[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE UPJOHN CO.]

The Structure of Novobiocin[1,2]

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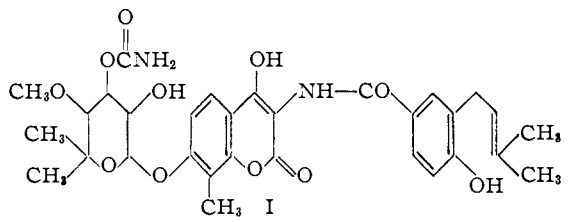
The antibiotic novobiocin[2] has been shown to be 7-[4-(carbamoyloxy)-tetrahydro-3-hydroxy-5-methoxy-6,6-dimethyl-pyran-2-yloxy]-4-hydroxy-3-[4-hydroxy-3-(3-methyl-2-butenyl)-benzamido]-8-methylcoumarin (I).

Novobiocin, elaborated by *Streptomyces niveus*, is a crystalline, acidic antibiotic of the composition $C_{31}H_{36}N_2O_{11}$. Studies on the fermentation,[3-4] extraction, purification and properties,[5,6] biological activity[7] and clinical usefulness[8] have been reported.

Because of its interesting antibacterial action and its clinical efficacy, the structure of novobiocin is of particular significance. Evidence is presented in this paper to show that novobiocin possesses structure I.



I

General Characteristics.—Novobiocin crystallized in two polymorphic modifications, one melting at 174–178° and the other at 152–156°.[5-6] The dibasic acid properties of the compound were indicated by the preparation of both acid and neutral salts. Potentiometric titrations and ultraviolet spectral shifts confirmed these observations and showed novobiocin to be a dibasic acid with pK'_a values of 4.3 and 9.1 in aqueous solution. The ultraviolet spectra in acid, neutral and alkaline solutions are given in Fig. 1. The ultraviolet spectra showed a characteristic hypsochromic shift of the phenolindophenol type in going from acid to alkaline solution. Isosbestic points linked the curves for acid to acid salt and acid salt to neutral salt transitions. Infrared spectra indicated the presence of a monosubstituted amide, hydroxyl groups, ether linkages, conjugate C=C unsaturation and possible isopropylidene and urethan groups. One methoxyl and at least two C-methyl groups were detected by analytical group determinations.

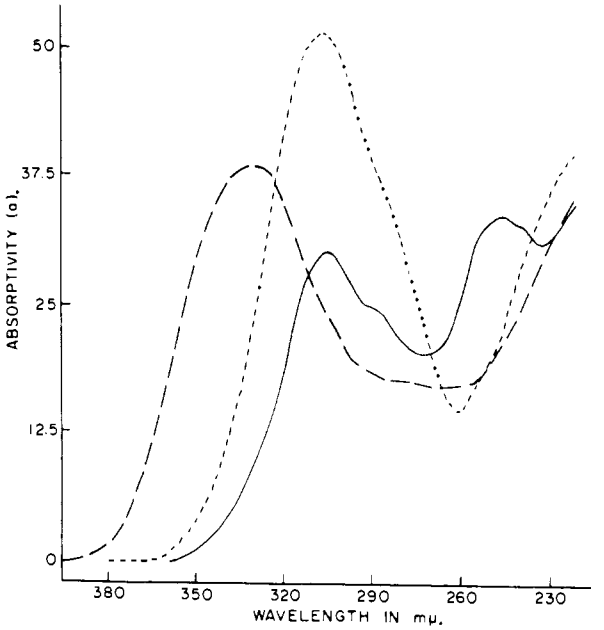


Fig. 1.—Ultraviolet spectra of novobiocin, solvents: ———, 70% ethanolic 0.01 *N* phosphate buffer. *p*H 7.5; - - - - - -, alkaline ethanol; – – – – – –, acid ethanol.

Investigation of the polarographic behavior of novobiocin revealed no groups reducible below −1.5 v. *vs.* S.C.E. in acid solution or below −2.0 v. *vs.* S.C.E. in neutral on alkaline solution. These findings virtually eliminated the possible presence of nitro groups, aromatic aldehyde or keto groups, conjugate unsaturation other than an aromatic system and conjugated aliphatic acid groups.

On reaction with one mole of diazomethane the stronger acid group was neutralized with the formation of a methyl derivative whose ultraviolet spectrum closely resembled the alkaline curve of novobiocin and which did not alter appreciably with change of *p*H. This observation, considered with the absence of carboxylate absorption in the infrared, indicated that the pK'_a 4.1 function was enolic. The pK'_a value of the other acid function was in the range for a phenol, although novobiocin failed to

(1) Novobiocin. IV. The investigations upon which this paper is based were first reported in a series of preliminary communications. (a) (THIS JOURNAL, **77**, 6710 (1955); (b) **78**, 1072; (c) **78**, 2019 (1956)). These findings are in agreement with those reported independently by the Merck group (d) (*ibid.*, **78**, 1770 (1956)).

(2) The Upjohn Co. Registered Trademark for novobiocin is Albamycin. Our former generic name, streptonivicin, has been abandoned.

(3) C. G. Smith, A. Dietz, W. T. Sokolski and G. M. Savage, *Antibiotics and Chemotherapy*, **6**, 135 (1956).

(4) H. Wallick, D. A. Harris, M. A. Reagan, M. Ruger and H. B. Woodruff, *Antibiotics Annual*, 909 (1955–1956).

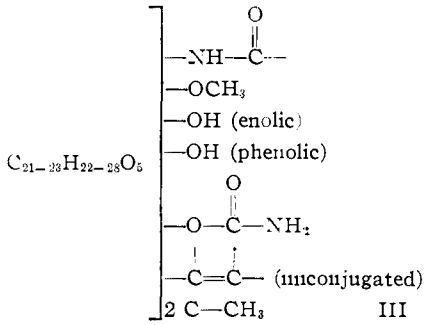
(5) H. Hoeksema, M. E. Bergy, W. G. Jackson, J. W. Shell, J. W. Hinman, A. E. Fonken, G. A. Boyack, E. L. Caron, J. H. Ford, W. H. DeVries and G. F. Crum, *Antibiotics and Chemotherapy*, **6**, 143 (1956).

(6) E. A. Kaczka, F. J. Wolf, F. Rathe and K. Folkers, THIS JOURNAL, **77**, 6404 (1955).

(7) (a) J. R. Wilkins, C. Lewis and A. R. Barbiers, *Antibiotics and Chemotherapy*, **6**, 149 (1956); (b) R. M. Taylor, W. T. Sokolski, G. M. Savage and M. J. Vander Brook, *ibid.*, **6**, 157 (1956); (c) E. J. Larson, N. D. Connor, O. F. Swoap, R. A. Runnells, M. C. Prestrud, T. E. Eble, W. A. Freyburger, W. Veldkamp and R. M. Taylor, *ibid.*, **6**, 226 (1956); (d) B. M. Frost, M. E. Valiant, L. McClelland, M. Solotorovsky and A. C. Cuckler, *Antibiotic Annual*, 918 (1955–1956); (e) W. F. Verwey, A. K. Miller and M. K. West, *ibid.*, 924 (1955–1956); (f) M. Finland, *ibid.*, 929 (1955–1956).

(8) (a) Feng-Kai Lin and L. L. Coriell, *ibid.*, 634 (1955–1956); (b) W. J. Martin, F. R. Heilman, D. R. Nichols, W. E. Wellman and J. E. Geraci, *Proc. Staff Meetings Mayo Clinic*, **30**, 540 (1955); (c) N. A. David and P. R. Burgner, *Antibiotic Medicine*, **2**, 219 (1956); (d) G. Lubash, J. Van Der Meulen, C. Berntsen, Jr., and R. Tompsett, *ibid.*, **2**, 233 (1956); (e) R. L. Nichols and M. Finland, *ibid.*, **2**, 241 (1956); (f) B. M. Limson and M. J. Romansky, *ibid.*, **2**, 277 (1956); (g) M. B. Milberg, R. D. Schwartz and J. N. Silverstein, *ibid.*, **2**, 286 (1956).

give a typical ferric chloride reaction for an unhindered phenol. Elemental analyses and molecular weight determinations on the intact molecule gave variable results because of solvation. For this reason only an approximate formula of $C_{30-31}H_{38-42}N_2O_{11}$ was established prior to degradation studies. Mild catalytic hydrogenation of I yielded a dihydro-derivative (II) whose ultraviolet and infrared spectra were almost identical with those of I indicating saturation of an unconjugated double bond. Dihydronovobiocin was found to be fully as active as novobiocin both *in vitro* and *in vivo*. The tentative findings on the intact molecule may be summarized by III.

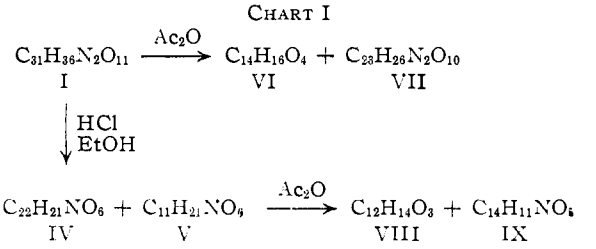


III

Preliminary Degradation Studies.—Cleavage of I into three conveniently sized moieties was accomplished by acid alcoholysis and by the action of hot acetic anhydride. Heating I in acid ethanol yielded IV, an optically inactive acid ($C_{22}H_{21}NO_6$),[9] and V, an optically active, neutral compound ($C_{11}H_{21}NO_6$) which appeared to be a glycoside. When an attempt was made to acetylate I using hot acetic anhydride in pyridine, an unexpected cleavage occurred. Instead of obtaining the acetylated antibiotic, two crystalline acetates were isolated: an optically inactive acid (VI) of the composition $C_{14}H_{16}O_4$ and an optically active neutral compound (VII) with the formula $C_{23}H_{26}N_2O_{10}$. In addition to splitting the molecule, it therefore appeared that acetic anhydride introduced three acetyl groups. Further study of the reaction showed that it proceeded in acetic anhydride alone but not with pyridine alone or with pyridine in combination with water, hydrochloric acid or glacial acetic acid. Carbon dioxide was not a product of the reaction. When the acid alcoholysis product, IV, was heated with acetic anhydride and pyridine, a similar cleavage and acetylation reaction took place to yield VIII ($C_{12}H_{14}O_3$), an optically inactive acid, and neutral IX ($C_{14}H_{11}NO_5$). These findings are summarized in Chart I.

Considering the composition of the various degradation products, VI through IX, and the methods by which they were obtained, the $C_{31}H_{36}N_2O_{11}$ formula for I was indicated. It also seemed likely that there were three main moieties in the molecule. Thus if I were represented by ABC, acetic anhydride cleaved the A–B linkage, and acid–ethanol the B–C linkage. The general properties of the degradation products indicated that those related to the A moiety were substitued benzoic acids, those related to B, an aromatic heterocyclic compound, and those related to C, some type of sugar.

CHART I



The Substituted Benzoic Acid.—Because of their apparent simplicity, the acetic anhydride degradation products VI and VIII were investigated first. The latter, a $C_{12}H_{14}O_3$ compound, m.p. 181–183°, titrated as a monobasic acid with pK'_a 6.3 in 66% ethanol. It gave a negative ferric chloride test. In the ultraviolet its spectrum showed a maximum at 258 mμ (a = 57.9) in alkaline ethanol and at 269 mμ (a = 67.2) in acid ethanol. An infrared doublet at 1385 and 1370 cm.$^{-1}$ in carbon tetrachloride solution suggested the presence of a *gem*-dimethyl group. This interpretation was consistent with a low C-methyl analysis. Compound VIII was identified as 2,2-dimethyl-6-carboxychroman[12] by comparison with an authentic sample.

