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[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE UPJOHN CO., KALAMAZOO, MICH.]

The Chemistry of Actinospectacin. IV. The Determination of the Structure of Actinospectacin[1]

BY PAUL F. WILEY, ALEXANDER D. ARGOUDELIS, AND HERMAN HOEKSEMA

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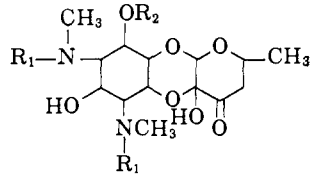
A structure is proposed for the antibiotic actinospectacin, and the work supporting the proposed structure is discussed.

Actinospectacin[2-5] is a broad spectrum antibiotic produced by an actinomycete, *Streptomyces spectabilis*. The structure of this antibiotic aside from stereochemistry has been determined to be that represented by I, and this paper discusses the evidence on which this proposed structure is based.



I, $R_1 = R_2 = H$
II, $R_1 = CH_3CO$, $R_2 = H$
III, $R_1 = R_2 = CH_3CO$
IV, $R_1 = H$, $R_2 = CH_3CO$
V, $R_1 = C_2H_5NHCO$, $R_2 = H$

Crystalline actinospectacin hexahydrate has the molecular formula $C_{14}H_{24}N_2O_7 \cdot 6H_2O$.[6] Both of the nitrogen atoms are basic having pK_a' values of 6.78 and 8.80. This is in agreement with the formation by actinospectacin of a dihydrochloride and a neutral sulfate.[4] Functional group analyses demonstrated the presence of one C-methyl group and two N-methyl groups, but methoxyl groups are absent. The infrared spectrum has several bands in the 3200–3500 cm.$^{-1}$ region attributable to hydroxyl and amino groups, but, in the hydrated state there is no absorption in the carbonyl region. However, the infrared spectra of rigorously dried actinospectacin and its salts have an absorption band at 1735 cm.$^{-1}$ indicative of a carbonyl group. The ultraviolet spectrum of actinospectacin shows end absorption and a small amount of absorption in the 290–300 mμ region which varies with the amount of moisture present. The variation of carbonyl absorption in infrared and ultraviolet spectra with the degree of hydration is attributed to hydration of a carbonyl group. The optical rotation of crystalline actinospectacin is slightly positive.

Both the previously mentioned infrared absorption at 1735 cm.$^{-1}$ and chemical data indicate the presence of a ketonic carbonyl in actinospectacin. This ketonic function does not react readily with most of the usual carbonyl reagents, but it does react with thiosemicarbazide to form a thiosemicarbazone. The product of this reaction is the thiosemicarbazone of actinospectacin which has lost a molecule of water, judging from analysis, resulting in an olefinic bond as evidenced by the appearance of a maximum in the ultraviolet region at 287 mμ (ϵ 15,900). Actinospectacin is readily reduced both by sodium borohydride and catalytically with hydrogen to give dihydroactinospectacin demonstrating that only one unsaturated site is reduced. This product no longer has the 1735 cm.$^{-1}$ infrared absorption band under any conditions, indicating reduction of the carbonyl group.

Actinospectacin forms a series of acetyl derivatives of which one (II) is the N,N′-diacetyl derivative. This compound has only two acetyl groups as determined by analysis and is neutral. These facts together with the presence of two N-methyl groups in the antibiotic establish that the nitrogen atoms must be present as methylamino groups. This is confirmed by periodate oxidation of actinospectacin to give, along with other products, two moles of methylamine. A triacetyl derivative of actinospectacin (III) is also readily obtained. This compound is neutral so it must be N,N′,O-triacetylactinospectacin. The infrared spectrum of III has absorption characteristic of ester and amide carbonyls, but it also still has infrared absorption in the hydroxyl region. Consequently, actinospectacin must have two or more hydroxyl groups.

Actinospectacin gave a negative iodoform reaction indicating that the C-methyl is not adjacent to carbonyl or a secondary hydroxyl group. Its action with Tollens reagent is somewhat ambiguous, but it had at best only a very weak reducing action.

Hydrolysis of actinospectacin with boiling 3–6 *N* hydrochloric acid gives a compound, designated actinamine, retaining both basic groups present in the antibiotic. The remainder of the molecule is converted to tarry products. This basic compound was isolated as a crystalline dihydrochloride which is easily converted to a crystalline base (VI) having the molecular formula $C_8H_{18}N_2O_4$ and pK_a' values of 7.2 and 8.9. The infrared spectrum of actinamine has bands at 3440 and 3330 cm.$^{-1}$ indicative of hydroxyl and/or amino groups; but there are no bands attributable to unsaturation of any type. The ultraviolet spectrum shows only end absorption. Actinamine as well as its salts and various derivatives (*vide infra*) is optically inactive in the range 310 to 589 mμ.

Acetylation of actinamine gives a hexaacetyl derivative (VII) which has both amide and ester groups present but no hydroxyl groups as shown by its infrared

(1) Preliminary reports of this work have been published and presented orally. See H. Hoeksema, A. D. Argoudelis, and P. F. Wiley, *J. Am. Chem. Soc.*, **84**, 1514, 3212 (1962); H. Hoeksema, Medicinal Chemistry Symposium, Boulder, Colo., June 18–21, 1962.

(2) The trademark of The Upjohn Co. for actinospectacin is Trobicin.

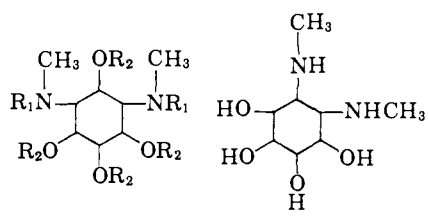
(3) D. J. Mason, A. Dietz, and R. M. Smith, *Antibiot. Chemotherapy*, **11**, 118 (1961).

(4) M. E. Bergy, T. E. Eble, and R. R. Herr, *ibid.*, **11**, 661 (1961).

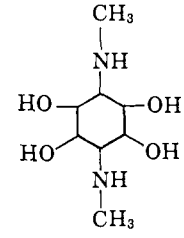
(5) A. C. Sinclair and A. F. Winfield, First Interscience Conference on Antimicrobial Agents and Chemotherapy, Oct. 31–Nov. 2, 1961, New York, N. Y.

(6) The molecular formula was previously erroneously reported as $C_{14}H_{26-28}N_2O_{7.4-5}$

spectrum and lack of basicity. This establishes that all of the oxygen atoms are present as hydroxyl groups. Actinamine consumes six moles of sodium metaperiodate with formation of two moles of methylamine but no formaldehyde. The formation of two moles of methylamine and of a neutral hexaacetyl derivative establishes the presence of two methylamino groups in actinamine. The failure of actinamine, which has six periodate-oxidizable groups on six adjacent carbon atoms, to form formaldehyde when oxidized with periodate indicates the presence of a cyclohexane ring. This combination of data is consistent only with a bis-(methylamino)-tetrahydroxycyclohexane structure for actinamine. Aside from stereoisomers the only possible structures for such a compound are VI, X, and XI.



X

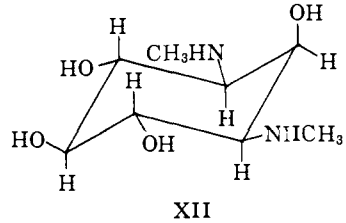


XI

VI, $R_1 = R_2 = H$
VII, $R_1 = R_2 = CH_3CO$
VIII, $R_1 = CH_3CO$, $R_2 = H$
IX, $R_1 = CH_3$, $R_2 = H$

That actinamine has the structure represented by VI was demonstrated by periodate oxidation of N,N′-diacetylactinamine (VIII) prepared by ammonolysis of hexaacetylactinamine. N,N′-Diacetylactinamine was characterized by analysis, its infrared spectrum, which has bands at 3280 and 3180 cm.$^{-1}$ indicative of hydroxyl groups and a band at 1635 cm.$^{-1}$ characteristic of amide groups, and titration. The diacetyl compound consumes two moles of periodate per mole with formation of formic acid. This combination of periodate consumption and acid formation would only be possible with the derivative of the isomer having structure VI which must be the correct structure of actinamine.

Actinamine and all of its derivatives are optically inactive indicating a *meso* compound for which there are eight possible stereoisomers. Slomp and MacKellar[7] have shown, using n.m.r. spectra, that the conformation of actinamine is as in XII. Some chemical evidence is



XII

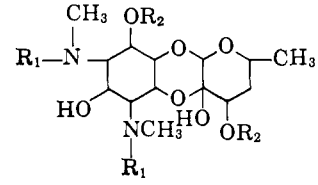
also available partially substantiating this conformamation. Because of the chemical relationship of actinamine to streptamine, it would appear *a priori* that the stereochemistry of the two compounds would be the same, *i.e.*, actinamine would be N,N′-dimethylstreptamine. That this is not the case was shown by conversion of actinamine to N,N′-dimethylactinamine dihydrochloride (IX) by Witkop's procedure[8] and comparison with N,N′-tetramethylstreptamine dihydrochloride.[8] The two compounds were not the same.

