mc) run in deuteriochloroform. (b) Chemical shifts reported in ppm downfield from tetramethylsilane.

(18) M. Karplus, *J. Am. Chem. Soc.*, **85**, 2870 (1963).

(19) (a) The H_8 proton of *syn*- and *anti*-bicyclo[3.2.1]oct-2-en-8-yl *p*-toluenesulfonates appears as a triplet (J = 5 cps) and singlet, respectively. Grateful acknowledgment is made to Professor Norman A. LeBel, Wayne State University, for these unpublished results. (b) Other authors confirm this observation in the bicyclo[3.2.1]octane series; see A. C. Oehschalger and L. H. Zalkow, *J. Org. Chem.*, **30**, 4205 (1965), and C. W. Jefford, B. Waegell, and K. Ramey, *J. Am. Chem. Soc.*, **87**, 2191 (1965).

(20) The signal for H_8 in the methyl ketal **6** overlaps those for H_5 and H_9.

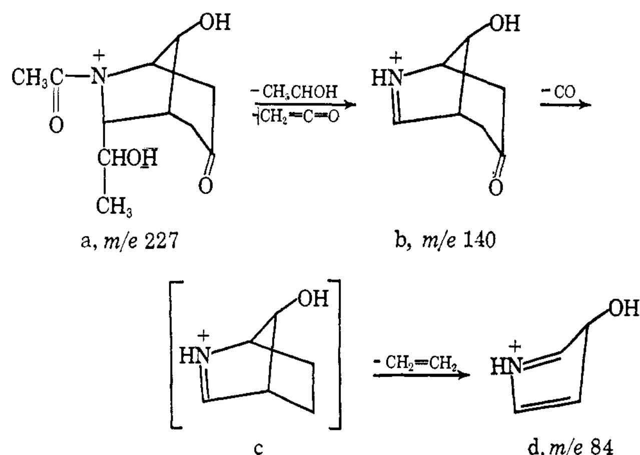
(21) The addition of base to the solution of N-ethylactinobolamine (**8**) in deuterium oxide does not alter the multiplicity of the H_8 signal. For structure **12** (C_2H_5 in place of Ac) a change in the multiplicity of the H_5 signal would be anticipated upon exchange of the hydrogen atoms on carbon adjacent the ketone carbonyl for deuterium.

(22) D. Gagnaire, E. Payo-Subiza, and A. Rousseau, "Nuclear Magnetic Resonance in Chemistry," B. Pease, Ed., Academic Press Inc., New York, N. Y., 1965, pp 165–171.

(23) The mass spectrum of the lycopodium alkaloid acrifoline, which has been shown to exist as a mobile equilibrium between two forms, the hydroxy ketone and its cyclic hemiketal,[24] has been similarly interpreted in terms of the collapse of molecular ions derived from both forms: D. B. MacLean, *Can. J. Chem.*, **41**, 2654 (1963).

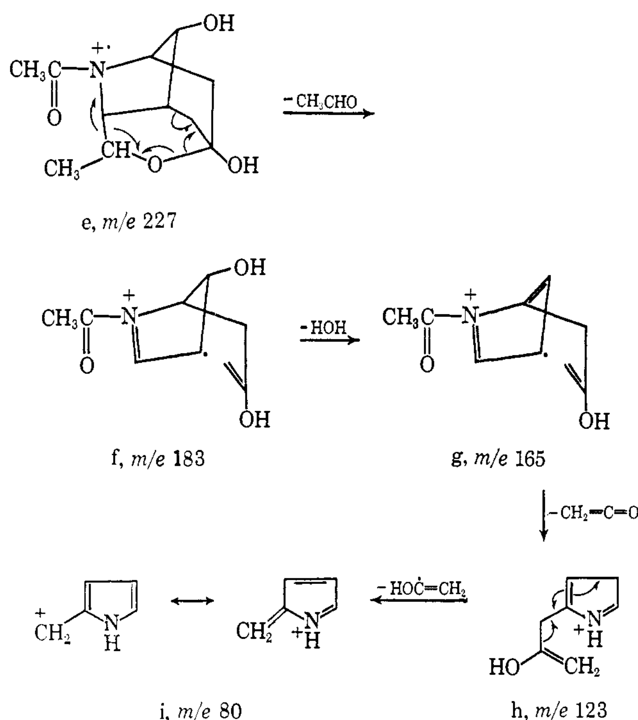
appears to control and direct the mass spectrometric fragmentation; therefore, for convenience in interpretation, the molecular ion (*m/e* 227) of the hydroxy ketone component is shown as the electron-deficient nitrogen species a in Scheme II. α Cleavage, with the ejection of a 1-hydroxyethyl radical (45 molecular units) and loss of the elements of ketene (42 molecular units) from the N-acetyl group, accounts for the appearance of a peak corresponding to *m/e* 140 in terms of the structure b. Loss of carbon monoxide from ion b followed by a reverse Diels–Alder cleavage of the favorably disposed 2-azabicyclo[2.2.2]oct-2-ene system c[25] generates an ion of *m/e* 84 which is pictured as d.