Compound VI, the $C_{14}H_{16}O_4$ acid obtained directly from I by the action of hot acetic anhydride, titrated as a monobasic acid with pK'_a 5.67 in 50% ethanol. The infrared spectrum, similar to that of acetylsalicylic acid, contained bands which could be accounted for by carbonyl, ester, ethylenic and C–O linkages. The ferric chloride test was negative, but hydrolysis with 0.5 N sodium hydroxide deacetylated it to a dibasic acid (XI) with pK'_a values of 6.2 and 11.0 in 66% ethanol. In the ultraviolet XI exhibited maxima at 260 mμ (a = 68.2) in acid ethanol and 288 mμ (a = 76.2) in alkaline ethanol characteristic of *p*-hydroxybenzoic acids (see Fig. 2). An infrared band at 842 cm.$^{-1}$ indicated the presence of an isopropylidene group. With ferric chloride XI gave a red-brown color suggesting steric hindrance of the phenolic hydroxyl. Successive oxidations of VI with osmium tetroxide and sodium periodate yielded acetone as a major product. The side-chain was shown therefore to be the pentenyl group. Its position on the aromatic ring was determined when XI was heated in 4 N hydrochloric acid in 70% ethanol and cyclized to 2,2-dimethyl-6-carboxychromane (VIII) *via* the ethyl ester.

Under mild hydrogenation conditions VI consumed one mole of hydrogen to yield X, $C_{14}H_{18}O_4$. This compound, with absorption spectra very similar to those of VI, was also obtained by the action of acetic anhydride on dihydronovobiocin (II). This conveniently established the site of hydrogenation in novobiocin. Thus, the structure 4-acetoxy 3-(3-methyl-2-butenyl)-benzoic acid was assigned

(9) Although first described by this Laboratory,[1a] this compound was named *cyclonovobiocic acid* and synthesized later by the Merck group.[10,11]

(10) C. F. Spencer, C. H. Stammer, J. O. Rodin, E. Walton, F. W. Holly and K. Folkers, THIS JOURNAL, **78**, 2655 (1956).

(11) E. A. Kaczka, C. H. Shunk, J. W. Richter, F. J. Wolf, M. M. Gasser and K. Folkers, *ibid.*, **78**, 4125 (1956).

(12) A. M. Lauer and O. Moe, *ibid.*, **65**, 289 (1943). We gratefully acknowledge receipt of a sample of this compound used for identification of VIII.

to VI, 4-acetoxy-3-(3-methylbutyl)-benzoic acid to X and 4-hydroxy-3-(3-methyl-2-butenyl)-benzoic acid to XI (see Chart II).

CHART II

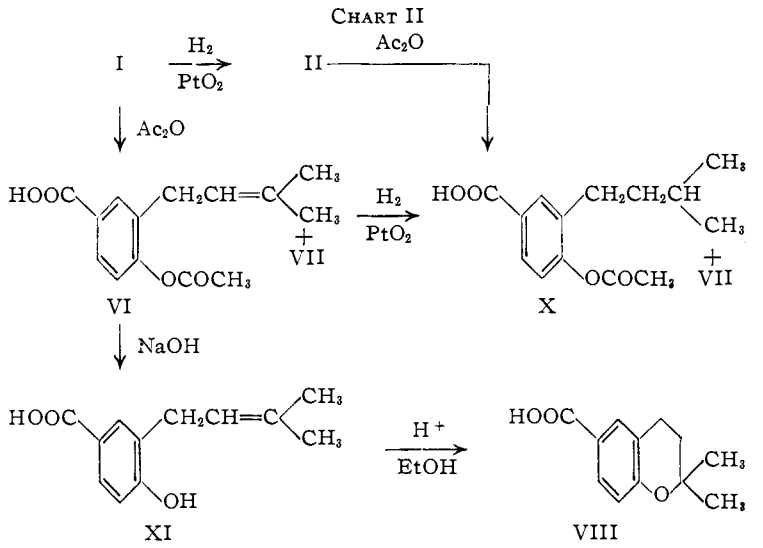
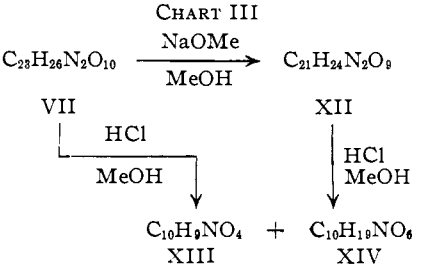


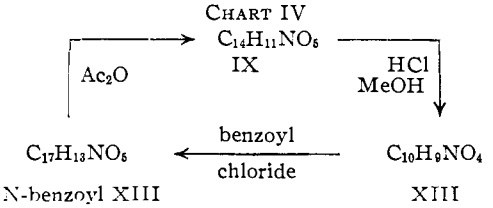
CHART III



The Heterocyclic Center Moiety.—As indicated in Chart I, the action of hot acetic anhydride on I provided, in addition to VI, an optically active, neutral compound, VII. This compound, m.p. 168–172°, solvated readily, especially when crystallized from acetone, but its composition was determined finally as $C_{23}H_{26}N_2O_{10}$. Its ultraviolet spectra showed characteristic fine structure between 280 and 340 mμ in neutral and acid solution and gave evidence of irreversible degradation in alkaline solution. Sodium methoxide in boiling methanol caused removal of one acetyl group with formation of another neutral compound, $C_{21}H_{24}N_2O_9$ (XII), m.p. 276–280°. The ultraviolet absorption was essentially the same as that of VII. Hot methanolic hydrogen chloride cleaved both VII and XII to optically inactive, amphoteric XIII and neutral, optically active XIV. These reactions are summarized in Chart III. The two methanolysis products were separated by fractional crystallization, XIII being the less soluble.

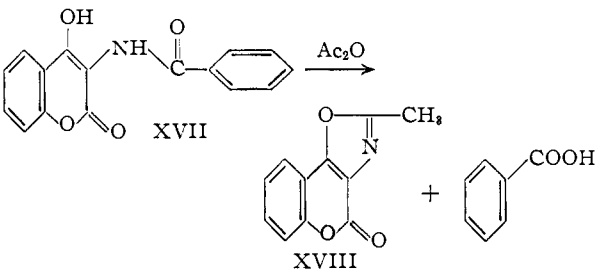
Compound XIII was isolated as the hydrochloride, but recrystallization from water or aqueous ethanol gave a halogen-free zwitterion-like material which decomposed above 200°. Both the hydrochloride and the free base gave a pink color with ferric chloride, a positive Benedict test, and an intense blue color with ninhydrin in pyridine. The ultraviolet spectra showed maxima at 238 mμ (a = 57.8) and 301 mμ (a = 34.5) and 347 mμ (a = 104.5) in alkaline ethanol. This same amphoteric material was obtained from IX (see Chart I) by acid hydrolysis (see Chart IV).

CHART IV



Benzoylation of XIII under Schotten–Baumann conditions gave an acidic monobenzoyl derivative of the composition $C_{17}H_{13}NO_5$. It appeared, therefore, that XIII had an aromatic nucleus substituted with an amino group and a strongly acid enol. Group analyses indicated the presence of one C-methyl group. Elemental analyses and ultraviolet absorption spectra indicated the presence of a neutral or phenolic hydroxyl group. On reaction with nitrous acid, XIII formed a yellowish diazo compound, $C_{10}H_7N_2O_4$ (XV). The diazo group was detected by intense infrared absorption at 2165 cm.$^{-1}$. The properties and reactions of XIII suggested a close resemblance to 3-amino-4-hydroxycoumarin[13] (XVI). The latter, generously supplied by Professor K. P. Link and also prepared according to his procedure,[13] served as a useful model compound.

Benzoylation of XVI provided 3-benzamido-4-hydroxycoumarin (XVII) whose ultraviolet spectra and other properties were similar to those of the unknown benzoyl derivative of XIII. Actually the spectra of 3-benzamido-4-hydroxycoumarin, which has no phenolic hydroxyl, exhibited the same unusual hypsochromic shift in going from acid to alkaline solution as did novobiocin itself. With the model compound the maxima occurred at 305 mμ in alkaline ethanol and at 320 mμ in acid ethanol *vs.* 308 and 333 mμ, respectively, for novobiocin. On heating with acetic anhydride, the model benzamido compound XVII yielded benzoic acid and a cyclized derivative whose properties and reactions



(13) C. F. Huebner and K. P. Link, THIS JOURNAL, **67**, 99 (1945).

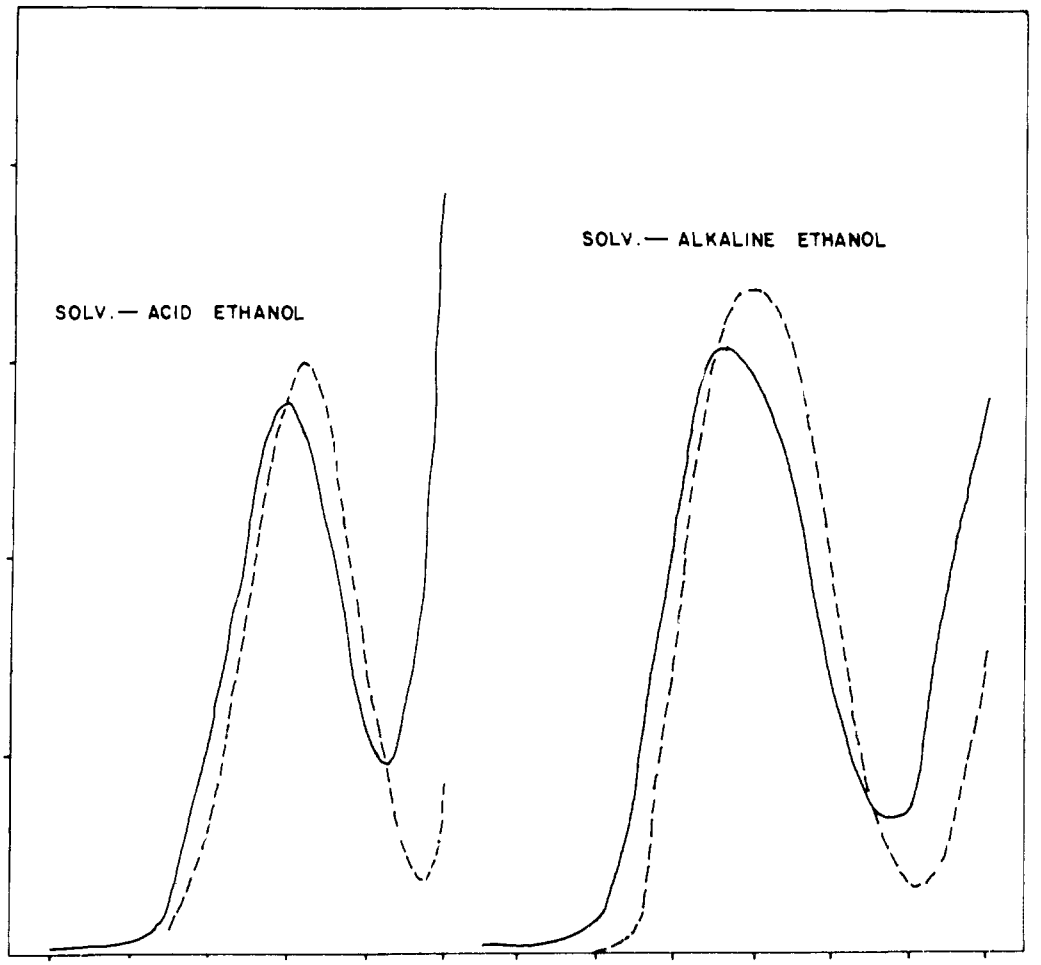
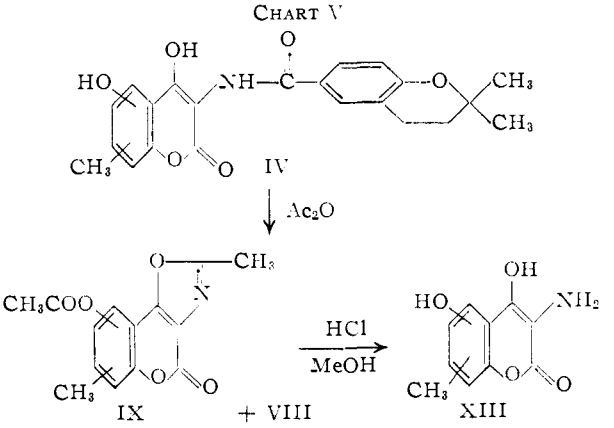


Fig. 2.—Ultraviolet spectra of *p*-hydroxybenzoic acid (- - - -) and compound XI (———).

showed it to be 2-methyl-4H[1]benzopyrano[3,4-d]oxazol-4-one (XVIII). The ultraviolet spectrum of XVIII in neutral and acid solution exhibited the same characteristic fine structure and irreversible destruction by alkali as did the spectra of the acetic anhydride products VII and IX (Fig. 3). Furthermore, treatment of N-benzoyl XIII with acetic anhydride caused elimination of benzoic acid and reformation of IX. This ready replacement of the benzoyl group by acetyl and cyclization to the oxazole derivative provided an explanation for the somewhat unexpected course of the acetic anhydride cleavage of I. It was concluded, therefore, that XIII was a hydroxyl- and methyl-substituted 3-amino-4-hydroxycoumarin and that IX was the acetoxy- and methyl-substituted analog of XVIII. Accepting these structures, the conversion of cyclonovobiocic acid (IV) to XIII was explained readily (Chart V).