(7) (a) G. Slomp and F. S. MacKellar, *Tetrahedron Letters*, **No. 12**, 521 (1962); (b) L. D. Colebrook and R. H. Gourlay, *Proc. Natl. Acad. Sci. U.S.*, **48**, 1693 (1962).

(8) G. F. Holland, R. C. Durant, S. L. Friess, and B. Witkop, *J. Am. Chem. Soc.*, **80**, 6031 (1958).

Methanolic hydrogen chloride, under various conditions, failed to cleave either actinospectacin or dihydroactinospectacin to an appreciable degree. Numerous types of acid hydrolyses and mercaptolyses gave actinamine, but the remaining six carbon atoms were isolated as intractable mixtures which suggested degradation and rearrangement. On the basis of the preceding data it seemed likely that actinospectacin would prove to be a glycoside of actinamine in which the glycosidic moiety, as it exists in actinospectacin, is a relatively unstable six-carbon sugar-like compound having a C-methyl group, one carbonyl group, and at least one hydroxyl group. The sugar-like compound, which would be obtained from the glycosidic moiety by hydrolysis without rearrangement or carbon chain cleavage, was designated actinospectose.

As a consequence of failure to isolate the six-carbon moiety without rearrangement, it was necessary to determine the structure of this fragment and its points of attachment to actinamine by periodate oxidation studies of actinospectacin, dihydroactinospectacin (XIII), and their acyl derivatives. Actinospectacin forms O-monoacetyl (IV), N,N′-diacetyl (II), and N,N′,O-triacetyl (III) derivatives as well as the N,N′-bis-(ethylcarbamoyl) derivative (V). Dihydroactinospectacin gave an N,N′-diacetyl (XIV), a mixture of triacetyl, and an N,N′,O,O′-tetraacetyl derivative (XV). The number of acetyl groups present and their types were determined from analytical, infrared, and titration data. The consumption of periodate by actinospectacin and these derivatives is shown in Table I.



XIII, $R_1 = R_2 = H$
XIV, $R_1 = CH_3CO$, $R_2 = H$
XV, $R_1 = R_2 = CH_3CO$

TABLE I

Compound	Moles of IO_4/mole
Actinospectacin	4
O-Monoacetylactinospectacin	3
N,N′-Diacetylactinospectacin	1
N,N′,O-Triacetylactinospectacin	1
N,N′-Bis-(ethylcarbamoyl)-actinospectacin	1
Dihydroactinospectacin	4
N,N′-Diacetyldihydroactinospectacin	1
N,N′,O,O′-Tetraacetyldihydroactinospectacin	0

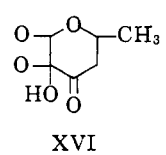
The consumption of four moles of periodate by actinospectacin with isolation of two moles of methylamine and two moles of formic acid suggests that only three carbon–carbon bonds in the actinamine moiety are attacked by periodate, and that the fourth mole attacks the actinospectose portion. Further oxidation of the actinamine ring would have resulted in formation of three moles of formic acid. This deduction was confirmed by isolation of actinamine from the periodate oxidation of N,N′-diacetylactinospectacin. Such behavior establishes that the six-carbon fragment of actinospectacin is attached to two adjacent oxygen atoms of actinamine.

The contrast between acetylation of actinospectacin and of dihydroactinospectacin and periodate oxidation of the resulting derivatives was very informative. The intact antibiotic formed only a triacetyl derivative while the reduced compound, in which only the carbonyl func-

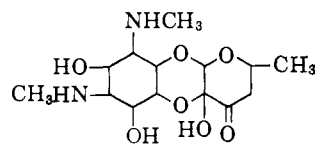
tion was reduced, could be acetylated at four sites. The former consumed one mole of periodate, but the latter consumed none. This can only be interpreted to mean that the hydroxyl formed by reduction is acetylatable and is adjacent to another carbon atom bearing a hydroxyl group which is not acetylated. Consequently the carbonyl function in actinospectacin is adjacent to a hydroxyl group. The α-hydroxy ketone system must contain a tertiary hydroxyl group since it does not give a bismuth oxide test for acyloins.[9]

The O-monoacetyl derivative of actinospectacin must have the acetyl group on the actinamine ring as indicated in structure IV on the basis of its periodate consumption of three moles. From the foregoing data this group cannot be at the tertiary hydroxyl group in actinospectose. If the acetyl group were at the other hydroxyl group in actinamine, IV would consume only two moles of periodate, while if the acetyl group were at some site in the actinospectose moiety other than the tertiary hydroxyl group, IV would consume four moles of periodate. By analogy, an acetyl group must be at the same position in N,N′,O-triacetylactinospectacin (III) and in N,N′,O,O′-tetraacetyldihydroactinospectacin (XV). Such a pattern of acetylation would be expected, as the unacylated hydroxyl in the actinamine moiety is axial, but the other one is equatorial.

Periodate oxidation of N,N′-diacetylactinospectacin (II) gave actinamine and glyoxylic acid which was also isolated from the dihydro analog XIV in addition to crotonaldehyde. A similar oxidation of N,N′-bis-(ethylcarbamoyl)-actinospectacin (V) gave crotonic acid. The aldehydes were isolated as 2,4-dinitrophenylhydrazones, and crotonic acid was identified as its *p*-bromophenacyl ester. The two- and four-carbon fragments must be derived from the actinospectose moiety and account for the carbon atoms present in that moiety. The unsaturation in the four-carbon compounds must arise by β-elimination of an oxygen at the hydroxyl level of oxidation since such unsaturation is absent in the starting compounds. The failure of actinospectacin to give an iodoform reaction and the failure of the eliminated oxygen atom to acetylate establish that this oxygen is present in an ether linkage which is part of a hemiacetal system. The carbonyl group of crotonic acid necessarily arises from the carbonyl function of actinospectacin as N,N′-diacetyldihydroactinospectacin gives crotonaldehyde. Consequently, the carboxyl group of glyoxylic acid must be formed from a carbonyl group masked as a hemiketal since it has already been established that there is no acid, ester, or second carbonyl function in actinospectacin, and that there is a tertiary hydroxyl group adjacent to the ketone carbonyl. All of these facts can only be explained by the partial structure XVI. The dihydro analog of this system, which is the form present in dihydroactinospectacin, has been reported as occurring in the cardiac glycoside, gomphoside.[10] The reported chemical behavior was identical with that found in actinospectacin and dihydroactinospectacin. The fragment XVI could be



XVI

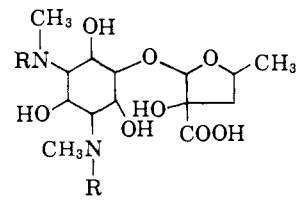


XVII

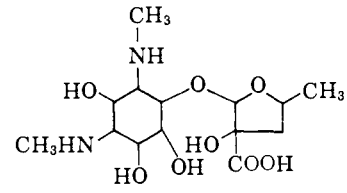
linked to actinamine either as in I or as in XVII. The choice between these two structures was made as a result of the base degradation products of actinospectacin and its N,N′-bis-(ethylcarbamoyl) derivative V.

Actinospectacin loses biological activity rapidly in mildly basic solutions forming an acid, which has been designated actinospectinoic acid, having the molecular formula $C_{14}H_{26}N_2O_8$. In addition to a carboxyl group (pK_a' 3.30) this compound still contains the two basic groups of actinospectacin, and it reduces four moles of periodate. Actinospectinoic acid no longer has infrared absorption at 1735 cm.$^{-1}$ due to ketonic carbonyl but does have bands at 1635 and 1595 cm.$^{-1}$ due to zwitterion formation. Such a spectrum indicates that the degradation reaction involves the ketonic carbonyl. The failure of dihydroactinospectacin to lose its biological activity in basic solution supports this viewpoint. Acid hydrolysis of actinospectinoic acid in the form of room temperature treatment with Brady reagent forms actinamine, carbon dioxide, and a five-carbon optically active compound which was isolated as its 1,2-bis-2,4-dinitrophenylhydrazone.[11,12] This derivative suggests that the five-carbon fragment is either a 1,2-dicarbonyl compound or an α-hydroxycarbonyl compound. When the acid hydrolysate of actinospectinoic acid was steam distilled, the five-carbon fragment was converted to an optically inactive α,β-unsaturated carbonyl compound which also was isolated as its 1,2-bis-2,4-dinitrophenylhydrazone. Sodium borohydride reduction of the steam distillate followed by periodate oxidation gave rise to crotonaldehyde and formaldehyde thus demonstrating that the distillable five-carbon compound is XXI.