Scheme II



Fragmentation of the molecular ion e derived from the hemiketal accounts for the formation of the balance of the prominent charge species (Scheme III). Expulsion of acetaldehyde (44 molecular units) from the molecular ion, shown as the concerted process e → f in the interest of mechanistic economy, defines the peak corresponding to *m/e* 183 as the allylic ion radical f. Additional stabilization is derived by the expulsion of water (18 molecular units) to yield the diallylic ion-radical g (*m/e* 165), the precursor of the fragment of *m/e* 123 (shown as structure h). The proposed sequential loss of the elements of water and ketene (*i.e.*, f → g → h) finds support in the appearance of metastable peaks corresponding to transformations 183 → 165 and 165 → 123, respectively. Finally, expulsion of the hydroxyethylene radical (43 molecular units) from h yields an ion pictured as the resonance-stabilized pyrrolecarbinyl carbonium ion i,[26] thus explaining the appearance of a prominent peak corresponding to *m/e* 80.

Scheme III



Lastly, a comment about the factors affecting the position of the equilibrium between hydroxy ketone and hemiketal in those derivatives of actinobolamine where equilibration is possible: although additional study is required, it appears as though the basicity of the nitrogen atom plays an influential role, *e.g.*, absorption characteristic of the carbonyl group is observed in the spectra of actinobolamine and N-ethylactinobolamine, but not in that of N-acetylactinobolamine.[27] This may, in part, be the result of the operation of two opposing forces: (1) intramolecular hydrogen bonding of hydroxyl hydrogen (of the hydroxyethyl side chain) to nitrogen,[28] the existence of which should favor a shift of the equilibrium to the hydroxy ketone, and (2) formation of a strain-free intramolecular hemiketal should be thermodynamically favored due to the presence of three carbon–oxygen single bonds compared to the one carbon–oxygen single bond and one carbon–oxygen double bond in the hydroxy ketone. In an apparent delicate balance, the stabilizing influence of hydrogen bonding may predominate in systems where nitrogen is basic. Some support for this interpretation may be found in the infrared spectrum[27] of the *p*-toluenesulfonic acid salt of N-ethylactinobolamine (**8**), a compound in which the existence of an intramolecular hydrogen bond is excluded. In contrast to the free base, the salt demonstrates *no* absorption characteristic of ketone carbonyl.

In summary, the chemical and spectral properties described require expression **1** for the actinobolamine molecule.

Experimental Section

All melting points are uncorrected and were taken on a Thomas-Hoover capillary melting point apparatus. Infrared spectra were determined on a Perkin-Elmer Model 137 or 237 B Infracord and ultraviolet spectra on a Cary Model 14 spectrophotometer. Nuclear magnetic resonance spectra were run in an appropriate solvent on a Varian Associates A-60 spectrometer with tetramethylsilane (TMS) or sodium 3-(trimethylsilyl)-1-propanesulfonate as internal standards and are reported in δ units. Rotations at the sodium D line were determined on a Rudolf Model 80 polarimeter. Whatman Grade No. 1 paper was employed in the paper chromatographic analyses. Thin layer chromatographic (tlc) plates

(24) The infrared spectrum of acrifoline in Nujol shows no carbonyl absorption, suggesting that it crystallizes as the hemiketal form. This parallels the behavior of N-acetylactinobolamine. See W. N. French and D. B. MacLean, *Can. J. Chem.*, **39**, 2100 (1961).

(25) There is no peak of significant intensity in the mass spectrum corresponding to ion c, hence the structure is bracketed.

(26) Loss of an acetyl radical from the tautomeric methyl ketone form of h also can account for the formation of ion i.

(27) It should be noted (see Experimental Section) that infrared spectra were determined on the solid state. Solution infrared studies, including an examination of very dilute solutions, are in progress.