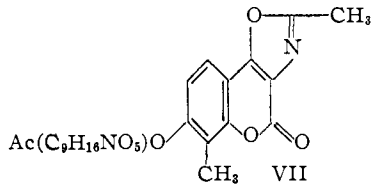
The assignment of the positions of the hydroxyl and methyl groups to the coumarin ring was made by converting XIII to a dihydroxymethylbenzoic acid. Hydrolysis of XIII with 1 *N* sodium hydroxide at room temperature or heating XIII in Benedict reagent gave complex colored mixtures which were separated by chromatography and the desired component purified by sublimation. The crystalline acid, obtained in 15–20% yields, melted with sublimation at 210–225°, gave a bluish-purple ferric chloride test and analyses indicated a formula of $C_8H_8O_4$ with one C–CH_3 group. Ultraviolet absorption studies indicated the presence of two phenolic hydroxyl groups.

CHART V



Attempts to decarboxylate the isolated dihydroxymethylbenzoic acid failed, but fusion of XIII in potassium hydroxide gave a 34% yield of a dihydroxytoluene melting at 115–120°. After sublimation, the colorless crystals melted at 120–122° and

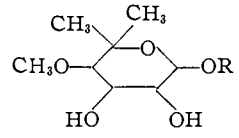
were identified as 2-methylresorcinol.[14] The identity of the isolated acid was confirmed as 2,4-dihydroxy-*m*-toluic acid by melting point and infrared comparison with an authentic sample[15] obtained by synthesis from 2,4-dihydroxybenzoic acid. Thus XIII was shown to be 3-amino-4,7-dihydroxy-8-methylcoumarin and VII to have the partial structure.



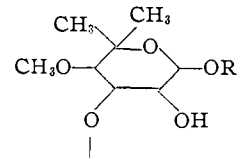
VII

The Sugar Moiety.—An optically active sugar-like degradation product was obtained either by heating I in acid ethanol[16] or by methanolic hydrogen chloride treatment of VII. The crystalline products $C_{11}H_{21}NO_6$ (V) and $C_{10}H_{19}NO_6$ (XIV) appeared to be ethyl and methyl glycosides, respectively, of the same $C_9H_{16}NO_6$ sugar. Neither compound was oxidized by periodate under the conditions of the Fleury and Lange procedure.[17] The preparation of monoacetate and monomesyl derivatives indicated the presence of one free hydroxyl group in V and XIV. The presence of one methoxyl in addition to the glycosidic alkoxyl was shown by analysis. A low C-methyl analysis (about 30% of the value calculated for one C-methyl) suggested the possibility of a *gem*-dimethyl group. A doublet at 1382 and 1366 cm.$^{-1}$ in the infrared spectra of these compounds supported this contention. Hydrolysis of XIV with hot aqueous acid caused loss of the nitrogen as ammonium chloride. This eliminated an acylamino group as a possibility for the nitrogen-containing function. When heated under reflux in methanolic hydrogen chloride, XIV yielded (in addition to ammonium chloride) a crystalline sugar derivative, $C_{10}H_{16}O_6$ (XIX), whose infrared spectrum showed a complete absence of OH/NH absorption and a strong band at 1785 cm.$^{-1}$ suggestive of a carbonate ester carbonyl. Upon treatment with barium hydroxide at room temperature, XIX gave nearly the theoretical yield of barium carbonate and a new glycoside, $C_9H_{18}O_5$ (XX). Repetition of this reaction with XIV and extending the reaction time somewhat gave ammonia, barium carbonate and a $C_9H_{18}O_5$ glycoside (XXI) as the products. These reactions and the physical-chemical data provided conclusive evidence for the presence of a carbamate group and further indicated the carbamate to be located on a carbon atom adjacent to the free hydroxyl group.

The isomeric methyl glycosides XX and XXI were shown to be anomers by hydrolysis to the same sugar aldehyde XXII. Both XX and XXI consumed one mole of periodate[17] cleanly and on oxidation with chromic acid gave acetone in 46% yield as the dinitrophenylhydrazone. This latter finding furnished confirmatory chemical evidence for the presence of the *gem*-dimethyl group. Analyses on the crystalline sugar aldehyde XXII were in agreement for the formula $C_8H_{16}O_5$ indicating retention of one methoxyl group under acid conditions. Positive reactions were obtained with Benedict and 2,4-dinitrophenylhydrazine reagents. Consumption of two moles of periodate with the formation of two moles of formic acid indicated the two free hydroxyl groups of XX and XXI to be adjacent to the no. 1 or glycosidic carbon atom of the sugar. It appeared, therefore, that these glycosides were α- and β-methyl 2,3-dihydroxy-4-methoxy-5,5-dimethylpyranoside or—to use the more convenient trivial nomenclature—α- and β-methyl novioside.[1c,18]

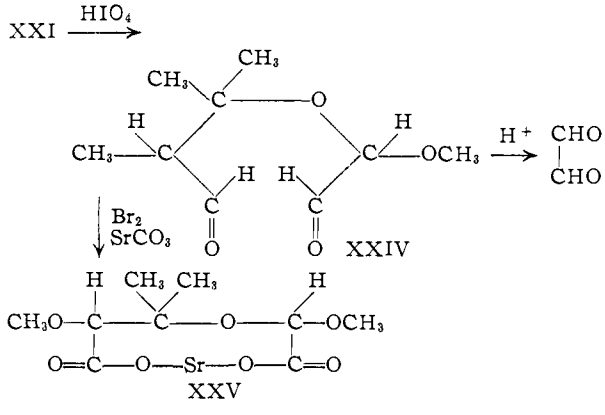


XX, XXI, R = Me
XXII, R = H



V, R = Et
XIV, R = Me
XXIII, R = H

Additional support for this structure was obtained by studying the oxidation products of XXI. Mild acid hydrolysis of the periodate oxidation product of XXI yielded glyoxal which was isolated as the 2,4-dinitrophenylosazone. Oxidation of the dialdehyde XXIV with bromine in the presence of strontium carbonate according to the method of Jackson and Hudson[19] gave the expected crystalline strontium salt XXV.



XXV

Hydrolysis of methyl 3-*O*-carbamylnovioside (XIV) with 0.5 *N* sulfuric acid removed the glyco-

(14) E. T. Jones and A. Robertson, *J. Chem. Soc.*, 1690 (1932).

(15) R. C. Shah and M. C. Laiwalla, *ibid.*, 1828 (1938).

(16) When Compound I was heated under reflux in alcohol with one mole of acetyl chloride or hydrogen chloride, the major products of the reaction were the glycoside (*i.e.*, V or XIV depending on the alcohol used) and 4,7-dihydroxy-3-[4-hydroxy-3-(3-methyl-2-butenyl)-benzamido]-8-methylcoumarin. For the latter we propose the trivial name *novobiocic acid*. Its structure was established by acetic anhydride cleavage to IX and 4-acetoxy-3-(3-methyl-2-butenyl)-benzoic acid (VI).

(17) P. F. Fleury and J. Lange, *J. pharm. chim.*, **17**, 107, 196 (1933).

(18) According to the Merck nomenclature,[1d] which evolved independently, these compounds would be called α- and β-methyl 4-methylnovobioside. This name seems undesirable because (1) the *biose* ending erroneously indicates the sugar to be a disaccharide and (2) precedent favors overwhelmingly the inclusion of the stable methoxyl group in the trivial name. This point has been discussed with the Merck group and they agree that the trivial name *novobiose* should be abandoned in favor of *noviose*. Recently the configuration of L-lyxose has been assigned to this sugar (E. Walton, J. O. Rodin, C. H. Stammer, F. W. Holly and K. Folkers, THIS JOURNAL, **78**, 5454 (1956)). Therefore, the glycosides in question may be named α- and β-methyl 4-*O*-methyl-5,5-dimethyl-L-lyxoside.

(19) E. L. Jackson and C. J. Hudson, *ibid.*, **59**, 994 (1937).

sidic methyl group to give 3-*O*-carbamylnoviose (XXIII) in 77% yield.[20] The carbamate group was assigned to the 3-position of the sugar because XXIII consumed one mole of periodate with the formation of one mole of formic acid. Since the type and position of the linkages between the three major moieties were established in cyclonovobiocic acid (IV) and degradation product VII, structure I could be assigned to novobiocin.

Acknowledgments.—The authors are indebted to Drs. J. L. Johnson, E. C. Olson, Mrs. G. S. Fonken and Mr. J. E. Stafford for certain physico-chemical determinations, and to Mr. W. A. Struck and his associates for micro-analytical work. Appreciation is expressed to Drs. M. Calvin, H. E. Carter, D. J. Cram, W. G. Jackson, M. S. Newman and D. I. Weisblat for their interest and assistance.

Experimental[21]

Novobiocin.—The crystalline novobiocin used in this study was prepared by methods described previously by this Laboratory.[3,5]

Sodium Acid Novobiocin.—To a solution of 6.18 g. (0.01 M.) of novobiocin in 200 ml. of acetone was added 1.67 ml. (0.01 M.) of 6 *N* sodium hydroxide. The mixture was seeded and stirred rapidly with a magnetic stirrer for 16–18 hr. at 30–40°. During this time the suspended alkali dissolved and crystalline sodium acid novobiocin separated. The crystals were collected, washed with acetone and dried to yield 5.9 g. (90%), m.p. 210–215° dec., $[\alpha]_D^{25}$ −34° (*c* 1.081 in water). The crystalline salt had a potency of approximately 1000 mcg./mg. by the *K. pneumoniae* novobiocin bioassay.[3]

Anal. Calcd. for $C_{31}H_{35}N_2O_{11}Na\cdot H_2O$: C, 57.05; H, 5.72; N, 4.29. Found: C, 57.04; H, 5.93; N, 4.06.