The six-carbon fragment of actinospectinoic acid was isolated intact by methanolysis of the acid to give actinamine and a neutral compound having the molecular formula $C_8H_{14}O_5$. This compound contained two methoxyl groups absent in actinospectinoic acid. One must be introduced by the cleavage of the glycosidic bond to actinamine, and the other is formed by esterification of the carboxyl group in the starting acid since the carboxyl group is no longer present, and the infrared spectrum of this neutral material exhibits absorption in the ester region. The isolation of these products can only be explained if actinospectinoic acid is represented by either structure XVIII or structure XX.



XVIII, R=H
XIX, R=C_2H_5NHCO



XX

The sequence of reactions to give the five-carbon and six-carbon compounds would be as shown in eq. 1 and 2, and the six-carbon neutral compound[11] would have structure XXII which is consistent with the properties of this compound and with the possible structures of actinospectacin. The formation of actinospectinoic acid is readily explained as being a special example of the tertiary ketol rearrangement,[13,14] while any other prod-

(9) W. Rigby, *J. Chem. Soc.*, 793 (1951).

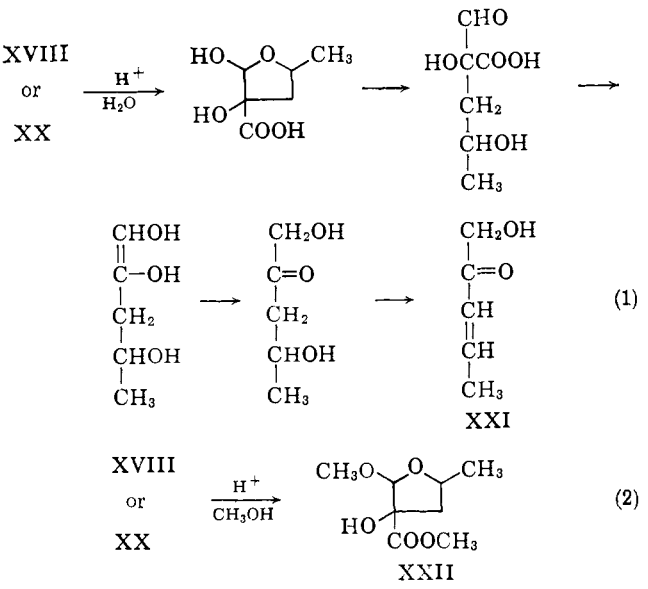
(10) R. G. Coombe and T. R. Watson, *Proc. Chem. Soc.*, 214 (1962).

(11) The racemic form of this compound has been reported by G. Hesse and G. Lettenbauer, *Ann.*, **623**, 142 (1959).

(12) The isolation and structure proof of XXII has also been reported by D. D. Chapman, R. L. Autrey, R. H. Gourlay, A. L. Johnson, J. Souto, and D. S. Tarbell, *Proc. Natl. Acad. Sci. U. S.*, **48**, 1108 (1962). These authors are in complete agreement with our structure for this degradation product.

(13) For a review on this subject see S. Selman and J. F. Eastham, *Quart. Rev.* (London), **14**, 221 (1960).

(14) Y. Mazur and M. Nussin, *Tetrahedron Letters*, **No. 22**, 817 (1961), and references cited therein.



uct would be theoretically implausible. Thus actinospectinoic acid must have either structure XVIII or XX depending on which of the two structures I or XIII is correct for actinospectacin.

A similar alkaline rearrangement of N,N′-bis-(ethylcarbamoyl)-actinospectacin gave N,N′-bis-(ethylcarbamoyl)-actinospectinoic acid (XIX). That this compound was the expected product was shown by its analysis, lack of basicity, acidic nature due to a carboxyl group, and the presence of an amide band in its infrared spectrum. The acid XIX consumed no periodate which would be the case only if it were a derivative of XVIII rather than of XX, and thus the structure of actinospectinoic acid is established as XVIII. Since a compound of structure XVIII can only be derived from actinospectacin if the latter has structure I, then the structure of actinospectacin must be represented by I.

Experimental[15]

Actinospectacin Hexahydrate (I).—Two grams of actinospectacin hydrochloride was dissolved in 30 ml. of ice-cold water, and the solution was passed over 30 ml. of Dowex 2-X8 resin surrounded by an ice-water cooling jacket. The column was washed with ice-water until the washings were neutral. The effluent was collected in a flask cooled in an ice-bath throughout. The combined effluent and washings were freeze-dried to give an amorphous material weighing 1.2 g.

The amorphous actinospectacin was crystallized by dissolving it in 4 ml. of water per gram and adding 5 volumes of acetone. Recrystallization gave 0.32 g., m.p. 65–72° to an opaque liquid which cleared at 97–100°. The infrared spectrum of this material had bands at 3520, 3380, 3300, and 3200 cm.$^{-1}$ but no bands in the carbonyl region. Drying of the crystalline material overnight at 60° under high vacuum caused reversion to an amorphous material which had a band at 1735 cm.$^{-1}$ in its infrared. Crystallization of the amorphous material from water–acetone gave crystalline actinospectacin. The crystalline material had pK_a' values of 6.78 and 8.80 and $[\alpha]^{25}D$ +7.6° (*c* 1, H_2O). It gave a negative iodoform reaction and a negative acyloin test with bismuth oxide.[9]

Anal. (*after drying*) Calcd. for $C_{14}H_{21}N_2O_7$: C, 50.44; H, 7.25; N, 8.40. Found: C, 50.26; H, 7.29; N, 8.76. *Anal.* (*before drying*) Calcd. for $C_{14}H_{21}N_2O_7 \cdot 6H_2O$: H_2O, 24.6; equiv. wt., 220.2. Found: H_2O, 24.4; equiv. wt., 221.1.

Periodate Oxidation of Actinospectacin. (a) Titration.—Actinospectacin was titrated by the Fleury–Lange procedure[16] using 0.08 *M* sodium metaperiodate solution. The consumption of periodate was, in hours (moles): 0.25 (3.0), 0.5 (3.18), 1 (3.22), 6 (3.45), 72 (3.8).

Actinospectacin sulfate was titrated in the same way. The consumption of periodate was, in hours (moles): 1 (4.07), 6 (4.36), 24 (4.17).

(b) Determination of Formic Acid.—One gram (4.7 mmoles) of sodium metaperiodate was added to a solution of 200.2 mg. (0.4 mmole) of actinospectacin sulfate tetrahydrate. After the solution had stood at room temperature for 2 hr., 35 ml. of saturated barium hydroxide solution was added, and the mixture was filtered. The filtrate was evaporated to dryness, and the residue was dissolved in 25 ml. of water. The resulting solution was adjusted to pH 2.0 with 10% phosphoric acid and distilled to dryness; 15 ml. of water was added to the residue, and the mixture was distilled to dryness. This procedure was repeated twice. A 5-ml. aliquot of the combined distillates (68 ml.) was titrated and found to contain 59 microequivalents of an acid of pK_a' 3.70 (lit. for formic acid is 3.75). This indicated 2.02 moles of formic acid per mole of actinospectacin. A repetition of this procedure gave 1.82 moles of formic acid per mole of actinospectacin.

(c) Isolation of Methylamine.—A solution of 1.0 g. (2.0 mmoles) of actinospectacin sulfate tetrahydrate and 3.2 g. (15 mmoles) of sodium periodate in 150 ml. of water was adjusted to pH 5.0 with sodium bicarbonate, and the solution was allowed to stand overnight at room temperature. The excess periodate and the iodate were precipitated by addition of 15 ml. of saturated barium hydroxide solution. The mixture was filtered through Celite, and the filtrate was steam distilled until the distillate was neutral. The distillate was collected in 50 ml. of 0.1 *N* hydrochloric acid. Titration of the acid solution with 0.1 *N* sodium hydroxide indicated that 2.65 mmoles of base had been distilled, 1.33 moles/mole of actinospectacin.

The distillate mixture was made strongly alkaline with sodium hydroxide and steam distilled into 50 ml. of water until the distillate was neutral. *p*-Hydroxyazobenzene-*p*′-sulfonic acid (0.72 g.) was added to the distillate, and it was freeze-dried. Recrystallization of the residue from water gave 0.52 g. of methylammonium *p*-hydroxyazobenzene-*p*′-sulfonate, m.p. 240°. A mixture melting point with an authentic sample was not depressed, and the infrared spectra of the two samples were identical.

Actinospectacin Thiosemicarbazone Dihydrochloride.—Twenty-five grams (0.05 mole) of actinospectacin hydrochloride was dissolved in a mixture of 800 ml. of ethanol and 1000 ml. of water, and 5.55 g. (0.061 mole) of thiosemicarbazide was added. After solution was complete, the reaction mixture was heated under reflux for 3 hr. and then concentrated under reduced pressure to a volume of 240 ml. The residue was cooled in an ice-bath, and 500 ml. of acetone and 240 ml. of concentrated hydrochloric acid were added. The crystals which formed were removed by filtration and dried; yield 20.5 g., m.p. 220–227°. One gram was recrystallized from water–acetone and absolute ethanol–ether mixtures to give 0.63 g., m.p. 225–230°. The ultraviolet spectrum in ethanol showed maxima at 230 mμ (ϵ 3800) and 287 mμ (ϵ 15,900).