(28) A vicinal amino alcohol system. Relatively strong hydrogen bonds have been reported in such systems. See M. Tichy, *Advan. Org. Chem.*, **5**, 155 (1965).

were prepared with Macherey, Nagel and Co. cellulose powder 300G (distributed by Brinkmann Instruments), Bio-Sil A (10–20 or 2-10 μ) with 5% binder (purchased from Bio-Rad Laboratories), and Adsorbosil-3 with 10% binder (purchased from Applied Science Laboratories, Inc.). Microanalyses were performed by Midwest Microlab, Inc., Indianapolis, Ind.

Solvent systems for paper chromatography and thin layer chromatography are as follows: A, 2-propanol–water (7:3, v/v); B, 2-propanol–acetic acid–pyridine–water (8:8:1:4, v/v/v/v); C, methyl ethyl ketone–propionic acid–water (75:25:30, v/v/v); D, 1-butanol–pyridine–water (6:4:3, v/v/v); E, pyridine–ethyl acetate–acetic acid–water (5:5:3:1, v/v/v/v); F, chloroform–diethylamine (7:3, v/v).

Acid Hydrolysis of Actinobolin Sulfate. A solution of 6.03 g (16.4 mmoles) of actinobolin sulfate in 90 ml of 4 *N* sulfuric acid was warmed on a steam bath for 15 hr. The pH of the resulting yellow solution was adjusted to 4.8 with a saturated solution of barium hydroxide. After filtering through Celite, the solution was passed over a column containing 200 ml of Amberlite IRA-400 ion-exchange resin (hydroxide form). Freeze drying of the water eluent yielded 2.33 g of a yellow amorphous solid, crude actinobolamine.

The basic resin column was next eluted with 10% acetic acid. These eluents were freeze dried to yield 1.37 g of a fluffy orange powder, found to be composed principally of alanine (94% crude yield) by cellulose tlc in three solvent systems (A, B, and C). Several recrystallizations from water–ethanol yielded a white crystalline solid, $[\alpha]^{26}D$ +2.6° (*c* 6.3, water) [lit.[29] $[\alpha]^{26}D$ +2.7° (*c* 10.5, water)]. Its nmr spectrum in deuterium oxide was identical with that of alanine.

Purification of Actinobolamine (1). A sample of crude actinobolamine, 0.84 g (4.5 mmoles), was prepared by the acid hydrolysis of 2.11 g (5.74 mmoles) of actinobolin sulfate as described above. This material was chromatographed on a column containing 10 g of Bio-Sil A, 100–200 mesh. Elution with ethyl acetate–ethanol (7:3 v/v) yielded, after evaporation of the solvents, 767 mg (72% from actinobolin sulfate) of actinobolamine, a colorless amorphous solid which resisted all crystallization attempts; $[\alpha]^{26}D$ +103° (*c* 1.7, methanol); infrared absorption, ν_{max} (thin film from methanol) 1715 cm^{-1} (C=O). The amorphous solid was shown to be homogeneous by cellulose tlc in four systems (B–E).

Preparation of N-Acetylactinobolamine (2). A solution of 0.89 g (4.8 mmoles) of crude actinobolamine in 30 ml of ethanol was treated with 1.1 ml of acetic anhydride for 9 hr at room temperature. The solution was evaporated to about half its original volume and Skellysolve B[30] was added to produce a turbidity. A white crystalline solid (649 mg) resulted, mp 192.5–193°. The mother liquors yielded an additional 102 mg of crystalline material, mp 191.5–192°, raising the total yield of N-acetylactinobolamine to 751 mg (69%). The melting point of analytically pure material was 193–194°; $[\alpha]^{25}D$ +105° (*c* 0.65, methanol); infrared absorption, ν_{max}^{KBr} 3400 (OH) and 1615 cm^{-1} (C=O characteristic of a tertiary amide).

Anal. Calcd for $C_{11}H_{17}NO_4$: C, 58.14; H, 7.54; O, 28.16; mol wt, 227. Found: C, 57.86; H, 7.79; O, 28.12; mol wt, 227 (mass spectroscopy).

Ozonolysis of N-Acetylactinobolamine. Oxygen gas containing 3% ozone[31] was bubbled through a solution containing 11.4 mg (0.05 mmole) of N-acetylactinobolamine in 50 ml of 95% ethanol maintained at −30°. After complete saturation of the solution, as determined by passing the exit gases into a potassium iodide–acetic acid solution, an 84% recovery of starting material, mp 189–191°, was obtained by evaporation of the solvent and crystallization of the residue from ethanol–Skellysolve B.