Disodium Novobiocin.—To a suspension of 10 g. (0.0162 M.) of novobiocin in 100 ml. of water was added slowly 32.4 ml. (0.0324 M.) of 1.0 *N* sodium hydroxide. The novobiocin dissolved during this treatment and the resulting solution was dried by evaporation from the frozen state. This white amorphous salt had a potency of about 960 mcg./mg. by the *K. pneumoniae* novobiocin assay.[3]

Anal. Calcd. for $C_{31}H_{34}N_2O_{11}Na_2\cdot 2H_2O$: C, 53.76; H, 5.53; N, 4.05; Na, 6.64. Found: C, 53.73; H, 5.63; N, 3.97; Na, 6.77.

Calcium Acid Novobiocin.—A solution of 4.0 g. (0.1 mole.) of sodium hydroxide in 50 ml. of water was added with stirring to a suspension of 61.8 g. (0.101 M.) of novobiocin in 250 ml. of acetone. The solution was filtered and 12.7 ml. of 4 *M* calcium chloride was added. The solution was warmed slightly and stirred until crystallization was well underway. With continued stirring, water was added slowly until a total volume of 600 ml. was obtained. The crystalline slurry was refrigerated overnight. The product was collected, washed with about 100 ml. of water and air-dried. After 24 hr. the salt dried *in vacuo* to provide 54.8 g. (86%) of nearly white microcrystalline powder. On the Kofler micro-hot-stage the crystals lost birefringence above 240° but did not melt up to 325°.

Anal. Calcd. for $C_{31}H_{35}N_2O_{11}Ca/2\cdot H_2O$: C, 57.31; H, 5.74; N, 4.31. Found: C, 57.12; H, 5.88; N, 4.21.

Diazomethane Methylation of Novobiocin.—The diazomethane from 6.9 g. (0.032 M.) of N-methyl-N-nitroso-*p*-toluenesulfonamide was distilled into a stirred suspension of 10 g. (0.016 M.) of novobiocin in 40 ml. of ether and 10 ml. of methanol at 4°. After all of the diazomethane was added (a 60% yield from the precursor was assumed), the reaction mixture was held at 25° for 4 hr. The precipitated product was collected, dissolved in 30 ml. of acetone then reprecipitated with 30 ml. of ether and 60 ml. of Skellysolve B. The oil which separated was dissolved in 35 ml. of acetone and precipitated with 6 volumes of Skellysolve B. The product weighed 2.6 g. and melted at 158–160° dec. This material was subjected to a 200-transfer countercurrent distribution analysis using the solvent system 20% water, 30% ethanol, 32.5% ethyl acetate and 17.5% cyclohexane. It was found to contain 5% unchanged novobiocin, but the remainder was homogeneous by this method. The peak fraction was isolated for analysis and physical constants. Its ultraviolet spectra showed maxima in basic ethanol at 314 mμ (a = 58.6) and in acid ethanol at 310 mμ (a = 27.2) and 290 mμ (a = 27.5).

Anal. Calcd. for $C_{32}H_{38}N_2O_{11}$: N, 4.47; OCH_3, 9.90. Found: N, 4.39; OCH_3, 9.61.

Dihydronovobiocin.—A solution of 40 g. (0.0654 M.) of novobiocin in 400 ml. of warm absolute ethanol was hydrogenated using 10 g. of Adams catalyst and 40 p.s.i.g. of hydrogen pressure for 1 hr. at 25–40°. The mixture was filtered through Celite, and 1400 ml. of water was added to the vigorously stirred filtrate. Addition of 20 ml. of 1 *N* hydrochloric acid to the resulting emulsion caused a white precipitate to form. This was collected and dried *in vacuo* to yield 36 g. (90%), m.p. 163–165° dec.

Anal. Calcd. for $C_{31}H_{40}N_2O_{11}$: C, 60.38, H, 6.54; N, 4.54. Found: C, 59.96; H, 6.44; N, 4.49.

Sodium Acid Dihydronovobiocin.—A solution of 4.0 g. (6.45 mM.) of dihydronovobiocin in 90 ml. of acetone was dried 30 minutes over magnesium sulfate. To the filtered solution was added 350 mg. (6.4 mM.) of sodium methoxide. The mixture was stirred 24 hr. at 25°. As the sodium methoxide dissolved, sodium acid dihydronovobiocin crystallized. The product was collected, washed with 90 ml. of dried acetone and dried at 25° *in vacuo* to yield 3.35 g. (81%), m.p. 225–235° dec.

Anal. Calcd. for $C_{31}H_{37}N_2O_{11}Na\cdot H_2O$: C, 56.87; H, 6.01; N, 4.28. Found: C, 56.98; H, 5.95; N, 4.47.

Calcium Acid Dihydronovobiocin.—To a solution of 250 g. (0.47 M.) of dihydronovobiocin in 1 l. of acetone was added 1200 ml. (0.47 M.) of a 0.00039 *N* sodium hydroxide solution. The solution was treated with 29.7 g. (0.20 M.) of calcium chloride dihydrate in 50 ml. of water and stirred 3 hr. The crystalline precipitate was washed with acetone and dried *in vacuo* at 25° to yield 190 g. (75%) of white crystals, m.p. 225–250° dec.

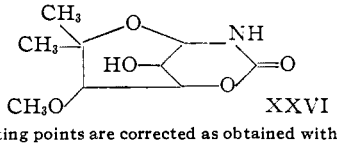
Anal. Calcd. for $(C_{31}H_{37}N_2O_{11})_2Ca\cdot 6H_2O$: C, 54.14; H, 6.30; N, 4.07; Ca, 2.91. Found: C, 54.57; H, 6.49; N, 4.24; Ca, 3.14.

Hydrolysis of Novobiocin in Acid Ethanol. A. Preparation of Cyclonovobiocic Acid (IV).—A solution of 10 g. (16.2 mM.) of novobiocin in 100 ml. of ethanol was heated to boiling. Concentrated hydrochloric acid (50 ml.) was added over a period of 7 minutes. The mixture was heated under reflux for about 30 minutes, then cooled. The solid (6.1 g., 95%) was collected, washed with water and recrystallized from 400 ml. of 1-butanol to yield 5.4 g. of IV. After a second recrystallization from ethanol the pale yellow crystals melted at 288–291°. In 75% DMF the compound titrated as a monobasic acid with pK'_a 6.3. Absorption maxima were observed at 252 mμ (a = 84.7) and 328 mμ (a = 68.2) in alkaline ethanol. In acid ethanol the intensity of the 252 mμ maximum was greatly diminished and the 328 mμ peak shifted to 331 mμ (a = 61.0).

Anal. Calcd. for $C_{22}H_{21}NO_6$: C, 66.82; H, 5.35; N, 3.54; 2 C-CH_3, 7.60. Found: C, 67.18; H, 5.40; N, 3.70; C-CH_3, 3.67.

B. **Preparation of Ethyl 3-*O*-Carbamyl-4-*O*-methyl-5,5-di-**

(20) Countercurrent distribution analysis of the hydrolysis mixture revealed the presence of three components: *ca.* 8% starting material, *ca.* 77% of the desired product (XXIII) and *ca.* 15% of a substance, which on the basis of its infrared spectrum (strong band at 1695 cm.$^{-1}$ and none in the 1600 cm.$^{-1}$ region), is believed to be XXVI.



XXVI

(21) All melting points are corrected as obtained with a Kofler micro-hot-stage. Specific rotation values were determined using a 2-decimeter tube. All ultraviolet spectra were obtained using Cary model 11 recording spectrophotometers; infrared measurements, with Perkin–Elmer recording spectrophotometers. With reference to solvents used for ultraviolet measurements, the term *acid ethanol* means 70% ethanol 0.01 *N* with respect to its sulfuric acid content, and *alkaline ethanol* means 70% ethanol 0.01 *N* with respect to its potassium hydroxide content. The abbreviation *a* (absorptivity) is the density of a 1-g./l. solution. The abbreviations M. and mM. are used for mole and millimole, respectively.

methyl-L-lyxoside (V).—The acid mother liquor from the above experiment was neutralized with 6 *N* sodium hydroxide and concentrated at reduced pressure to a small volume. The precipitated sodium chloride was removed and the filtrate was taken to dryness. The residue was extracted with acetone and the extract diluted with Skellysolve B to yield 316 mg. of ethyl 3-*O*-carbamyl-4-*O*-methyl-5,5-dimethyl-L-lyxoside (V), m.p. 172–176°. Recrystallization from acetone–Skellysolve B gave a 72% recovery of material melting at 173–175°, $[\alpha]_D^{25}$ −36° (*c* 1.0 in ethanol).

Anal. Calcd. for $C_{11}H_{21}NO_6$: C, 50.18; H, 8.04; N, 5.32; OCH_3, 11.79; OEt, 17.11. Found: C, 50.68; H, 8.16; N, 5.25; OCH_3, 11.71; OEt, 17.01.

Cleavage of Novobiocin to Novobiocic Acid and Ethyl 3-*O*-Carbamyl-4-*O*-methyl-5,5-dimethyl-L-lyxoside (V).—To a solution of 10 g. (0.0162 M.) of novobiocin in 100 ml. of boiling absolute ethanol was added slowly 1.1 g. (0.014 M.) of acetyl chloride. After 2 hr. of heating under reflux the solution was cooled, then treated with 300 ml. of water to precipitate 6.0 g. (94% yield) of crystalline 4,7-dihydroxy-3-[4-hydroxy-3-(3-methyl-2-butenyl)-benzamido]-8-methylcoumarin (novobiocic acid). This product was recrystallized from acetone, then purified in a 424-transfer countercurrent distribution in a solvent system containing ethyl acetate, ethanol, water and cyclohexane. The main peak fraction, representing 63% of the reaction product, was recovered and analyzed. Prolonged heating *in vacuo* at 100° was necessary to remove solvent of crystallization.

Anal. Calcd. for $C_{22}H_{21}NO_6$: C, 66.82; H, 5.35; N, 3.50. Found (average of three analyses): C, 66.47; H, 5.66; N, 3.50.

The filtrate from the reaction mixture was distilled *in vacuo* to a volume of 100 ml., then neutralized with 1*N* sodium hydroxide. The distillation was continued until the volume was 25 ml. This was then chilled to precipitate 3.0 g. of crude crystalline V, which was recrystallized from a acetone–Skellysolve B solution to yield 1.14 g. (27%) of ethyl glycoside identical with that described above.

Cleavage of Novobiocic Acid with Acetic Anhydride to VI and IX.—A solution containing 2 g. (0.005 M.) of novobiocic acid, 20 ml. of pyridine and 4 g. (0.039 M.) of acetic anhydride was heated under reflux 4 hr. It was cooled to 4°, diluted with 25 ml. of water and acidified to *p*H 2 with 22 ml. of concentrated hydrochloric acid. The resulting precipitate was filtered and dried to yield 2.6 g. of material. This was leached three times with 30 ml. of ether. The residue (1.5 g.) was crystallized from ethanol and water and the crystalline product dried *in vacuo* to yield 0.75 g. (55%). Its melting point, ultraviolet and infrared spectra were identical with those of IX.

The ether leachings were combined and evaporated to dryness to yield 0.9 g. of solid. This was crystallized from ethanol and water to yield 0.72 g. (57%) of white crystalline product which was identified as VI by its ultraviolet spectra and melting point.

Reaction of Novobiocin with Acetic Anhydride: Preparation of VI and VII.—A solution of 100 g. (0.162 M.) of novobiocin in 1 l. of pyridine and 216 g. (2.14 M.) of acetic anhydride was heated under reflux 4 hr. The solution was cooled to 5°, 1500 ml. of water and 1030 ml. of 12 *N* hydrochloric acid were added. The precipitate which formed was collected, washed with water and dried to yield 110 g. of partially crystalline solid. This was extracted with ether and the extract was evaporated to yield crude VI. After recrystallization from 400 ml. of ethanol and 600 ml. of water, 26 g. (60%) of VI, m.p. 100–113°, was obtained. The ether-insoluble fraction (51 g.) was recrystallized from 750 ml. of ethanol and 200 ml. of water to yield 46.5 g. (65%) of VII, m.p. 167–172°. Recrystallization from boiling acetone gave solvated crystals which melted at 150–170°. Another recrystallization from ethanol and water gave material which melted at 169–172°.