Anal. Calcd. for $C_{15}H_{29}Cl_2N_5O_6S$: C, 37.66; H, 6.11; N, 14.64; Cl, 14.82; S, 6.70. Found: C, 37.93; H, 6.04; N, 13.43; Cl, 14.59; S, 6.64.

Dihydroactinospectacin (XIII). (a) By Sodium Borohydride Reduction.—Twenty-five grams (0.075 mole) of actinospectacin was dissolved in 1250 ml. of methanol, and 5.8 g. (0.15 mole) of sodium borohydride was added gradually. After the reaction mixture had stood at room temperature for 2.5 hr., it was adjusted to pH 3.0 with 6.0 *N* hydrochloric acid. The solution was evaporated to dryness under reduced pressure at room temperature. The residue was mixed thoroughly with 625 ml. of dry methanol, and the mixture was filtered. The filtrate was evaporated to dryness under reduced pressure. This procedure was repeated three times using successively 625 ml., 375 ml., and 125 ml. of dry methanol. The final residue was dissolved in 125 ml. of dry methanol and the solution was filtered. Four volumes of ether was added to the filtrate, and the precipitate was collected by filtration and dried in a vacuum desiccator. The product was dissolved in 80 ml. of 3.0 *N* hydrochloric acid, and acetone was added until slight turbidity appeared. Refrigeration gave 14.1 g. of crystalline product, m.p. 205–208° dec., $[\alpha]^{25}D$ +28.1° (*c* 1, H_2O).

Two grams of dihydroactinospectacin dihydrochloride was dissolved in 20 ml. of water and passed over a column of 30 ml. of Dowex 2-X8 resin followed by thorough washing. The total effluent was freeze-dried. The residue was recrystallized twice from a water–acetone mixture. The final melting point was 83–84°, pK_a' values 6.69 and 8.06. The infrared spectrum had bands at 3560, 3380, 3320, 3240, 1620, 1480, 1110, 1050, and 1025 cm.$^{-1}$ (unchanged after drying).

Anal. Calcd. for $C_{14}H_{26}N_2O_7$: C, 50.29; H, 7.84; N, 8.38; mol. wt., 334. Found: C, 50.53; H, 8.24; N, 8.20; mol. wt. (electr. titr.), 332.

One gram of this material was converted to its sulfate by solution in 3 ml. of 1.0 *N* sulfuric acid and precipitation by addition

(15) Part of these melting points were determined using a capillary and part using a Kofler hot-stage apparatus. Melting points are uncorrected.

(16) J. R. Dyer, "Methods of Biochemical Analysis," Vol. 3, Interscience Publishers, Inc., New York, N. Y., 1956, p. 111.

of acetone. Three recrystallizations from water–acetone gave a compound, m.p. 214–216° dec.

Anal. Calcd. for $C_{14}H_{26}N_2O_7 \cdot H_2SO_4$: C, 38.89; H, 6.53; N, 6.48; S, 7.42. Found: C, 39.03; H, 6.46; N, 6.43; S, 7.96.

(b) **By Catalytic Reduction.**—A suspension of 2.7 g. (0.0067 mole) of actinospectacin dihydrochloride in 10 ml. of 50% ethanol was shaken with 300 mg. of platinum oxide under hydrogen at 40 p.s.i. for 3 days. The catalyst was removed by filtration, and the filtrate was evaporated to dryness. The residue was crystallized from water–acetone; yield 1.45 g., m.p. 203–210°; m.p. 205–208° after recrystallization. The infrared spectrum of thoroughly dried sample was identical with the dihydrochloride prepared by sodium borohydride reduction.

Anal. (*dried at 50°*) Calcd. for $C_{14}H_{26}N_2O_7 \cdot 2HCl \cdot H_2O$: C, 39.51; H, 7.11; N, 6.59; Cl, 16.68. Found: C, 39.46, 40.15, 40.04; H, 7.06, 6.19, 7.20; Cl, 17.55, 17.67, 17.40; N, 6.81, 6.89. *Anal.* (*dried at 100°*) Calcd. for $C_{14}H_{24}N_2O_7 \cdot 2HCl$: C, 41.28; H, 6.93; Cl, 17.41. Found: C, 41.78; H, 7.12; Cl, 17.39.

Periodate Oxidation of Dihydroactinospectacin.—Dihydroactinospectacin was titrated by the Fleury–Lange[16] procedure using 0.08 *M* sodium metaperiodate solution. The consumption of periodate was, in hours (moles): 0.5 (3.48), 2 (3.61), 4 (3.93), 6 (3.92), 24 (3.85).

Actinamine Dihydrochloride.—A solution of 25.0 g. of actinospectacin sulfate tetrahydrate in 250 ml. of 6.0 *N* hydrochloric acid was heated under reflux for 6 hr. The cooled mixture was filtered, and the filtrate was extracted with three 125-ml. portions of chloroform, which were discarded. The aqueous solution was evaporated to dryness under reduced pressure. The residue was slurried with 100 ml. of methanol, and the methanol was removed by evaporation under reduced pressure. This procedure was repeated. The residue was again slurried with methanol and filtered. The filter cake was washed with cold methanol. There was obtained 14.6 g. of crystalline product decomposing without melting at 310° with prior darkening.

One-half gram of this material was dissolved in 10 ml. of water, and the solution was put on a column containing 10 ml. of Dowex 2. The column was washed with water until the washings were neutral. The effluent was collected while it was basic. This solution was freeze-dried. The residue was dissolved in 10 ml. of water, and the solution was put on a column containing 10 ml. of IRC-50. This column was washed thoroughly with water and eluted with 100 ml. of 1.0 *N* hydrochloric acid. The eluate was evaporated to dryness under reduced pressure. The excess hydrochloric acid was removed by twice slurrying with methanol and evaporating to dryness under reduced pressure. The final residue was again slurried with methanol and filtered. The filter cake was washed with methanol. This material was recrystallized twice by dissolving it in water, adding acetone to turbidity and refrigerating. The final product decomposed at 315° with prior darkening. The ultraviolet spectrum of this compound showed only end absorption. The infrared spectrum had bands at 3200 cm.$^{-1}$ characteristic of hydroxyl groups and at 1595 cm.$^{-1}$ characteristic of ammonium ions, but absorption in the carbonyl region was absent. Titration in water gave pK_a' values of 7.22 and 8.90. Optical rotation was zero.

Anal. Calcd. for $C_8H_{18}N_2O_4 \cdot 2HCl$: C, 34.41; H, 7.12; N, 10.05; Cl, 25.45; O, 22.93; mol. wt., 279. Found: C, 34.46, 34.39; H, 7.12, 7.07; N, 10.02, 9.83; Cl, 25.31; O, 21.23; mol. wt. (elect. titr.), 280.

Actinamine (VI).—A solution of 2.0 g. of actinamine dihydrochloride in 30 ml. of water was passed over 30 ml. of Dowex 2-X8. The column was washed with water until the washings were neutral. The effluent was collected while it was basic and evaporated to dryness under reduced pressure. The residue was recrystallized three times from a water and acetone mixture. The final melting point was 129° with preliminary softening beginning at 117°. The ultraviolet spectrum of actinamine showed only end absorption. The infrared spectrum had bands at 3440 and 3330 cm.$^{-1}$ characteristic of hydroxyl and imino or amino groups. There was no absorption in the carbonyl region. The optical rotation was zero between 310 and 589 mμ.

Anal. Calcd. for $C_8H_{18}N_2O_4$: C, 46.60; H, 8.74; N, 13.59; O, 31.07; mol. wt., 206. Found: C, 46.99; H, 8.99; N, 14.06; O, 31.00; mol. wt. (elect. titr.), 204.

Periodate Oxidation of Actinamine Hydrochloride. (a) Titration and Attempted Isolation of Formaldehyde.—Actinamine dihydrochloride was titrated by the Fleury–Lange procedure[16] using 148.8 mg. in 0.1 *M* sodium metaperiodate solution. The consumption of periodate was, in hours (moles): 0.5 (5.0), 1 (5.2), 2 (5.4), 4 (6.0), 22 (6.2). The remainder of the reaction mixture was used to determine formaldehyde formed by the procedure recommended by Dyer.[16] No formaldehyde was detected.

(b) **Isolation of Methylamine.**—Sodium metaperiodate (6.4 g., 30 mmoles) was dissolved in 300 ml. of water. In this solution was dissolved 1.0 g. (3.6 mmoles) of actinamine dihydrochloride. The pH was adjusted to 5.1 with sodium bicarbonate and maintained at that pH by further addition of sodium bicarbonate. After the solution had stood at room temperature overnight, it was made strongly alkaline by the addition of 250 ml. of saturated barium hydroxide solution. The precipitate which formed was removed by filtration through Celite. The filtrate was steam distilled into 100 ml. of 0.1 *N* hydrochloric acid until no more basic material was distilling. The hydrochloric acid solution was titrated with 0.1 *N* sodium hydroxide solution; 39 ml. of base was required to titrate the amine salt present. This corresponded to 7.1 mmoles of base (theoretical 7.2 mmoles).