Catalytic Hydrogenation of N-Acetylactinobolamine. A solution of 70 mg (0.31 mmole) of N-acetylactinobolamine, mp 190–191°, in glacial acetic acid was hydrogenated over a prereduced platinum oxide catalyst[32] at room temperature and atmospheric pressure for 3.5 hr. During this time no uptake of hydrogen was observed (in contrast, a similar solution containing cyclohexene consumed the theoretical quantity of hydrogen in 10 min). Filtration of the solution followed by removal of the acetic acid by freeze drying and crystallization of the residue yielded 60 mg (86%) of recovered N-acetylactinobolamine, mp 191.5–192.5°.

N-Acetylactinobolamine Di-*p*-nitrobenzoate (3). A solution of 120 mg (0.530 mmole) of N-acetylactinobolamine and 208 mg (1.12 mmoles) of *p*-nitrobenzoyl chloride in 5 ml of dry pyridine was warmed at 50° for 5 hr. The brown reaction mixture was poured into 10 ml of cold 1 *N* hydrochloric acid. The resulting tan precipitate was filtered, washed with water, and dried *in vacuo.* Several recrystallizations from acetone–Skellysolve B yielded 152 mg (55%) of analytically pure N-acetylactinobolamine di-*p*-nitrobenzoate (3), mp 191.5–192°; $[\alpha]^{26}D$ −45.5° (*c* 0.59, chloroform); infrared absorption, ν_{max}^{KBr} 1735 (ester C=O), 1715 (ketone C=O), and 1650 cm^{-1} (amide C=O). The nmr spectrum of **3** ($DCCl_3$) suggests esterification of the hydroxy ketone form **2a** because of the appearance of the H_9 proton as a quintet (centered at δ 5.78,[17b] $J \simeq 6.5$ cps) rather than a quartet (see Discussion).

Anal. Calcd for $C_{25}H_{23}N_3O_{10}$: C, 57.14; H, 4.41; O, 30.45. Found: C, 57.00; H, 4.63; O, 30.58.

N-Acetyldihydroactinobolamine (4). A solution of N-acetylactinobolamine (334 mg; 1.47 mmoles) and sodium borohydride (298 mg; 7.90 mmoles) in 15 ml of water was allowed to stand at room temperature for 23 hr.[33] Amberlite IR-120 ion-exchange resin (20 ml, acid form) was added. After the evolution of hydrogen ceased, the supernatant solution was introduced onto a column containing 75 ml of the acid resin. The aqueous eluent was evaporated to dryness under reduced pressure. The residue was dissolved in methanol and evaporated to dryness, leaving a residue of 203 mg. Crystallization from ethanol–Skellysolve B yielded 154 mg (47%) of white crystals, mp 205–206°. Several recrystallizations yielded analytically pure material, mp 215–217°; $[\alpha]^{26}D$ +64.7° (*c* 0.45, methanol).

Anal. Calcd for $C_{11}H_{19}NO_4$: C, 57.62; H, 8.35; N, 6.11. Found: C, 57.82; H, 8.32; N, 6.14.

Upon addition of a few drops of deuterium oxide to an nmr sample tube containing compound **4** in dimethyl-d_6 sulfoxide, three one-proton signals in the region for hydroxyl hydrogen disappeared.

N-Acetylactinobolamine Ethanedithiol Ketal (5). N-Acetylactinobolamine (381 mg; 1.68 mmoles), ethanedithiol (0.45 ml; 5.4 mmoles), and 2.5 ml of concentrated hydrochloric acid were stirred at 4° for 24 hr. The reaction mixture was diluted to 400 ml with methanol and 30 ml of Amberlite IRA-400 ion-exchange resin (hydroxide form, methanol washed) was added. The solution was then passed over a column containing 100 ml of the basic resin. The eluent was evaporated to dryness under reduced pressure. Crystallization of the residue from ethanol–Skellysolve B yielded 376 mg (74%) of N-acetylactinobolamine ethanedithiol ketal, mp 226–226.5°. The melting point of analytically pure material was also 226–226.5°; $[\alpha]^{26}D$ +54.9° (*c* 0.68, methanol). A second crop of crystalline material (67 mg) was obtained from the mother liquors, mp 225–225.5°, bringing the total yield to 443 mg (87%).

Anal. Calcd for $C_{13}H_{21}NO_3S_2$: C, 51.46; H, 6.98; N, 4.62; O, 15.82; mol wt, 303. Found: C, 51.35; H, 6.90; N, 4.39; O, 15.80; mol wt, 303 (mass spectroscopy).