Compound VI, an optically inactive acid (pK_a' 5.67 in 50% ethanol), exhibited absorption maxima at 235 mμ (a = 51.6), 275 mμ (a = 5.35) and at 285 mμ (a = 4.65) in acid ethanol. In ethanolic base an irreversible shift of the maximum to 288 mμ (a = 63.2) occurred.

Optically active, $[\alpha]_D^{25}$ −94.4 (*c* 2% in DMF), VII titrated as a neutral compound which appeared to be unstable in alkali. Its ultraviolet absorption spectrum in neutral or acid ethanol showed characteristic peaks at 292 mμ (a = 25.3), 315 mμ (a = 39.7), 238 mμ (a = 33.6), 321 mμ (a = 35.0) and 329 mμ (a = 32.3). In alkaline ethanol the spectrum was irreversibly changed to end absorption with inflection points at about 240, 290 and 340 mμ.

Anal. Calcd. for $C_{14}H_{16}O_4$ (VI): C, 67.72; H, 6.50; acetyl, 17.3; 2 C-CH_3, 12.08; equiv. wt., 248.27. Found: C, 67.75; H, 6.56; acetyl, 16.3; C-CH_3, 8.84; equiv. wt., 254. Calcd. for $C_{23}H_{26}N_2O_{10}$ (VII): C, 56.33; H, 5.35; N, 5.71. Found: C, 56.65; H, 5.44; N, 5.74.

Subsequent experiments showed that this cleavage does not occur in pyridine, aqueous pyridine, pyridine with hydrochloric acid or with pyridine and glacial acetic acid. No carbon dioxide was formed during the reaction.

Reaction of Cyclonovobiocic Acid (IV) with Acetic Anhydride: Formation of VIII and IX.—A solution of 10 g. (0.025 M.) of IV in 80 ml. of pyridine and 10 ml. (0.10 M.) of acetic anhydride was heated under reflux for 3 hr. After addition of 2 ml. of water to the hot solution, it was cooled to 5° and acidified to *p*H 2 with 80 ml. of 12 *N* hydrochloric acid. The precipitate was dissolved in 400 ml. of hot 97% ethanol. On cooling, 4.3 g. of crude IX separated. The filtrate was diluted with 800 ml. of water to precipitate VIII (4.6 g.). Both compounds were recrystallized twice from ethanol. Compound VIII, m.p. 184°, was obtained in 90% yield and was identified as 2,2-dimethyl-6-carboxychromane by comparison with an authentic sample.[12] Compound IX, m.p. 203–206°, obtained in 62% yield, was neutral, optically inactive and unstable in alkali. Its ultraviolet spectrum is given in Fig. 3.

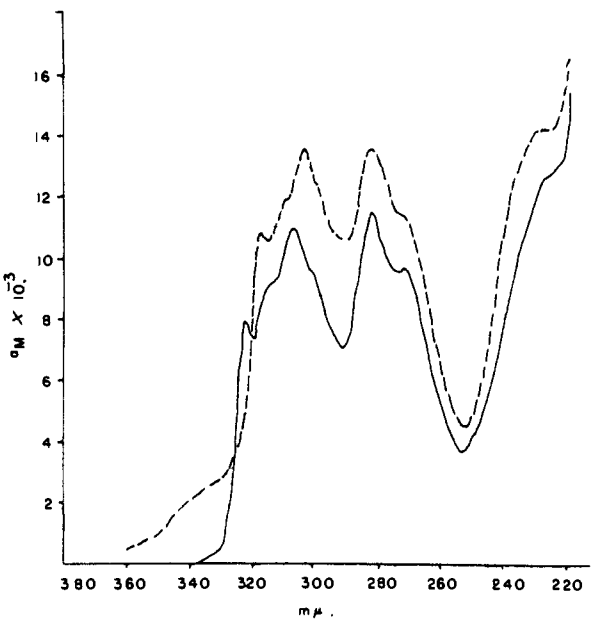


Fig. 3.—Ultraviolet spectra of 2-methyl-4H-[1]benzopyrano [3,4-d]oxazol-4-one (XVIII) (———) and compound IX (- - - - -): solvent and acid ethanol.

Anal. Calcd. for $C_{12}H_{14}O_3$ (VIII): C, 69.88; H, 6.84. Found: C, 69.80; H, 6.71. Calcd. for $C_{14}H_{11}NO_5$ (IX): C, 61.54; H, 4.06; N, 5.13. Found: C, 61.59; H, 3.77; N, 5.06.

Deacetylation of VI; Preparation of 4-Hydroxy-3-(3-methyl-2-butenyl)-benzoic Acid (XI).—A solution of 0.5 g. (2 mM.) of VI, 20 ml. of 1 *N* sodium hydroxide and 50 ml. of ethanol was allowed to stand at 25° for 3 hr. After acidification to *p*H 2 with 6 *N* hydrochloric acid, the solution was distilled at reduced pressure to an aqueous concentrate. This was extracted with ether, the extract dried and evaporated. The residue was crystallized from acetone and water by rapid evaporation of the acetone to yield 350 mg. (85%) of XI, m.p. 103–106°; ultraviolet spectra are given in Fig. 2.

Anal. Calcd. for $C_{12}H_{14}O_3$: C, 69.88; H, 6.84; 2 C-CH_3, 14.6; equiv. wt., 206.23. Found: C, 69.89; H, 6.94; C-CH_3, 6.94; equiv. wt., 208.

Osmium Tetroxide–Sodium Periodate Oxidation of VI.—To a solution of 3 g. (12.1 mM.) of VI in 60 ml. of ether (dried by distillation from methylmagnesium iodide) was

added 3 g. (11.8 mM.) of osmium tetroxide in 20 ml. of dry ether at 0°. A heavy green precipitate formed immediately. The mixture was allowed to warm slowly to 24° and was stored for 3 days at this temperature. The precipitate was separated, the ethereal solution evaporated and the residue and precipitate combined. The solid was added to 100 ml. of ethanol, 80 ml. of water and 18 g. of sodium sulfite and the mixture heated under reflux for 30 minutes. The mixture was filtered, the precipitate washed with 200 ml. of hot ethanol and the washings added to the filtrate. The latter was evaporated to 25 ml. of aqueous solution, acidified with hydrochloric acid to *p*H 2 and extracted with ether. The dried ether extract was evaporated to yield 1.82 g. of hygroscopic solid. The ultraviolet spectra were similar to those of the unsaturated starting material, but the infrared absorption showed greatly increased hydroxyl content. A 1.72-g. quantity of this solid was dissolved in 17.2 ml. of methanol, and 1.5 g. of sodium metaperiodate in 30 ml. of water was added. After 1.5 hr. at 25° the solution was neutralized to *p*H 6.0 with sodium hydroxide and distilled as rapidly as possible into ethanolic 2,4-dinitrophenylhydrazine to yield 200 mg. of a mixture of products. This material (90 mg.) was chromatographed using a 1″ × 40″ silicic acid column (dry-packed) which was developed with a Skellysolve B–ether mixture (the ether content progressively increased from 2 to 6%). The column was dried, extracted, cut into fractions and the products isolated by elution with chloroform. After recrystallization from ethanol and water 40 mg. of formaldehyde 2,4-dinitrophenylhydrazone, m.p. 155–165°, and 30 mg. of acetone dinitrophenylhydrazone, m.p. 115–125°, were obtained. The compounds were identified by their infrared spectra in chloroform solution.

Conversion of VI to 2,2-Dimethyl-6-carboxychromane.—A solution of 1.0 g. (4 mM.) of VI in 10 ml. of absolute ethanol was heated to boiling. Hydrochloric acid (5 ml. of 12 *N*) was added and the solution was heated under reflux for 1.5 hr. After cooling, the solution was added to 35 ml. of water and extracted with one-half its volume of ether. Evaporation of the dried extract gave an oil whose ultraviolet spectra indicated it to be the ester of VIII. The oil was dissolved in 35 ml. of ethanol and 10 ml. of 1 *N* sodium hydroxide. After standing 3 days at 25° the solution was acidified with 100 ml. of 0.1 *N* hydrochloric acid. The white crystals which formed were recrystallized from 20 ml. of 50% ethanol to yield 0.55 g. (67%) of VIII, m.p. 181–183°.

Hydrogenation of VI to 4-Acetoxy-3-(3-methylbutyl)-benzoic Acid (X).—A solution of 2 g. of VI in 50 ml. of absolute ethanol was hydrogenated 1 hour at 40 p.s.i.g. of hydrogen with 1 g. of Adams catalyst. The catalyst was removed by filtration and the filtrate was diluted with 150 ml. of water. The crude product (1.3 g.) which separated was recrystallized from warm aqueous ethanol to yield 1.06 g. of X, m.p. 136–144°, identical with the material obtained by acetic anhydride cleavage of dihydronovobiocin.

Cleavage of Dihydronovobiocin with Acetic Anhydride; Preparation of X and VII.—A solution of 2 g. (3.2 mM.) of dihydronovobiocin, 20 ml. of pyridine and 4 g. (39 mM.) of acetic anhydride was heated under reflux 3 hr. The solution was diluted with 25 ml. of water, chilled to 5°, brought to *p*H 1 with 20 ml. of 12 *N* hydrochloric acid and extracted with three 50-ml. portions of ether. The combined extracts were washed with 150 ml. of water, dried and evaporated to a residue. Crystallization from 150 ml. of 30% ethanol yielded 0.32 g. of X, m.p. 135–144°. The ultraviolet spectra were similar to those of VI.

Anal. Calcd. for $C_{14}H_{18}O_4$: C, 67.18; H, 7.25. Found: C, 67.45; H, 6.93.

The material which was not extracted with ether was separated by filtration, crystallized and recrystallized from ethanol to yield 0.68 g. of VII identical with that obtained from novobiocin.

Deacetylation of VII.—A suspension of 2.0 g. (4 mM.) of VII in 50 ml. of commercial anhydrous methanol and 1 *N* sodium methoxide in methanol was heated under reflux for 30 minutes. During this time all of the original solid dissolved and the product separated as white crystals (1.43 g.), m.p. 268–272°. For analysis a portion of the material was recrystallized from a large volume of boiling acetone. This material (XII) was neutral, non-reducing, ferric chloride and ninhydrin negative and exhibited ultraviolet spectra qualitatively identical with those of VII.

Anal. Calcd. for $C_{21}H_{24}N_2O_9$: C, 56.34; H, 5.40; N, 6.25. Found: C, 56.07; H, 5.54; N, 6.25.

Methanolysis of XII; Preparation of 3-Amino-4,7-dihydroxy-8-methylcoumarin (XIII) and Methyl 3-*O*-Carbamyl-4-*O*-methyl-5,5-dimethyl-L-lyxoside (XIV).—A suspension of 2.0 g. (4.45 mM.) of XII (VII could also be used) in 400 ml. of methanol and 100 ml. of 3.15 *N* methanolic hydrogen chloride was heated under reflux with frequent swirling. After about 2 hr. all of the crystals dissolved and the colorless solution was cooled, filtered and concentrated *in vacuo*. The two products, XIII and XIV, crystallized and were separated by fractional crystallization from methanol and ether to yield 1.9 g. of XIII as the hydrochloride and 435 mg. of XIV. The former, less soluble in methanol, decomposed above 200°, gave a pink color with ferric chloride and a strong positive reaction with ninhydrin in pyridine in the cold.