The distillate was made strongly alkaline with sodium hydroxide and steam distilled until all volatile base was removed. Two grams of *p*-hydroxyazobenzene-*p'*-sulfonic acid was added to the distillate and it was evaporated to dryness under reduced pressure. The residue was recrystallized from water. There was obtained 1.48 g. of the methylamine salt of *p*-hydroxyazobenzene-*p'*-sulfonic acid, m.p. 241° dec., no depression in melting point when mixed with an authentic sample and identical infrared curves. The yield was 69% calculated for two methylamino moieties in actinamine.

Hexaacetylactinamine (VII) was prepared by the procedure of Peck, *et al.*[17] A mixture of 2.0 g. (7.2 mmoles) of actinamine dihydrochloride, 1.2 g. (14.4 mmoles) of anhydrous sodium acetate, and 120 ml. of acetic anhydride was heated under reflux for 2 hr. The mixture was evaporated to dryness under reduced pressure, The residue was extracted with 100 ml. of chloroform which was filtered, and the filtrate was evaporated to dryness. The residue was dissolved in 10 ml. of chloroform and ether was added until crystals began to form. Refrigeration gave 2.7 g., m.p. 190–194°.

One gram of this was recrystallized four times from chloroform–ether; m.p. 196–198°. The infrared spectrum of this compound had no absorption in the hydroxyl region but did have ester carbonyl absorption at 1750 cm.$^{-1}$ and amide carbonyl absorption at 1650 cm.$^{-1}$. There was no titratable group present. The optical rotation was zero.

Anal. Calcd. for $C_{20}H_{30}N_2O_{10}$: C, 52.40; H, 6.53; N, 6.11; O, 34.93; CH_3C (6), 19.6. Found: C, 52.34; H, 6.71; N, 6.20; O, 34.06; CH_3C, 18.9.

N,N'-Diacetylactinamine (VII).—Anhydrous methanol (500 ml.) was cooled in an ice-bath and saturated with ammonia. Five and three-tenths grams of hexaacetylactinamine was dissolved in the methanol, and the solution was allowed to stand at room temperature for 24 hr. The volatile material was removed by evaporation under reduced pressure. The residue was dissolved in boiling methanol, and the methanolic solution was concentrated until crystallization began. Refrigeration gave 1.45 g. of crystalline N,N'-diacetylactinamine, m.p. 243–247° dec. Two recrystallizations from methanol raised the melting point to 250–252° dec. There was no titratable group present. The infrared spectrum absorbed in the hydroxyl region at 3280 and 3180 cm.$^{-1}$ and in the amide carbonyl region at 1635 cm.$^{-1}$. The optical rotation was zero.

Anal. Calcd. for $C_{12}H_{22}N_2O_6$: C, 49.65; H, 7.58; N, 9.65; CH_3CO (2), 29.6. Found: C, 49.77; H, 7.65; N, 9.47; CH_3CO, 23.3.

Periodate Oxidation of N,N'-Diacetylactinamine. (a) Titration.—N,N'-Diacetylactinamine was titrated by the Fleury–Lange procedure[16] using a solution of 302.3 mg. in 50 ml. of 0.1 *M* sodium periodate and titrating 5-ml. aliquots. The consumption of periodate, in hours (moles): 0 (0.44), 0.5 (1.26), 1 (1.60), 2 (1.94), 4 (2.08), 8 (2.08), 24 (2.52).

(b) **Determination of Formic Acid.**—N,N'-diacetylactinamine (580 mg., 2.0 mmoles) was dissolved in 80 ml. of 0.1 *M* sodium periodate solution. Titration of a 10-ml. aliquot of this solution, after it had stood in the dark for 4 hr., indicated a periodate consumption of 2.1 moles per mole of N,N'-diacetylactinamine; 0.3 ml. of ethylene glycol was added to the remainder of the solution, and after 0.5 hr. a second 10-ml. aliquot was titrated with 0.1 *N* sodium hydroxide solution. This titration indicated that 0.88 mole of acid had been formed per mole of starting material.

Saturated barium hydroxide solution (100 ml.) was added to the remaining reaction mixture, and the mixture was filtered. The filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in 50 ml. of water, and the solution was again evaporated to dryness under reduced pressure. This procedure was repeated twice. The residue was dissolved in 20 ml. of water, and the solution was adjusted to pH 2.0 with 10% phosphoric acid. The acidic solution was distilled to dryness under reduced pressure. The residue was twice dissolved in 20 ml. of water and distilled to dryness in the same fashion. One-

(17) R. L. Peck, C. F. Hoffhine, E. W. Peel, R. R. Graber, F. W. Holly, R. Mozingo, and K. Folkers, *J. Am. Chem. Soc.*, **68**, 776 (1946).

fourth of the distillate was adjusted to pH 8.0 with 0.1 *N* sodium hydroxide solution, and the solution was evaporated to dryness under reduced pressure. The residue was dissolved in 150 ml. of water, and 20 ml. of 10% mercuric chloride solution, 10 ml. of saturated sodium acetate solution, and 2.0 ml. of 4.0 *N* hydrochloric acid was added. The resulting solution was heated in the dark on the steam-bath for 1 hr. Refrigeration, filtration, and drying gave 141 mg. of mercurous chloride. Calculated on the basis of starting material, this gave 1.57 mmoles (78%) of formic acid formed from 2.0 mmoles of N,N′-diacetylactinamine.

N,N′-Tetramethylstreptamine dihydrochloride was prepared as described by Witkop.[8] The melting point was 273° dec. (lit.[8] 275–276°).

N,N′-Dimethylactinamine dihydrochloride (IX) was prepared by Witkop's procedure.[8] The yield of methylated material melting at 253° dec. from 1.5 g. of actinamine was 1.65 g. This was recrystallized from methanol–acetone and methanol. The final melting point was 256–258° dec. with partial melting at 113–115° and resolidification. The product was optically inactive. The pK_a' values were 6.85 and 8.78.

Anal. Calcd. for $C_{10}H_{22}N_2O_4 \cdot 2HCl$: C, 39.09; H, 7.82; N, 9.13; CH_3N, 19.54; mol. wt., 307. Found: C, 38.91; H, 7.85; N, 9.01; CH_3N, 18.3; mol. wt. (elect. titr.), 318.

N,N′-Bis-(carbobenzoxy)-actinospectacin.—A solution containing 1.7 g. (5.3 mmoles) of actinospectacin in 50 ml. of pyridine was evaporated under reduced pressure to a volume of 25 ml. The residual solution was cooled to 0°, and 1.7 g. (10 mmoles) of benzyl chloroformate dissolved in 10 ml. of chloroform was added slowly. The mixture was slowly warmed to room temperature and allowed to stand overnight, then heated to 50° for 1 hr. After the solvent had been removed by evaporation under reduced pressure, the residue was slurried with water, and the mixture was extracted with ethyl acetate. The ethyl acetate solution was dried over magnesium sulfate and diluted with petroleum ether (60–70°) to give 1 g. of a tan powder. A 200-transfer countercurrent distribution of 750 mg. of the material in the system cyclohexane–ethyl acetate–ethanol–water (5:5:6:4) gave 54% of the starting material as a main peak (K = 0.32) which gave a fair fit to the theoretical curve. The product was isolated by evaporation of the solvent, solution of the residue in ethyl acetate and precipitation as above.

Anal. Calcd. for $C_{30}H_{36}N_2O_{11}$: C, 59.99; H, 6.04; N, 4.67. Found: C, 59.59; H, 6.24; N, 4.71.

N,N′-Bis-(carbobenzoxy)-O-acetylactinospectacin.—A dry pyridine solution of 5.0 g. (8.3 mmoles) of N,N′-bis-(carbobenzoxy)-actinospectacin was treated at room temperature with 2.5 ml. (2.5 mmoles) of acetic anhydride for 7 days. The mixture was stirred for 1 hr. with 1.2 ml. of water and evaporated to dryness under high vacuum. The residue was dissolved in ethyl acetate, and after the solution was washed several times with water it was dried over sodium sulfate. Part of the solvent was removed by evaporation, and the residue was diluted with petroleum ether (60–70°), precipitating 5.0 g. of solid. This material was purified by a 200-transfer countercurrent distribution using the solvent system 95% ethanol–water–ethyl acetate–cyclohexane (30:20:22:28). Only one major peak was located (K = 0.61), and 58% of the starting material was recovered under this peak by solvent evaporation. The infrared spectrum of this product had a new carbonyl band at 1755 cm.$^{-1}$.