A solution of N-acetylactinobolamine ethanedithiol ketal (84 mg; 0.28 mmole) and *p*-nitrobenzoyl chloride (141 mg; 0.760 mmole) in 3 ml of dry pyridine was warmed at 50° for 5 hr. The yellow-orange solution was poured into 20 ml of cold 3 *N* hydrochloric acid. The tan precipitate which formed was filtered, washed with water, and dried *in vacuo*. Crystallization from acetone–Skellysolve B yielded 160 mg (96%) of orange crystals, mp 204.5–205°. Recrystallization gave analytically pure, yellow crystals of N-acetylactinobolamine ethanedithiol ketal di-*p*-nitrobenzoate, mp 206–207°; $[\alpha]^{26}D$ −89.1° (*c* 0.59, chloroform).

Anal. Calcd for $C_{27}H_{27}N_3O_9S_2$: C, 53.90; H, 4.52; N, 6.99. Found: C, 54.18; H, 4.74; N, 6.73.

A solution of 75.0 mg (0.25 mmole) of N-acetylactinobolamine ethanedithiol ketal in 7 ml of 4 *N* sulfuric acid was refluxed for 4 hr under a nitrogen atmosphere.[34] The acid solution was passed over a column containing 40 ml of Bio-Rad AG 21-K anion-exchange resin (hydroxide form), and the water eluent was freeze dried yielding a residue of 49.6 mg. Acylation with acetic anhydride (0.5 ml) in ethanol (10 ml) at room temperature for 4 hr followed by evaporation of all volatile components produced a residue of

(29) "Handbook of Chemistry and Physics," 43rd ed, Chemical Rubber Publishing Co., Cleveland, Ohio, 1961–1962, p 1764.

(30) A petroleum ether fraction, bp 65–68°, marketed by Skelly Oil Co.

(31) Generated by an Orec Model 03C6 ozonator, Ozone Research and Equipment Corp., Phoenix, Ariz.

(32) Platinum oxide catalyst purchased from Englehard Industries, Inc., Newark, N. J.

(33) M. L. Wolfrom and H. Thompson, "Methods in Carbohydrate Chemistry," Vol. II, Academic Press Inc., New York, N. Y., 1963, p 65.

(34) Studied by Mr. Denny B. Nelson and Mr. Frederick J. Antosz.

67.1 mg. Preparative tlc on a 0.75-mm Absorbosil-3 plate (developed with acetone) revealed four zones which were mechanically separated and eluted from the solid support by washing with ethyl acetate–ethanol (1:1, v/v). Of the four fractions, 1–4 [in order of decreasing R_f values, 7.0, 5.2, 10.9, and 12.3 mg, respectively, representing a recovery of 35.4 mg (60%) of the material spotted on the chromatogram], fractions 2 and 3, identified as N-acetylactinobolamine ethanedithiol ketal (mp 222.5–224°) and N-acetylactinobolamine (mp 189.5–191°; infrared identical with authentic sample), respectively, appeared to be essentially homogeneous by analytical tlc (Absorbosil-3, acetone as developer). Of the product recovered from the preparative chromatogram, 31% is N-acetylactinobolamine and 15% is N-acetylactinobolamine ethanedithiol ketal. The remaining fractions, 1 and 4 (55% of the product), were not identified, but were shown to be sulfur containing.[35]

N-Acetylactinobolamine Methyl Ketal (6). A solution of N-acetylactinobolamine (534 mg; 2.25 mmoles), trimethyl orthoformate (1.3 ml; 12 mmoles), and 15 drops of boron trifluoride etherate in 15 ml of methanol was stirred at room temperature for 15 hr. The solution was passed onto a column containing 10 ml of Bio-Rad AG 21K resin (hydroxide form, methanol washed). The eluent was evaporated to dryness under reduced pressure. Crystallization of the syrup from ethyl acetate–Skellysolve B gave 494 mg (87%) of N-acetylactinobolamine methyl ketal, mp 121–121.5°. The mother liquors yielded an additional 50 mg (9%), mp 118.5–119.5°.

Anal. Calcd for $C_{12}H_{19}NO_4$: C, 59.73; H, 7.94; N, 5.81. Found: C, 59.94; H, 8.21; N, 5.88.