Anal. Calcd. for $C_{10}H_{10}NO_4Cl$: C, 49.49; H, 4.15; N, 5.77; Cl, 14.61. Found: C, 48.52; H, 4.79; N, 5.44; Cl, 12.06.

Recrystallization of XIII hydrochloride from water or aqueous ethanol yielded the free-base or zwitterion compound.

Anal. Calcd. for $C_{10}H_9NO_4$: C, 57.99; H, 4.38; N, 6.77. Found: C, 57.69; H, 4.32; N, 6.25.

Final purification of XIV was accomplished by recrystallization from acetone and Skellysolve B. The colorless platelets were neutral, ninhydrin-negative, showed only end absorption in the ultraviolet and melted at 194–195°, $[\alpha]_D^{25}$ − 24.7° (*c* 1 in 95% ethanol).

Anal. Calcd. for $C_{10}H_{18}NO_8$: C, 48.19; H, 7.68; N, 5.62. Found: C, 48.11; H, 7.71; N, 5.61.

Reaction of IX with Methanolic Hydrogen Chloride. Preparation of 3-Amino-4-hydroxy-8-methylcoumarin Hydrochloride (XIII).—A solution of 2 g. (7.3 mM.) of IX in 100 ml. of 3.5 *N* methanolic hydrogen chloride and 400 ml. of methanol was heated under reflux 3 hr., then stored at 24° 16 hr. Concentration of the solution to 100 ml. by distillation *in vacuo* and chilling to −10° yielded 1.5 g. of white precipitate. Recrystallization from 100 ml. of absolute ethanol and 200 ml. of ether gave 1.15 g. which decomposed over the range 240–310°. This material was ninhydrin-positive in the cold and gave a transient pink color with ferric chloride.

Anal. Calcd. for $C_{10}H_{10}NO_4Cl$: C, 49.49; H, 4.15; N, 5.77. Found: C, 49.88; H, 5.30; N, 5.18.

Recrystallization of this hydrochloride from hot 30% ethanol gave XIII free base identical with that obtained from XII.

3-Benzamido-4,7-dihydroxy 8-methylcoumarin (N-Benzoyl XIII).—Benzoyl chloride (0.6 ml.) was added portionwise and with vigorous shaking to a solution of 0.46 g. of XIII in 30 ml. of 1 *N* sodium hydroxide at 0–5°. After 2 hr., the solution was filtered free of a trace of insoluble material and acidified with 2 *N* hydrochloric acid. The orange precipitate which formed was filtered off, washed with water and dried. The crude product was extracted with four 30–40-ml. portions of boiling Skellysolve B to remove benzoic acid then recrystallized from 95% ethanol using Darco G-60 for decolorization. The yield of pale yellow product was 0.39 g., m.p. 309–310°. For analysis, a sample was recrystallized again from ethanol.

Anal. Calcd. for $C_{17}H_{13}NO_5$: C, 65.59; H, 4.21; N, 4.50. Found: C, 65.63; H, 4.19; N, 4.42.

Conversion of N-Benzoyl-XIII to IX by the Action of Acetic Anhydride.—A solution of 515 mg. of 3-benzamido-4,7-dihydroxy-8-methylcoumarin in 45 ml. of pyridine and 15 ml. of acetic anhydride was held at 25° for 1 hr. and heated under reflux for 2 hr. After cooling, water and solid potassium bicarbonate were added and the solution taken to dryness *in vacuo*. The residue was taken up in water and chloroform. After shaking, the layers were separated and the aqueous phase extracted several times with chloroform. The combined, dried extracts were evaporated and the residue crystallized from aqueous ethanol to yield 100 mg. of nearly colorless crystals, m.p. 201–203°. Comparison with IX obtained by the other route by means of ultraviolet and infrared spectra and mixed melting point established the identity.

Anal. Calcd. for $C_{14}H_{11}NO_5$: C, 61.54; H, 4.06; N, 5.13. Found: C, 61.84; H, 3.80; N, 5.12.

3-Benzamido-4-hydroxycoumarin (XVII).—Using the same procedure as employed for the benzoylation of XIII, 1.0 g. of 3-amino-4-hydroxycoumarin[13] was benzoylated with benzoyl chloride in 1 *N* sodium hydroxide at 5°. The benzoic acid-free product was recrystallized from ethanol to yield 700 mg. of benzamide, m.p. 207–208° dec.

Anal. Calcd. for $C_{16}H_{11}NO_4$: C, 68.48; H, 3.94; N, 4.98. Found: C, 68.31; H, 4.04; N, 4.88.

2-Methyl-4H-[1] benzopyrano[3,4-d]oxazol-4-one (XVIII).—A solution of 200 mg. (0.14 mM.) of 3-benzamido-4-hydroxycoumarin in 2 ml. of pyridine was heated under reflux for 3 hr. with 5.0 g. (50 mM.) of acetic anhydride. After addition of 0.4 ml. of water and cooling, the solution was diluted with one volume of water and acidified to *p*H 2 with hydrochloric acid. The precipitate was filtered, washed with water and dried. The crude product was washed with ether and recrystallized from absolute ethanol to yield 80 mg. of white crystals. m.p. 195° dec. The ultraviolet spectrum was almost identical with that of IX (see Fig. 3).

Anal. Calcd. for $C_{11}H_7NO_3$: C, 65.67; H, 3.51; N, 6.96. Found: C, 65.83; H, 3.55; N, 6.94.

3-Diazo-7-hydroxy-8-methyl-2,4-chromandione (XV).—To an ice-cold solution of 200 mg. of XIII in 20 ml. of glacial acetic acid and 15 ml. of water was added 200 mg. of sodium nitrite. The solution was stirred until the nitrite crystals dissolved. After 1.5 hr. the solution was allowed to warm to room temperature and was taken to dryness *in vacuo*. The oily yellow residue was triturated with water and the product crystallized. The crude crystals (80 mg.) were recrystallized from ethanol and water using Darco G-60 to yield 60 mg. of peach-colored crystals which melted at 195–197° with explosive decomposition. The infrared spectrum showed a prominent band at 2165 cm.$^{-1}$ for the diazo group. Ultraviolet maxima occurred at 250 mμ (a = 46.2) and 312 mμ (a = 69.9) in acid ethanol and at 260 mμ (shoulder) (a = 37.8) and 366 mμ (a = 95.0) in alkaline ethanol.

Anal. Calcd. for $C_{10}H_7N_2O_4$: C, 54.79; H, 3.22; N, 12.78. Found: C, 55.28; H, 3.19; N, 12.91.

2,4 Dihydroxy-*m*-toluic Acid from 3-Amino-4,7-dihydroxy-8-methylcoumarin (XIII).—A solution of 0.50 g. (2.05 mM.) of XIII hydrochloride in 30 ml. of 1 *N* sodium hydroxide was allowed to stand at 25° for 4 days. The dark solution, which smelled of ammonia, was acidified with 2 *N* hydrochloric acid. Following the vigorous evolution of carbon dioxide, the solution was extracted several times with ether. The combined, dried extract was evaporated to yield 0.21 g. of red-brown solid. This material was dissolved in ethanol, stirred with Darco G-60 and the filtered solution evaporated to dryness. Most of the residue dissolved in saturated aqueous bicarbonate. The solution was filtered, acidified and extracted with ether. After drying, the ether extract was concentrated to a volume of about 10 ml. and passed through a $^3/_8'' \times 10''$ column of Florisil (Upjohn 6565a) previously dry-packed and wet-down with ether. As the column was developed with ether, most of the colored impurities were held near the top. The ferric chloride-positive material passed through with little or no adsorption. Fractions of 15–20 ml. were collected and evaporated. The residues from the first two fractions were crystallized from ether and Skellysolve B to provide 65.8 mg. (20%) of pale yellow acid which melted with sublimation at 210–215°. Final purification by sublimation at 200–220° and atmospheric pressure gave 50.4 mg. of colorless plates, m.p., with sublimation, 210–215°. In ethanol solution the spectrum showed maxima at 262.5 mμ (a_M = 12,950) and 298 mμ (a_M = 4,175); in acid ethanol, 264 mμ (a_M = 14,300) and 298 mμ (a_M = 4,250); and in alkaline ethanol, 237 mμ (a_M = 12,350) and 282 mμ (a_M = 14,375). This substance was identified as 2,4-dihydroxy-*m*-toluic acid by direct comparison with a synthetic sample.[15]

Anal. Calcd. for $C_8H_8O_4$: C, 57.14; H, 4.80; C-CH_3, 8.93. Found: C, 57.28; H, 5.24; C-CH_3, 8.16.

2-Methylresorcinol from 3-Amino-4,7-dihydroxy-8-methylcoumarin (XIII).—Three grams (12.3 mM.) of XIII hydrochloride was added to 20 g. of molten potassium hydroxide with stirring and continued heating on the hot-plate. The mixture became very dark and bubbled vigorously as ammonia evolved. After 30 minutes the reaction mixture was cooled to room temperature. Water (35 ml.) was added to the dark solid and the mixture was stirred until a uniform gelatinous slurry was obtained. The coal-black slurry was acidified with concentrated hydrochloric acid and extracted several times with ether. After drying over magnesium sulfate, the solvent was evaporated and the residue crystallized from benzene. Two crops totaling 0.52 g. (34%) of yellow crystals, m.p. 115–120°, were obtained. Part of this material was sublimed at *ca.* 125° and atmospheric pressure to yield colorless crystals which melted at 120–122°. When dissolved in ethanol and treated with 1% aqueous ferric chloride, little or no color change was noted. In aqueous solution alone (*i.e.*, no ethanol) a fleeting purple color was observed. This is in agreement with the properties recorded by Jones and Robertson,[14] for 2-methylresorcinol. When mixed with tolu-*p*-quinol, the m.p. was depressed to 100–105°.

Anal. Calcd. for $C_7H_8O_2$: C, 67.73; H, 6.49; C-CH_3, 12.1. Found: C, 67.89; H, 6.21; C-CH_3, 9.18.

Methyl 2-*O*-Acetyl-3-*O*-carbamyl-4-*O*-methyl-5,5-dimethyl-L-lyxoside.—A solution of 201 mg. (0.8 mM.) of XIV in 2.0 ml. of pyridine and 0.5 ml. of acetic anhydride was allowed to stand at 25° for 48 hr. The solution was cooled in an iced bath and diluted with 10 ml. of water. After 10–15 minutes the reaction mixture was extracted twice with an equal volume of chloroform. The dried, pooled extracts were evaporated to yield 230 mg. of colorless oil which was crystallized and recrystallized from Skellysolve B; yield 202 mg., m.p. 44–48°, $[\alpha]^{24}_D$ −14.0 (c 1.136 in 95% ethanol).

Anal. Calcd. for $C_{12}H_{21}NO_7$: C, 49.47; H, 7.27; N, 4.81. Found: C, 49.67; H, 7.49; N, 4.96.

Methyl 3-*O*-Carbamyl-2-*O*-mesyl-4-*O*-methyl-5,5-dimethyl-L-lyxoside.—A solution of 916 mg. (3.67 mM.) of XIV in 15 ml. of chloroform and 3 ml. of pyridine was chilled in an ice-bath and swirled with 0.3 ml. of methanesulfonyl chloride. After standing in a glass-stoppered flask at 4° for 22 hr., the reaction mixture was filtered. The filtrate was cooled to 0–5°, shaken with water and the layers separated. The chloroform layer was washed with ice-cold 0.5 *N* sulfuric acid, water, saturated sodium bicarbonate solution and finally with water. After drying over anhydrous magnesium sulfate, the solution was evaporated to dryness to yield 610 mg. (61%) of oil which crystallized quite readily. On recrystallization from water, the colorless crystals melted at 149–151°.

Anal. Calcd. for $C_{11}H_{21}NO_8S$: S, 9.79; OCH_3, 19.0. Found: S, 9.82; OCH_3, 18.5.