Anal. Calcd. for $C_{32}H_{38}N_2O_{12}$: C, 59.80; H, 5.96; N, 4.36. Found: C, 59.73; H, 5.90; N, 4.22.

O-Acetylactinospectacin (IV).—A solution of 3 g. (4.7 mmoles) of N,N′-bis-(carbobenzoxy)-O-acetylactinospectacin in 100 ml. of ethylene glycol dimethyl ether was shaken with 0.3 g. of Pd–C catalyst under hydrogen at 40 p.s.i. for 3 hr. The mixture was filtered, and the filtrate was evaporated to dryness leaving 1.6 g. of residue as a white glass. The residue was dissolved in ethyl acetate and precipitated by addition of petroleum ether (60–70°). The product was a white amorphous solid having an infrared spectrum with a strong carbonyl band at 1730 cm.$^{-1}$ superimposed over the usual medium actinospectacin band and having absorption at 1225 cm.$^{-1}$ not usually present in actinospectacin.

Anal. Calcd. for $C_{16}H_{26}N_2O_8$: C, 51.35; H, 7.00; N, 7.48; O, 34.19. Found: C, 51.68; H, 7.09; N, 6.46; O, 34.11.

It was not definitely established that this compound still retained the ketonic carbonyl, but these conditions do not reduce actinospectacin.

Periodate Oxidation of O-Acetylactinospectacin.—O-Acetylactinospectacin was titrated by the Fleury–Lange procedure[16] using 0.08 *M* sodium metaperiodate solution. The consumption of periodate was: hours (moles): 0.25 (2.40), 2 (2.94).

N,N′-Diacetylactinospectacin (II).—A solution of 0.85 ml. (9 mmoles) of acetic anhydride in 10 ml. of pyridine was slowly added to a solution of 1 g. (3 mmoles) of actinospectacin in 20 ml. of pyridine cooled to 5°. After the solution had stood at room temperature for 7 days, it was evaporated to dryness under high vacuum. The residue was dissolved in ethyl acetate and precipitated with petroleum ether (60–70°). The precipitate was a white amorphous solid, wt. 1.02 g. A 200-transfer countercurrent distribution of this material in the system 1-butanol–water gave two materials (K = 0.11 and K = 0.38). The slower moving material was isolated by evaporation to give 160 mg. of amorphous solid. The product had no titratable groups and no absorption in the infrared spectrum in the region of 1745 cm.$^{-1}$.

Anal. Calcd. for $C_{18}H_{28}N_2O_9 \cdot H_2O$: C, 49.76; H, 6.96; N, 6.45; O, 36.83; CH_3C (3), 10.4. Found: C, 49.53, 49.20; H, 7.39, 7.07; N, 6.42; O, 36.66; CH_3C, 10.3.

Periodate Oxidation of N,N′-Diacetylactinospectacin. (a) Titration.—N,N′-Diacetylactinospectacin was titrated by the Fleury–Lange procedure[16] using 0.08 *M* sodium periodate solution. The consumption of periodate was, in hours (moles): 1 (0.69), 3 (0.93), 4 (0.88), 34 (0.86), 48 (1.1).

(b) Isolation of Actinamine.—Sodium metaperiodate (11.1 g., 0.051 mole) was added gradually over a period of 24-hr. to a solution of 1.1 g. (0.0025 mole) of N,N′-diacetylactinospectacin. The excess periodate was removed by precipitation with 15 g. of barium hydroxide octahydrate. Excess barium hydroxide was removed by precipitation with carbon dioxide. The mixture was filtered, and the filtrate was passed through a 2.5 cm. × 25 cm. column of Dowex 2 (Cl^-) resin to give a solution which gave a negative starch–iodide test. This solution was evaporated to 15 ml. under reduced pressure and acidified with 15 ml. of concd. hydrochloric acid. The resulting solution was heated under reflux for 2 hr. and allowed to stand overnight at room temperature. Addition of 350 ml. of acetone and seeding with actinamine dihydrochloride gave 410 mg. (60%) of actinamine dihydrochloride identified by comparison of its infrared spectrum with that of an authentic sample.

(c) Isolation of Glyoxylic Acid.—One gram (2.4 mmoles) of N,N′-diacetylactinospectacin and 1.07 g. (5.0 mmoles) of sodium periodate were dissolved in 25 ml. of water. After the solution had stood at room temperature for 2 hr., sufficient sodium bisulfite (about 2 g.) was added to decolorize the reaction mixture. The solution was filtered, and one-half of it was added to 250 ml. of Brady reagent. The resulting solution was heated for 0.5 hr. on the steam-bath and refrigerated for 36 hr. The yield of solid, after filtration and drying, was 0.20 g. (66%), m.p. 190–192°. A mixture melting point with an authentic sample of glyoxylic acid 2,4-dinitrophenylhydrazone gave no depression. The infrared spectrum of the product was also identical with an authentic sample.

Anal. Calcd. for $C_8H_6N_4O_6$: C, 37.80; H, 2.38; N, 22.04. Found: C, 37.67; H, 1.78; N, 22.29.

N,N′,O-Triacetylactinospectacin (III).—The faster moving fraction (K = 0.38) from the countercurrent distribution described in the preparation of N,N′-diacetylactinospectacin yielded 220 mg. of white amorphous solid upon evaporation of the solvent. This was dissolved in ethyl acetate and precipitated by addition of petroleum ether (60–70°). The product showed ester carbonyl absorption at 1745 cm.$^{-1}$ in the infrared. No titratable groups were present.

Anal. Calcd. for $C_{20}H_{30}N_2O_{10} \cdot H_2O$: C, 50.41; H, 6.77; N, 5.80; CH_3C (4), 12.6. Found: C, 50.76; H, 6.79; N, 5.97; CH_3C, 12.1.

Periodate Oxidation of N,N′,O-Triacetylactinospectacin.—N,N′,O-Triacetylactinospectacin was titrated by the Fleury–Lange procedure[16] using 0.08 *M* sodium periodate solution. The consumption of periodate was, in hours (moles): 1 (0.75), 4 (0.76).

N,N′-Bis-(ethylcarbamoyl)-actinospectacin (V).—A mixture of 10 g. (0.03 mole) of anhydrous actinospectacin and 4.3 g. (0.06 mole) of ethyl isocyanate in 1 l. of dry chloroform was stirred for 48 hr. The reaction mixture was filtered, and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in 30 ml. of warm acetone, and about 125 ml. of Skellysolve B was added. The precipitate was triturated until it had become solid and was removed by filtration. The dried product was distributed through 500 transfers in a countercurrent distribution apparatus using a 1-butanol–water system. The material from tubes 130–180 (K = 0.44) was combined and evaporated to dryness under reduced pressure. The residue was dissolved in 100 ml. of water, and the solution was filtered and freeze-dried. The residue melted at 210–218° dec. with prior softening at 188°, wt. 5.1 g., $[\alpha]^{25}D$ −8° (*c* 0.84, H_2O). The infrared spectrum had peaks at 3365, 1740, 1615, 1535, 1169, 1125, and 1158 cm.$^{-1}$. This compound had no titratable groups.

Anal. Calcd. for $C_{20}H_{34}N_4O_9$: C, 50.63; H, 7.23; N, 11.81; O, 30.3. Found: C, 50.51; H, 7.20; N, 12.01; O, 30.01.

Periodate Oxidation of N,N′-Bis-(ethylcarbamoyl)-actinospectacin. (a) Titration.—N,N′-Bis-(ethylcarbamoyl)-actinospectacin was titrated by the Fleury–Lange procedure[16] using 474 mg. (1.0 mmole) in 30 ml. of 0.1 *M* sodium metaperiodate solution. The consumption of periodate was, in hours (moles): 0 (0.93), 0.5 (0.98), 1 (0.95), 3.5 (1.08), 16 (1.24).

(b) **Isolation of Crotonic Acid.**—N,N′-Bis-(ethylcarbamoyl)-actinospectacin (0.95 g., 2.0 mmoles) was dissolved in 40 ml. of 0.0925 *M* sodium metaperiodate solution. After the solution had stood at room temperature for 2 hr., 25 ml. of 0.44 *N* barium hydroxide solution was added. The mixture was filtered, acidified with 25 ml. of concd. sulfuric acid, and steam distilled until 1.1 l. of distillate had been collected. The distillate was titrated with 0.1 *N* sodium hydroxide solution to a pH of 8.5. This required 12.1 ml., indicating 1.21 mmoles of volatile acid had been formed. The titrated solution was evaporated to dryness under reduced pressure. The residue was mixed with 0.3 g. of *p*-bromophenacyl bromide and 10 ml. of alcohol, and the mixture was heated under reflux for 3 hr. Dilution of the reaction mixture with water, refrigeration, and filtration gave 80 mg. of crystalline product. Recrystallization of the product from alcohol gave a product, m.p. 94–95°. This material had an infrared spectrum identical with the spectrum of an authentic sample of *p*-bromophenacyl crotonate, and a mixture melting point with an authentic sample showed no depression.