N-Acetylactinobolamine methyl ketal (29 mg; 0.12 mmole) was dissolved in 1 ml of concentrated hydrochloric acid. The solution was kept at 0° for 24 hr, then diluted with methanol, and passed over a column containing 25 ml of Amberlite IRA-400 resin (hydroxide form, methanol washed). The eluent was evaporated under reduced pressure, leaving a residue which was crystallized from ethanol–Skellysolve B to yield 14 mg (52%) of N-acetylactinobolamine, mp 187–188°. The infrared spectrum was identical with that of N-acetylactinobolamine.

N-Acetylactinobolamine methyl ketal (75 mg; 0.31 mmole) and *p*-nitrobenzoyl chloride (67 mg; 0.36 mmole) in 2 ml of dry pyridine were stirred at room temperature for 28 hr. The brown mixture was poured into 20 ml of cold water. This mixture was extracted three times with 25-ml portions of chloroform. The chloroform extracts were washed first with three 25-ml portions of a saturated solution of sodium bicarbonate, then with water, then dried over magnesium sulfate, and evaporated to dryness. The residue yielded, after two recrystallizations from acetone–Skellysolve B, 84 mg (69%) of N-acetylactinobolamine methyl ketal *p*-nitrobenzoate, mp 211°; $[\alpha]^{26}D$ −17.4° (*c* 0.42, chloroform). The mother liquors yielded an additional 10 mg (8%), mp 208–209°. The infrared spectrum contained no absorption characteristic of the hydroxyl group but indicated ester carbonyl absorption (1730 cm^{-1}).

Anal. Calcd for $C_{19}H_{22}N_2O_7$: C, 58.45; H, 5.68; N, 7.18. Found: C, 58.56; H, 5.71; N, 7.03.

N-Ethylactinobolamine Methyl Ketal (7). A solution of N-acetylactinobolamine methyl ketal (554 mg; 2.30 mmoles) in 20 ml of tetrahydrofuran (freshly distilled from lithium aluminum hydride) was added over a 15-min period to 18 ml of 1 *M* borane[36] in tetrahydrofuran at 0° under a nitrogen atmosphere.[37] The solution was refluxed for 3 hr and cooled, and 30 ml of water was cautiously added. The volume was reduced to about 10 ml *in vacuo*. After diluting with methanol, the solution was passed onto a column of 60 ml of Amberlite IRA-400 resin (hydroxide form, methanol washed). The eluent was evaporated to dryness under reduced pressure. Crystallization of the residue from acetone–Skellysolve B yielded 495 mg of crystalline material, mp 144–150° Recrystallization gave 386 mg (74%) of analytically pure N-ethylactinobolamine methyl ketal, mp 159–160°; $[\alpha]^{26}D$ +42.6° (*c* 0.39, methanol).

Anal. Calcd for $C_{12}H_{21}NO_3$: C, 63.40; H, 9.31; O, 21.12. Found: C, 63.34; H, 9.49; O, 21.10.

N-Ethylactinobolamine methyl ketal (88 mg; 0.39 mmole) and *p*-toluenesulfonic acid monohydrate (80 mg; 0.42 mmole) were dissolved in 5 ml of hot ethanol. The addition of Skellysolve B produced 126 mg (81%) of white crystals, mp 193–196°. Two recrystallizations yielded the *p*-toluenesulfonate salt as analytically pure material, mp 210–210.5°.

Anal. Calcd for $C_{19}H_{29}NO_6S$: C, 57.12; H, 7.32; N, 3.51. Found: C, 57.15; H, 7.34; N, 3.78.

N-Ethylactinobolamine (8). N-Ethylactinobolamine methyl ketal (259 mg; 1.14 mmoles) in 2 ml of concentrated hydrochloric acid was allowed to stand at 5° for 25 hr. The solution was diluted with cold methanol and passed over a column containing 90 ml of Bio-Rad AG 21K resin (hydroxide form, methanol washed). The eluent was evaporated and the residue crystallized from acetone–Skellysolve B to yield 197 mg (81%) of a white crystalline solid, mp 159–160°. Several recrystallizations gave analytically pure material, mp 160°; $[\alpha]^{26}D$ +71.1° (*c* 0.49, methanol); infrared absorption ν_{max}^{KBr} 1720 cm^{-1} (C=O).

Anal. Calcd for $C_{11}H_{19}NO_3$: C, 61.94; H, 8.98; O, 22.50. Found: C, 62.00; H, 8.82; O, 22.52.