Methyl 4-*O*-Methyl-5,5-dimethyl-L-lyxoside 2,3-Cyclic Carbonate (XIX).—A solution of 1.03 g. (4 mM.) of XIV in 175 ml. of commercial absolute methanol and 40 ml. of 3 *N* methanolic hydrogen chloride was heated under reflux for 3.5 hr. The solution was concentrated to dryness *in vacuo*. The residue was slurried in 10–12 ml. of acetone, and 98.4 mg. of insoluble ammonium chloride was removed. The filtrate was evaporated to 5 ml. and 245 mg. of XIV recovered. The filtrate was concentrated to dryness under nitrogen and the remaining hydrogen chloride dispelled by vacuum drying. The residue was extracted with 15 ml. of hot water. As the solution cooled colorless crystals formed, 109 mg., m.p. 122–128°. After sublimation at 120° and 20–25 mm. the carbonate ester melted at 132–132.5°, $[\alpha]^{25}_D$ +117.7° (c 0.998 in 95% ethanol).

Anal. Calcd. for $C_{10}H_{16}O_6$: C, 51.72; H, 6.95; OCH_3, 26.7. Found: C, 51.97; H, 6.91; OCH_3, 27.6.

Methyl 4-*O*-Methyl-5,5-dimethyl-L-lyxoside (XX).—Three ml. of saturated aqueous barium hydroxide solution was added to a solution of 76 mg. (0.328 mM.) of XIX in 12 ml. of carbon dioxide-free water. A white precipitate of barium carbonate separated immediately. After 2 hr. the precipitate (58.25 mg., 90%) was removed and the filtrate was neutralized with solid carbon dioxide. Again the barium carbonate was removed and the filtrate was concentrated *in vacuo* to a colorless sirup (48.26 mg., 71%). Crystallization and recrystallization from Skellysolve B gave colorless platelets, m.p. 61–68°, $[\alpha]^{25}_D$ +113.8° (c 1.0 in water).

Anal. Calcd. for $C_9H_{18}O_5$: C, 52.41; H, 8.80. Found: C, 52.42; H, 8.57.

Preparation of Anomeric Methyl 4-*O*-Methyl-5,5-di-

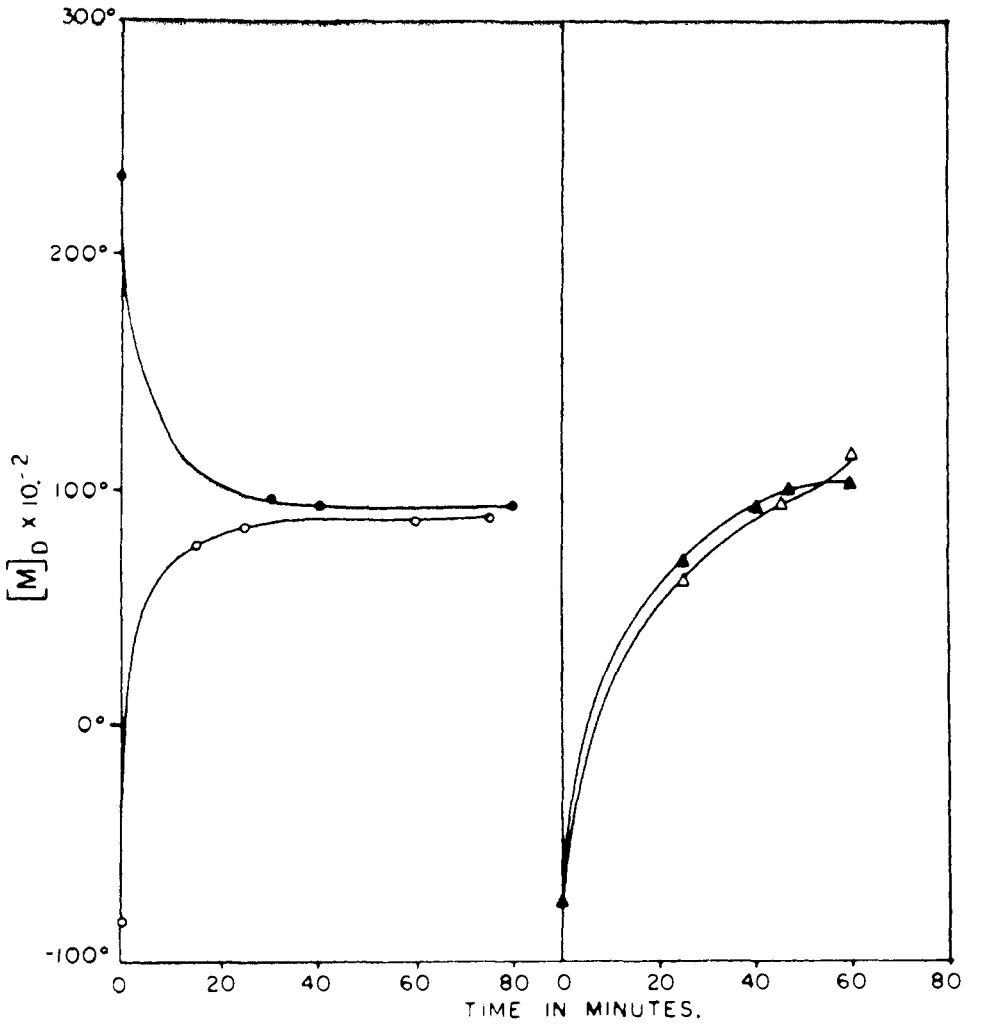


Fig. 4.—Rotatory changes during the hydrolysis of methyl glycosides in 0.5 *N* sulfuric acid at *ca.* 80°: ●, methyl novioside, anomer XXI; ○, methyl novioside, anomer XX; ▲, methyl 3-*O*-carbamylnovioside, 30 ml. of solution; △, methyl 3-*O*-carbamylnovioside, 250 ml. of solution.

methyl-L-lyxoside (XXI) from XIV.—A solution of 2.0 g. (8 mM.) of XIV in 75 ml. of water was treated with 55 ml. of a saturated solution of barium hydroxide and allowed to react at 25° for 16 hr. The precipitate of barium carbonate was removed by filtration through a pad of Celite, and the filtrate (which smelled strongly of ammonia) was neutralized with carbon dioxide. The second precipitate was removed in the same fashion and the filtrate was taken to dryness *in vacuo*. The residue was dissolved in ether, filtered free of the last traces of barium carbonate and the solution evaporated to yield 1.6079 g. of a clear oil, which crystallized on standing. After recrystallization from Skellysolve B the dense, colorless rods melted at 65–70° and depressed the m.p. of XX. The infrared spectrum of XXI was very similar to that of XX but not identical, $[\alpha]^{24}D$ −63.6° (*c* 0.621 in 95% ethanol). −63.6° (*c* 1.0 in water).

Anal. Calcd. for $C_8H_{18}O_5$. C, 52.41; H, 8.80; OCH_3, 30.0; C-CH_3, 14.7. Found: C, 52.67; H, 8.88; OCH_3, 29.9; C-CH_3, 2.17.

Methyl 2,3-Di-*O*-acetyl-4-*O*-methyl-5,5-dimethyl-L-lyxoside.—Using the procedure described for the acetylation of XIV, 201.58 mg. (0.975 mM.) of XXI was acetylated using 3 ml. of pyridine and 1 ml. of acetic anhydride. The product was crystallized and recrystallized from Skellysolve B to yield 217 mg. of dense rods, m.p. 62–64°, $[\alpha]^{24}D$ −20.4° (*c* 0.973 in 95% ethanol).

Anal. Calcd. for $C_{13}H_{22}O_7$: C, 53.78; H, 7.64; OAc, 29.66. Found: C, 54.16; H, 7.59; OAc, 28.07.

4-*O*-Methyl-5,5-dimethyl-L-lyxose (XXII).—A solution of 840 mg. (4.07 mM.) of methyl novioside (XXI) in 84 ml. of 0.5 *N* sulfuric acid was heated on the steam-bath at 80–85° for 1.25 hr. Previous studies had shown that this was sufficient time for completion of the reaction as indicated by the change in rotation (see Fig. 4). The solution was cooled and stirred with an excess of barium carbonate. The neutralized mixture was filtered free of insoluble salts and concentrated *in vacuo* to dryness. The residue was dissolved in absolute ethanol and the solution filtered to remove traces of salts. Evaporation of the filtrate gave a nearly quantitative yield of noviose as a colorless sirup which crystallized on standing. After recrystallization from ethanol–ether, acetone–Skellysolve B and finally from ethyl acetate, the fine colorless needles melted at 128–130°, $[\alpha]^{24}D$ +19.9° (*c* 0.950 in 50% ethanol).

Anal. Calcd. for $C_8H_{16}O_5$: C, 49.99; H, 8.39; OCH_3, 16.15. Found: C, 49.71; H, 8.50; OCH_3, 16.23.

Noviose, identical with the above, was obtained by similar treatment of XX. This proved the α-, β-methyl glycoside relationship between XX and XXI.

Chromic Acid Oxidation of Methyl 4-*O*-Methyl-5,5-dimethyl-L-lyxoside (Methyl Novioside).—To 300 mg. (1.45 mM.) of XXI was added 10 ml. of a solution containing 7.5 ml. of 5 *N* chromic acid and 2.5 ml. of concd. sulfuric acid. The solution was distilled rapidly into ethanolic 2,4-dinitrophenylhydrazine. Two additional 5-ml. portions of the oxidizing mixture and 15 ml. of water were added during the distillation. The yield of re-