N,N′-Diacetyldihydroactinospectacin (XIV).—A solution of 10 g. (0.03 mole) of amorphous dihydroactinospectacin in 300 ml. of dry pyridine was concentrated to 100 ml. under reduced pressure. The residue was diluted with 200 ml. of dry pyridine, and 5.8 ml. (0.06 mole) of acetic anhydride was added. The solution was allowed to stand at room temperature for 8 days and distilled to dryness under reduced pressure (1 mm.). The residue was extracted with ethyl acetate. The solvent was removed from the extract by evaporation under reduced pressure. The residue was dissolved in 20 ml. of ethyl acetate, and this solution was diluted with petroleum ether (60–70°). The 900 mg. of tan powder so obtained was subjected to a 500-transfer countercurrent distribution in the solvent system 1-butanol–water. A single major peak was observed ($K = 0.2$). The product, a white amorphous powder, wt. 500 mg., was isolated from this fraction, $[\alpha]^{25}D$ +8.7° (*c* 1, ethanol).

Anal. Calcd. for $C_{18}H_{30}N_2O_9$: C, 51.66; H, 7.23; N, 6.70; CH_3C (3), 10.8. Found (cor. for water content): C, 51.40; H, 7.61; N, 7.18; CH_3C, 10.9.

Periodate Oxidation of N,N′-Diacetyldihydroactinospectacin. (a) Titration.—N,N′-Diacetyldihydroactinospectacin was titrated by the Fleury–Lange procedure[16] using 0.08 *M* sodium metaperiodate solution. After 4 hr., 1 mole of periodate was consumed. Three runs gave the same result.

(b) **Isolation of Crotonaldehyde and Glyoxylic Acid.**—Sodium metaperiodate (0.63 g., 3 mmoles) was added to a solution of 0.5 g. (1.2 mmoles) of N,N′-diacetyldihydroactinospectacin, purified by countercurrent distribution, in 30 ml. of water. After the solution had stood at room temperature for 2 hr., 0.75 g. of sodium bisulfite was added. The iodine was removed by filtration followed by extraction of the filtrate with chloroform; 400 ml. of Brady reagent was added to the colorless solution which was then heated for 15 min. After the solution had cooled to room temperature, filtration gave 240 mg. of orange crystals. Further standing and cooling gave 100 mg. of pale yellow crystals. Chromatography of the orange crystals over a silicic acid column, 2.5 × 75 cm., developed with the solvent system ether–benzene–petroleum ether (60–70°) (33:30:50) showed only one major component. From the extruded column 50 mg. of crystalline product was isolated. The ultraviolet and infrared spectra of this product were identical with those of authentic crotonaldehyde 2,4-dinitrophenylhydrazone.

Anal. Calcd. for $C_{10}H_{10}N_4O_4$: N, 22.39. Found: N, 22.14.

Recrystallization of the lighter colored crystals from water gave 40 mg. of product, m.p. 190°. Its ultraviolet and infrared spectra were identical with those of authentic glyoxylic acid 2,4-dinitrophenylhydrazone.

Anal. Calcd. for $C_8H_6N_4O_6$: N, 22.00. Found: N, 21.51.

N,N′,O,O′-Tetraacetyldihydroactinospectacin (XV).—The insoluble residue from the ethyl acetate extraction in the preparation of N,N′-diacetyldihydroactinospectacin was dissolved in 100 ml. of dry pyridine and 5.8 ml. of acetic anhydride was added. The solution was allowed to stand at room temperature for 8 days and was heated to 80° for 1 hr. The volatile material was removed by evaporation under reduced pressure. The residue was extracted with ethyl acetate, and the extract was diluted with petroleum ether (60–70°). The precipitate was a tan powder, wt. 8.5 g. This was subjected to countercurrent distribution in the system 1-butanol–water for 500 transfers. A major peak ($K = 1.42$) was located, and its solid content was isolated by evaporation under reduced pressure. The product was a white powder, wt. 1.65 g., no titratable groups, $[\alpha]^{25}D$ −8° (*c* 1, chloroform).

Anal. Calcd. for $C_{22}H_{34}N_2O_{11}$: C, 52.58; H, 6.82; N, 5.58; CH_3C (5), 14.9; CH_3CO (4), 33.50. Found: C, 52.39; H, 6.87; N, 5.37; CH_3C, 16.3; CH_3CO, 32.80.

Periodate Oxidation of N,N′,O,O′-Tetraacetyldihydroactinospectacin.—N,N′,O,O′-Tetraacetyldihydroactinospectacin was titrated by the Fleury–Lange procedure[16] using 0.08 *M* sodium metaperiodate solution. There was no consumption of periodate in 24 hr.

Actinospectinoic Acid (XVIII).—A solution of 5.0 g. of actinospectacin in 250 ml. of 0.1 *N* barium hydroxide solution was allowed to stand at room temperature for 24 hr. The barium ion was precipitated by addition of 2.0 *N* sulfuric acid, and the precipitate was removed by centrifugation. The supernatant was evaporated to dryness under reduced pressure. The residue was dissolved in 15 ml. of water and 250 ml. of acetone was added. Refrigeration and filtration gave 4.5 g. of crystalline product, m.p. 233–235° dec. Two recrystallizations from the same solvent did not change the melting point, $[\alpha]^{25}D$ −89° (*c* 1, H_2O); pK_a' values 3.3, 7.37, 9.33. The infrared spectrum had bands at 3440, 3340, 3150, 1635, 1595, 1485, 1225, 1160, 1125, 1080, 1070, 1055, and 1030 cm.$^{-1}$.

Anal. Calcd. for $C_{14}H_{26}N_2O_8$: C, 48.00; H, 7.43; N, 8.02; O, 36.55; mol. wt., 350. Found: C, 47.73; H, 7.35; N, 8.17; O, 37.23; mol. wt. (electr. titr.), 369.

Periodate Titration of Actinospectinoic Acid.—Actinospectinoic acid was titrated by the Fleury–Lange procedure[16] using 106.4 mg. (0.3 mmole) dissolved in 20 ml. of 0.1 *M* sodium periodate solution. The consumption of periodate was, in hours (moles): 0 (3.20), 0.75 (3.60), 2 (3.70), 4 (3.96), 8 (4.12), 24 (4.47).

Acid Hydrolysis of Actinospectinoic Acid without Steam Distillation.—A solution of 2 g. of actinospectinoic acid in 35 ml. of 1 *N* hydrochloric acid was mixed with 1.7 l. of Brady reagent, and the reaction mixture was allowed to stand at room temperature for 93 hr. The orange precipitate which formed was removed after 21, 43, and 93 hr. The combined yield was 1.15 g. Each precipitate was recrystallized separately from acetone, and all the recrystallized materials had the same melting points and the same ultraviolet and infrared spectra. The first fraction melted at 247–249°, $[\alpha]^{25}D$ +345° (*c* 0.454, nitrobenzene). The ultraviolet spectrum had λ_{sh} 244 mμ (ε 16,450), λ_{sh} 255 mμ (ε 15,350), λ_{sh} 295 mμ (ε 4280), λ_{max} 400 mμ (ε 33,800), and λ_{max} 437.5 mμ (ε 35,900). The infrared spectrum had bands at 3620, 3535, 3485, 3200, 3100, 1655, 1612, 1596, 1578, 1525, 1500, 1255, 1218, 1140, 1082, 1050, and 740 cm.$^{-1}$.

Anal. Calcd. for $C_{17}H_{16}N_8O_9$: C, 42.89; H, 3.40; N, 23.54; O, 30.25; CH_3C (1), 3.15. Found: C, 42.98; H, 3.71; N, 22.69; O, 30.57; CH_3C, 3.6.

Acid Hydrolysis of Actinospectinoic Acid with Steam Distillation.—A solution of 5 g. of actinospectinoic acid in 300 ml. of 1.33 *N* sulfuric acid was steam distilled until a total of 1800 ml. of distillate had been collected. The distillate was mixed with 6.75 l. of Brady reagent and allowed to stand at room temperature for 24 hr. Filtration of the reaction mixture gave 2.25 g. of orange solid. Two recrystallizations of a portion of the product from acetone gave a m.p. of 223–225°. This material was optically inactive. Its ultraviolet spectrum had absorption at λ_{sh} 244 mμ (ε 4320), λ_{sh} 296 mμ, λ_{max} 400 mμ (ε 8300), and λ_{max} 430 mμ (ε 8560). The infrared spectrum had significant bands at 3295, 3260, 3095, 1612, 1590, and 1540 cm.$^{-1}$.

Anal. Calcd. for $C_{17}H_{14}N_8O_8$: C, 44.58; H, 3.10; N, 24.47; O, 27.95. Found: C, 44.76; H, 3.92; N, 21.88; O, 28.46.