N-Ethylactinobolamine (28 mg; 0.13 mmole) and *p*-toluenesulfonic acid monohydrate (29 mg; 0.15 mmole) were dissolved in 10 ml of acetone. The solution was evaporated to dryness, and the residue was crystallized from ethanol–Skellysolve B to give 41 mg (82%) of the *p*-toluenesulfonate salt, mp 173.5–174.5°. The infrared absorption spectrum displayed no bands characteristic of the carbonyl group.

Anal. Calcd for $C_{18}H_{27}NO_6S$: C, 56.08; H, 7.06; O, 24.90. Found: C, 56.37; H, 7.17; O, 25.22.

Dibenzylidene-N-acetylactinobolamine (10). The procedure of Johnson was used.[38] Crude actinobolamine, prepared from the acid hydrolysis of 3.04 g (8.26 mmoles) of actinobolin sulfate as previously described, was added to a solution containing 5 ml of benzaldehyde and 5 ml of 33% aqueous sodium hydroxide in 60 ml of methanol. This solution was stirred at room temperature in a nitrogen atmosphere for 2.5 hr, then concentrated to about 20 ml under reduced pressure at room temperature. The solution was diluted with 75 ml of water and extracted with four 75-ml portions of chloroform. The chloroform solution was extracted with four 50-ml portions of 2 *N* hydrochloric acid. The aqueous acid layer was made basic with powdered sodium bicarbonate and extracted with four 75-ml portions of chloroform. The chloroform extracts were evaporated to dryness, and the residue was dissolved in 100 ml of ethanol and 3 ml of acetic anhydride. This solution was allowed to stand at room temperature overnight, then evaporated to dryness. Crystallization from acetone–Skellysolve B yielded 486 mg (12%) of yellow crystals. Further recrystallizations gave analytically pure, pale yellow crystals of dibenzylidene-N-acetylactinobolamine monoacetonate, softening at 134° and becoming clear at 158°; ultraviolet absorption, λ_{max} (methanol) 314 mμ (log ϵ 4.33);[39] infrared absorption, ν_{max}^{KBr} 1715 (acetone C=O) and 1685 cm^{-1} (conjugated ketone C=O). The nature of the stereochemistry about the carbon–carbon double bonds or the configurational homogeneity of the analytical sample were not determined.

Anal. Calcd for $C_{25}H_{25}NO_4 \cdot C_3H_6O$: C, 72.87; H, 6.77. Found: C, 73.10; H, 6.76.

Dibenzylene-N-ethylactinobolamine (11). A solution containing 117 mg (0.55 mmole) of N-ethylactinobolamine, 0.5 ml (5.0 mmoles) of benzaldehyde, and 1.0 ml of 33% aqueous sodium hydroxide solution in 20 ml of methanol was stirred at room temperature in a nitrogen atmosphere for 6.5 hr. The volume of the solution was reduced to about 5 ml under reduced pressure at room temperature and then diluted to 25 ml with chloroform. Water (50 ml) was added and the chloroform layer separated. The aqueous phase was extracted with two additional 25-ml portions of chloroform. The chloroform extracts were combined and extracted four times with 25-ml portions of 1 *N* hydrochloric acid. The acid solution was made basic with powdered sodium bicarbonate, then extracted three times with 50-ml portions of chloroform. The chloroform was evaporated under reduced pressure leaving 149 mg of yellow-orange crystals. Recrystallization from ethyl acetate–Skellysolve B yielded 39 mg (13%) of dibenzylidene-N-ethylacetinobolamine. Three additional recrystallizations gave analytically pure material, mp 220° dec; ultraviolet absorption, λ_{max} (methanol) 313 mμ (log ϵ 4.26);[40] infrared absorption, ν_{max}^{KBr} 1685 cm^{-1} (conjugated

(35) F. Feigl, "Spot Tests in Organic Analysis," 6th ed, Elsevier Publishing Co., New York, N. Y., 1960, p 93.

(36) Metal Hydrides Division of Ventron, Beverly, Mass.

(37) H. C. Brown and P. Heim, *J. Am. Chem. Soc.*, **86**, 3566 (1964).

(38) W. S. Johnson, W. A. Vredenburgh, and J. E. Pike, *ibid.*, **82**, 3409 (1960).

(39) H. S. French and L. Wiley, *ibid.*, **71**, 3702 (1949), report λ_{max} (ethanol) 330 mμ (log ϵ 4.40) for 2,6-dibenzylidenecyclohexanone.

(40) The ultraviolet absorption spectrum of 2,4-dibenzylidenetropinone[41] was examined; λ_{max} (methanol) 329 mμ (log ϵ 4.35).