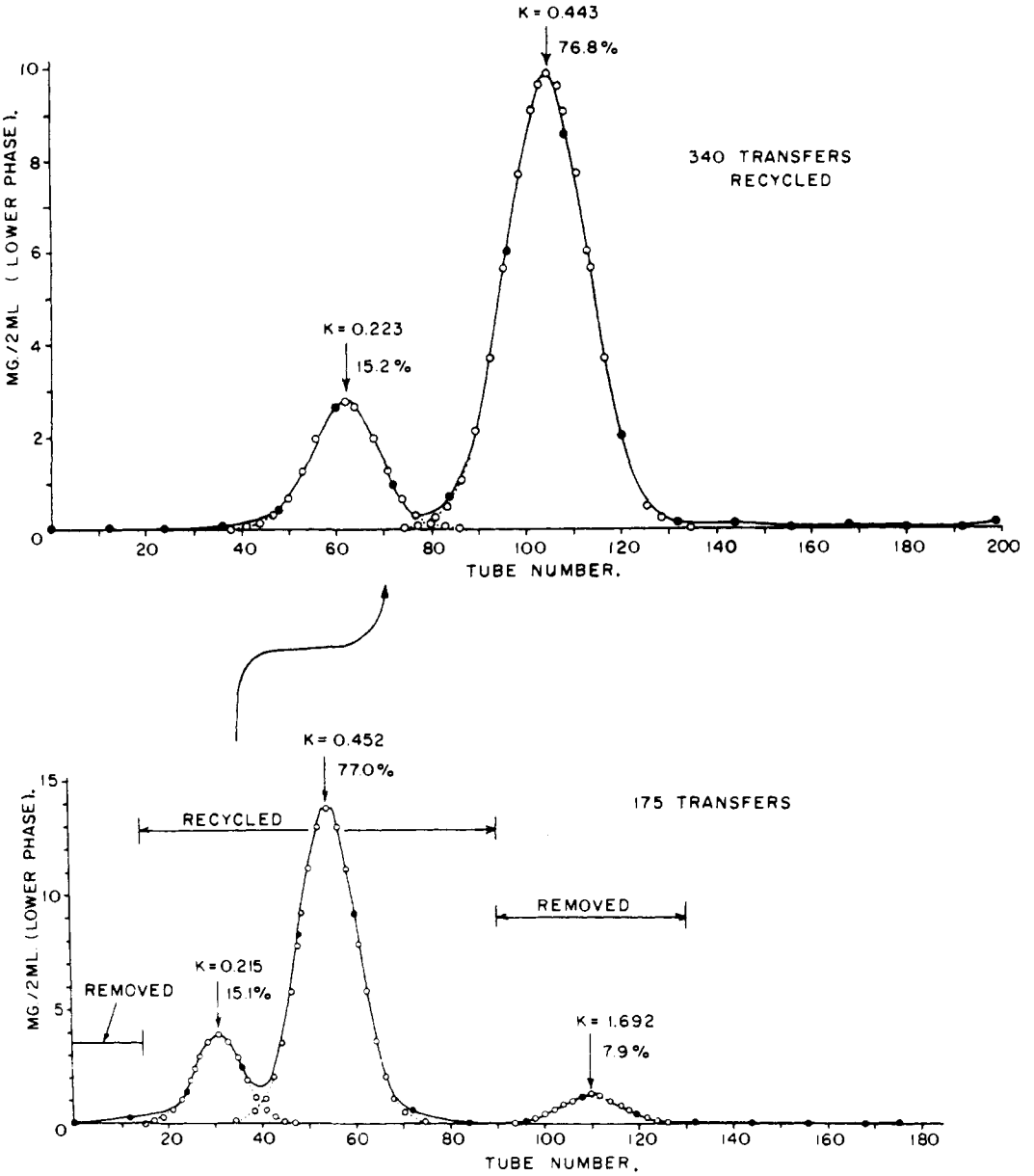


Fig. 5.—Countercurrent distribution analysis of methyl 3-*O*-carbamylnovioside hydrolysate; ●, observed; O, calculated.

crystallized acetone 2,4-dinitrophenylhydrazone was 160 mg. (46%), m.p. 125–127°. The sample was further identified by infrared comparison with authentic acetone 2,4-dinitrophenylhydrazone.

Quantitative Periodate Oxidation Studies.—Using the procedure of Fleury and Lange,[17] 0.1-mM. quantities of each compound were oxidized with sodium metaperiodate in aqueous solution in the dark at room temperature. Formic acid was determined by potentiometric titration, conversion of mercuric chloride to calomel[22] or by isolation as the water-soluble but alcohol-insoluble strontium formate. Results for pertinent compounds are given in Table I.

Glyoxal from Periodate Oxidation of Methyl Novioside (XXI).—To 249 mg. of XXI in 50 ml. of water was added 25 ml. of a 50 mg./ml. solution of sodium metaperiodate. After 2 hr. in the dark at 25°, the iodate and excess periodate were precipitated with barium hydroxide (*p*H 7). The neutral solution was evaporated *in vacuo* to 25 ml. and sodium bicarbonate added to precipitate the remaining barium ions. The filtrate was evaporated to dryness at reduced pressure and the residue extracted with ethanol. Removal of the solvent left a residue of 225 mg. which was dissolved in 10 ml. of 0.5 *N* hydrochloric acid. The solution was heated at 80° for 15 minutes then added to a hot solution of 730 mg. of 2,4-dinitrophenylhydrazine, 1 ml. of concd. hydrochloric acid and 100 ml. of ethanol. This mixture was boiled 5 minutes and an additional 5 ml. of 2 *N* hydrochloric acid was added. On cooling, 400 mg. of glyoxal dinitrophenylosazone (m.p. 345°) separated. Its identity was confirmed by infrared comparison with an authentic sample.

TABLE I

Compound	$M \cdot IO_4^-$ consumed per M. cpd. 30 min.	60 min.	100 min.	M. formic acid formed per M.cpd.
Methyl 3-*O*-carbamylnovioside (XIV)	0.0	0.0	0.0	0
Methyl novioside (XX)	0.98	0.98	..	0
Methyl novioside (XXI)	0.96	0.98	0.99	0
Noviose (XXII)	1.87	1.96	1.98	1.9
3-*O*-Carbamylnoviose (XXIII)	0.95	0.97	0.98	0.8

(22) L. Ahlen and O. Samuelson, *Anal. Chem.*, **25**, 1263 (1953).

Strontium 3-(Carboxymethoxymethoxy)-2-methoxy-3-methylbutyrate (XXV).—A solution containing 1.24 g. (6 mM.) of methyl novioside (XXI) and 2.57 g. (12 mM.) of sodium metaperiodate in 150 ml. of water was allowed to react in the dark at room temperature for 1 hr. and 10 minutes. A hot solution of 1.595 g. of strontium hydroxide octahydrate in 25–30 ml. water was added portionwise along with 24 ml. of 0.5 *N* hydrochloric acid. The *p*H was maintained between 5 and 6. After 30 minutes the precipitated salts were removed by filtration through a Celite pad. The filtrate and water wash were combined, 0.4 g. of strontium carbonate was added and the suspension concentrated *in vacuo* to about one-third volume. The salts were removed by filtration and the filtrate was taken to dryness. The residue was extracted with absolute ethanol and the filtered extract made up to 50 ml. with absolute ethanol. Forty ml. of this solution was evaporated to dryness to yield 694 mg. of the dialdehyde XXIV as a pale yellow oil. The oil was dissolved in 75 ml. of water; 8 g. of strontium carbonate and 1 ml. of bromine were added. The mixture was swirled and shaken every few minutes for 0.5 hr., then allowed to react in the dark for 23 hr. The excess bromine was removed by aeration, the strontium carbonate by filtration and the bromide by precipitation with silver carbonate (8 g.). The filtrate was freed of silver ions with hydrogen sulfide and the excess hydrogen sulfide removed by aeration. The colorless solution was concentrated at the water-pump to a volume of 3–4 ml. Ethanol (5 ml.) was added and the product separated as needles. A total of 807.23 mg. (77%) of the crystalline salt was obtained. For analysis a sample was recrystallized from 50% ethanol, $[\alpha]^{24}D$ +10° (*c* 0.734 in water).

Anal. Calcd. for $C_9H_{14}O_7Sr \cdot 1.5H_2O$: C, 31.00; H, 4.91; OCH_3, 17.39; Sr, 25.12. Found: C, 31.18; H, 5.07; OCH_3, 19.65; Sr, 25.68.

Hydrolysis of Methyl 3-*O*-Carbamyl-4-*O*-methyl-5,5-dimethyl-L-lyxoside (XIV) to 3-*O*-Carbamyl-4-*O*-methyl-5,5-dimethyl-L-lyxose (XXIII).—A solution of 303.5 mg. (0.928 mM.) of XIV in 30 ml. of 0.5 *N* sulfuric acid was heated at *ca.* 80°. At 15–30 minute intervals the solution was cooled quickly to room temperature and the specific rotation was determined. After about an hour of heating there appeared to be no further change in rotation (Fig. 4). The solution was neutralized with excess barium carbonate, filtered and concentrated to dryness *in vacuo*. The residue was taken up in ethanol, the solution was filtered and the clear, colorless filtrate evaporated to yield 257 mg. of colorless glass. The product gave a positive Benedict reaction and consumed one mole of periodate with the formation of one mole of formic acid (Table I). After thorough drying the material gave satisfactory elemental analyses but failed to crystallize.

Anal. Calcd. for $C_9H_{17}NO_6$: C, 45.95; H, 7.29; N, 5.96. Found: C, 45.95; H, 7.66; N, 5.92.

To check the homogeneity of amorphous XXIII, the hydrolysis was repeated on larger scale using 2.50 g. of XIV in 250 ml. of 0.5 *N* sulfuric acid. The rotation *vs.* time plot for this experiment (Fig. 4) gave an indication that a secondary reaction was starting toward the end of the one-hour reaction time. The reaction mixture was worked up as before and the amorphous product was subjected to countercurrent distribution analysis using 1-butanol and water as the solvent system (Fig. 5). After 175 transfers in the 200-tube machine, analysis by solids measurement revealed the presence of three components: a well-resolved, fast-moving minor component (K = 1.692), the major component (K = 0.452) and a minor component (K = 0.215) incompletely resolved. The fast-moving component was removed from the machine yielding 200 mg. of colorless crystals identified as methyl 3-*O*-carbamylnovioside (XIV). The empty tubes were refilled with fresh solvent and the distribution continued to a total of 340 transfers. At this point the remaining two components were resolved. Contents of appropriate tubes were pooled and worked up to yield 1.5595 g. (*ca.* 76% over-all yield from XIV) of 3-*O*-carbamylnoviose and 336 mg. of material, presumably the monohydrate of XXVI. After prolonged drying *in vacuo*, 3-*O*-carbamylnoviose became birefringent and melted at 124–126°. The compound gave a positive Benedict reaction and consumed one mole of periodate, $[\alpha]^{26}D$ +45.3° (*c*, 0.971 in 95% ethanol).

Anal. Calcd. for $C_9H_{17}NO_6$: C, 45.95; H, 7.29; N, 5.96. Found: C, 46.15; H, 7.75; N, 5.63.

The third component obtained as an amorphous, hygroscopic glass, was not fully characterized. Its infrared absorption with a strong band at 1695 $cm.^{-1}$ assured the presence of a carbonyl, probably in a cyclic system. The absence of absorption in the 1600 $cm.^{-1}$ region indicated that the simple carbamate structure had been altered. Elemental analyses showed that the nitrogen was retained and that the gummy product was solvated, $[\alpha]^{26}D$ +63° (*c*, 0.96 in 95% ethanol).

Anal. Calcd. for $C_9H_{15}NO_5 \cdot H_2O \cdot 1/2C_2H_5OH$: C, 46.50; H, 7.81; N, 5.42. Found: C, 46.95; H, 7.45; N, 5.61.

KALAMAZOO, MICHIGAN

[CONTRIBUTION FROM AVERY LABORATORY, UNIVERSITY OF NEBRASKA]

6-Phenylthieno[2,3-b]pyridine from 2-Nitro-3-thenaldehyde

BY WILLIAM J. RAICH[1] AND CLIFF S. HAMILTON

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3-Methylthiophene was converted to 3-thenyl acetate which on nitration gave a mixture of 2- and 5-nitro-3-thenyl acetates. The readily isolated 2-isomer was hydrolyzed to the alcohol, nitric acid oxidation of which gave 2-nitro-3-thenaldehyde. The aldehyde condensed readily with malonic acid and with acetophenone. The product from the latter reaction was reduced and cyclized to 6-phenylthieno[2,3-b]pyridine.

Recently, an interest developed in the synthesis of compounds containing fused thiophene rings of the C_4N–C_4S or C_4S–C_5N systems in which the sulfur and nitrogen atoms are in the peri position. Of particular interest as an intermediate was 2-nitro-3-thenaldehyde (I) which by analogy to *o*-nitrobenzaldehyde would be a satisfactory starting material for the preparation of a number of the desired type compounds. Using commercially available 3-methylthiophene (VI), 2-nitro-3-thenaldehyde (I) was prepared in an over-all yield of 5%. The aldehyde was converted *via* the condensation product with acetophenone to 6-phenylthieno[2,3-b]-pyridine (XII), the thiophene analog of 2-phenylquinoline.

3-Methylthiophene (VI) was treated with N-bromosuccinimide as described by Campaigne and Tuller[2] to give 3-thenyl bromide which without isolation was converted immediately to 3-thenyl acetate (IV) in 58% over-all yield. The small amount of 2-bromo-3-methylthiophene formed in the bromination step was removed readily from the ester by distillation. The physical properties of the ace-

(1) Parke, Davis Fellow, 1952–1955. The Dow Chemical Company, Midland, Michigan.

(2) E. Campaigne and B. F. Tuller, *Org. Syntheses*, **33**, 96 (1953).