The residue from the steam distillation was filtered and mixed with 1.5 l. of ethanol. After 4 hr. refrigeration the crystalline precipitate was isolated by filtration. The filter cake was washed with 50 ml. of ethanol and 50 ml. of ether and dried; wt. 4.4 g. The infrared spectrum had bands at 3320, 3080, 2720, 2500, 2415, 1627, 1595, 1192, 1105, 1045, and 1024 cm.$^{-1}$, but no maximum appeared in the ultraviolet spectrum above 220 mμ. The pK_a' values were 7.32 and 9.55.

Anal. Calcd. for $C_8H_{18}N_2O_4 \cdot H_2SO_4$: C, 31.61; H, 6.63; N, 9.22; O, 42.11; S, 10.55; mol. wt., 304. Found: C, 32.28; H, 6.42; N, 8.62; O, 43.03; S, 10.54; mol. wt. (electr. titr.), 304.

Carbon Dioxide from Acid Hydrolysis of Actinospectinoic Acid.—Nitrogen was passed through a solution of 2 g. of actinospectinoic acid in 1 l. of Brady reagent and into a trap containing saturated aqueous barium hydroxide solution for 48 hr. The precipitate was removed from the trap and dried; wt. 450 mg. The product was identified as barium carbonate by its infrared spectrum. The yield was 39.5%.

Crotonaldehyde and Formaldehyde from Actinospectinoic Acid.—A solution of 5 g. of actinospectinoic acid in 300 ml. of 1.33 *N* sulfuric acid was steam distilled until a total of 1800 ml. distillate was collected. The distillate showed substantial ultraviolet absorption at 230 mμ. The distillate was adjusted to pH 8.5 with sodium bicarbonate, mixed with a solution of 5 g. of sodium borohydride in 50 ml. of water, and allowed to stand at room temperature for 2 hr. The reaction mixture was adjusted to pH 1.0 with 6 *N* hydrochloric acid. The acidic solution showed no ultraviolet maximum at 230 mμ. After the solution had been adjusted to pH 6.5, a solution of 10 g. of sodium metaperiodate in 200 ml. of water was added. After the reaction mixture had stood at room temperature for 40 min., it was adjusted to pH 9.5 with saturated barium hydroxide solution. The precipitate

was removed by filtration, and the filtrate was adjusted to pH 7.0. The neutral solution was steam distilled, and seven 100-ml. fractions were collected. The first fraction (λ_{max} 227 mμ, a 394) was mixed with 1.5 l. of Brady reagent and allowed to stand at room temperature for 12 hr. The precipitate was isolated by filtration and recrystallized once from acetic acid and a second time from benzene–petroleum ether (60–70°); m.p. 184–186° (lit. for crotonaldehyde 2,4-dinitrophenylhydrazone, 187–188°). The infrared spectrum was identical with that of an authentic sample of crotonaldehyde 2,4-dinitrophenylhydrazone.

The fourth fraction from the steam distillation was combined with 500 ml. of Brady reagent and allowed to stand at room temperature for 12 hr. The precipitate was isolated by filtration and recrystallized from ethanol, m.p. 164–166° (lit. for formaldehyde 2,4-dinitrophenylhydrazone, 164–166°). The infrared spectrum was identical with that of an authentic sample of formaldehyde 2,4-dinitrophenylhydrazone.

Methyl Actinospectoic Acid Methyl Ester (XXII).—Ten grams of actinospectinoic acid was added to a mixture of 95 ml. of acetyl chloride and 600 ml. of methanol, and the solution was allowed to stand at room temperature for 48 hr. The precipitated actinamine dihydrochloride (wt. 8.05 g.) was removed by filtration. The filtrate was mixed with an equal volume of ether and allowed to stand at room temperature for 24 hr. A second precipitate had formed and was removed by filtration. The filtrate was concentrated to a volume of 340 ml. under reduced pressure and adjusted to a pH of 6.4 with methanolic sodium hydroxide prepared by dissolving 12 g. of sodium hydroxide in 400 ml. of absolute methanol. After the precipitate had been removed by filtration, the filtrate was concentrated to 450 ml. and again filtered. This filtrate was concentrated to 150 ml., and 500 ml. of ether and 300 ml. of acetone were added. The mixture was allowed to stand at room temperature for 12 hr. and filtered. The organic solvents were removed from the filtrate by evaporation under reduced pressure, leaving 6.0 g. of viscous liquid. The liquid was fractionated, collecting the material boiling at 51–54° at 0.05 mm. The infrared spectrum of the distillate had absorption bands at 3450 and 1750–1740 cm.$^{-1}$ and $[\alpha]^{25}D$ −65.5° (c 0.998, 95% ethanol).

Anal. Calcd. for $C_8H_{14}O_5$: C, 50.57; H, 7.43; O, 42.11; $CH_3C(3)$, 7.85; $CH_3O(2)$, 32.6. Found: C, 49.49; H, 7.75; O, 43.31; CH_3C, 6.8; CH_3O, 26.62.

N,N′-Bis-(ethylcarbamoyl)-actinospectinoic Acid (XIX).—A solution of 5.0 g. of N,N′-bis-(ethylcarbamoyl)-actinospectacin in 25 ml. of 0.1 N barium hydroxide was allowed to stand at room temperature for 5 hr. Sufficient 0.1 N sulfuric acid was added to remove barium ion, and the precipitate was removed by centrifugation. The supernatant was filtered and freeze-dried. The residue was distributed through 500 transfers in a countercurrent distribution apparatus using a 1-butanol–water system. Tubes 180–260 were combined and evaporated to dryness under reduced pressure. The residue was dissolved in 50 ml. of water, and the solution was freeze-dried giving 1.35 g. of amorphous solid, m.p. 165–171° dec., $[\alpha]^{25}D$ −63° (c 1, H_2O). The pK_a' of this material was 3.57. The infrared spectrum had bands at 3390, 1720, 1606, 1535, 1245, 1190, 1055, and 1023 cm.$^{-1}$.

Anal. Calcd. for $C_{20}H_{36}N_4O_{10}$: C, 48.77; H, 7.37; N, 11.40; mol. wt., 474. Found: C, 49.22; H, 7.34; N, 11.06; mol. wt. (elect. titr.), 481.

Periodate Titration of N,N′-Bis-(ethylcarbamoyl)-actinospectinoic Acid.—This was run by the Fleury–Lange procedure[16] using 492 mg. (1.04 mmoles) dissolved in 30 ml. of 0.1 M sodium periodate solution. There was no periodate consumption in 3.5 hr.

[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF PITTSBURGH SCHOOL OF MEDICINE, PITTSBURGH 13, PENNA.]

Insulin Peptides. VII. The Synthesis of Two Decapeptide Derivatives Containing the C-Terminal Sequence of the B-Chain of Insulin[1]

BY PANAYOTIS G. KATSOYANNIS AND (IN PART) KENJI SUZUKI

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The partially protected decapeptide γ-methyl-L-glutamyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-alanine methyl ester dihydrochloride and the fully protected decapeptide N-carbobenzoxy-γ-benzyl-L-glutamyl-N^{ω}-tosyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-alanine methyl ester have been synthesized and their chemical and stereochemical homogeneity has been established. These decapeptide derivatives contain amino acid sequences found in the C-terminal portion of the B-chain of insulin.

Studies are under way in this laboratory directed toward the synthesis of the A- and B-chains of insulin and eventually to the total synthesis of this protein.[2] To this end several peptide derivatives embodying within their structures amino acid sequences found in the insulin chains have been prepared and the detailed synthesis of a number of these derivatives has been already reported.[3–8]

As a step toward the synthesis of the B-chain of insulin the preparation of certain derivatives of the decapeptide L-glutamyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-L-lysyl-L-alanine was desired. This decapeptide contains the C-terminal sequence of the B-chain of insulin. Peptide derivatives containing some of the amino acid sequences of the above decapeptide have been prepared by other investigators.[9–11] In the present communication the synthesis of the partially protected decapeptide γ-methyl-L-glutamyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-alanine methyl ester dihydrochloride (V) and of the fully protected decapeptide N-carbobenzoxy-γ-benzyl-L-glutamyl-N^{ω}-tosyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-alanine methyl ester (VII) is reported.

The key intermediate in the synthesis of these derivatives was the protected heptapeptide N-carbobenzoxy-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-alanine methyl ester, whose preparation has been reported previously.[5] Removal of the carbobenzoxy group from the protected heptapeptide by catalytic hydrogenation and coupling of the resulting product with the N-carbobenzoxyglycine p-nitrophenyl ester[12] afforded the protected octapeptide N-carbobenzoxyglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-alanine methyl ester (I) in 80% yield. The

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(2) A preliminary report of the work described in this paper and of the work carried out thus far in our laboratory which is directed toward the synthesis of insulin has been presented (P. G. K.) in the Eighth National Medicinal Chemistry Symposium of the American Chemical Society held in Boulder, Colo., June 18–20, 1962.

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