(41) R. G. F. Manske and H. L. Holmes, "The Alkaloids," Vol. I, Academic Press Inc., New York, N. Y., 1950, p 367.

ketone C=O). This compound showed a single ultraviolet sensitive spot on Absorbosil-3 thin-layer microslides in systems C and F.

Anal. Calcd for $C_{25}H_{27}NO_3$: C, 77.08; H, 6.99; N, 3.60. Found: C, 76.84; H, 7.04; N, 3.75.

Periodate Oxidations of N-Acetylactinobolamine (2), N-Acetyldihydroactinobolamine (4), and N-Ethylactinobolamine (8). One milliliter of 0.45 *M* sodium metaperiodate was added to a solution of 15 mg of the compound in a few milliliters of water. The solution was diluted to 10 ml in a volumetric flask, and the reaction was allowed to proceed at room temperature. At measured time intervals 1-ml aliquots were withdrawn, and the periodate uptake was determined by the standard procedure.[42] A blank and a control sample of α-methyl D-glucopyranoside were analyzed with each unknown sample. Table II summarizes the number of moles of periodate reduced at various time intervals.

Table II

Time, hr	Moles of periodate reduced[a] 2	4	8
1	0.00	0.29	2.02
2	0.08	0.00	1.79
4	0.03		2.18
8	0.00	0.13	2.60
10			2.36
20		0.00	
24	0.00	0.22	
48	0.32	0.14	

[a] Study indicates that the values of moles of periodate reduced obtained by titration[42] are accurate to about ±0.25 mole

Permanganate Oxidation of N-Acetylactinobolamine. N-Acetylactinobolamine (350 mg; 1.54 mmoles) was dissolved in 20 ml of water and warmed to 80–90°. A solution of 17 ml of 0.5 *M* potassium permanganate solution was added dropwise with stirring over a 10-min period. The addition of another 1 ml of oxidant required several minutes to decolorize. The solution was cooled and filtered through Celite. To the filtrate was added Dowex 50 ion-exchange resin (acid form) until gas evolution ceased. The solution was passed over a column containing 17 ml of Dowex 50 (acid form), and the column was eluted with water until the washings were neutral. The strongly acidic eluent was concentrated to dryness *in vacuo*. The residual yellow oil was dissolved in 10 ml of 3 *N* sulfuric acid and heated on a steam bath for 3 hr. The solution was neutralized with barium hydroxide and the barium sulfate removed by filtration. The neutral filtrate was passed over a column containing 10 ml of ZeoRex (Permatit, acid cycle), and the column was rinsed with water. The column was then eluted with 0.15 *N* aqueous ammonia solution, and 8-ml fractions were collected on a fraction collector. Fractions 12–15 showed an acidic and neutral amino acid by paper electrophoresis in 0.05 *M* pyridine acetate buffer, pH 5.0. The acidic zone corresponded in mobility to that of aspartic acid.

The ninhydrin-positive fractions (12–15) were combined and evaporated *in vacuo*, affording 138 mg. The residue was dissolved in water and passed over a column containing 10 ml of Dowex 2 ion-exchange resin (acetate form), and the column was washed with water until the effluent was free of ninhydrin-positive material. The effluent was concentrated to dryness, affording 38 mg of residue. The residue was digested with ethanol and finally recrystallized from ethanol–water to yield 8 mg of an amino acid which was identical with L-threonine by infrared absorption and paper chromatography (developing solvents A, B, and D); $[\alpha]^{22}D$ −28.6° (*c* 1.0, water) [lit.[43] $\alpha^{26}D$ −28.3° (*c* 1.1, water)].

The Dowex 2 column was next eluted with 0.2 *M* citric acid, and the main ninhydrin-positive fractions were evaporated to dryness *in vacuo*. The residue (19 mg) was recrystallized two times from ethanol–water, affording pure D-aspartic acid (infrared spectrum identical with L isomer); $[\alpha]^{26}D$ −20° (*c* 0.5, 6 *N* hydrochloric acid) [lit.[43] $[\alpha]^{24}D$ +24.6° (*c* 2.0, hydrochloric acid) for L-aspartic acid].

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(42) R. D. Guthrie in "Methods in Carbohydrate Chemistry," Vol. I, R. L. Whistler and M. L. Wolfram, Ed., Academic Press Inc., New York, N. Y., 1962, p 435.

(43) "The Merck Index," 7th ed, Merck and Co., Inc., Rahway, N. J., 1960, p 1043.