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Chemistry of the Neomycins. VII. Compounds Obtained from Methyl Neobiosaminide C[1]

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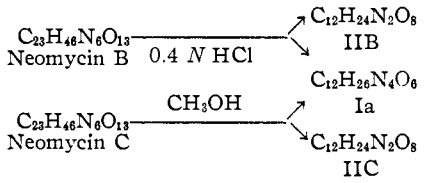
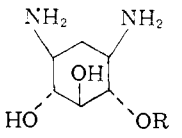
Derivatives and degradation products of neobiosamine C, a major fragment of neomycin C, are described; included is neosamine C, a diaminohexose. Neobiosamine C is shown to be a neosaminido-D-ribose.

The "neomycin complex" of antibiotics has been the subject of considerable chemical and clinical interest since its discovery by Waksman and Lechevalier in 1949.[3] It consists of two major isomeric components, neomycins B and C, together with neamine (formerly "neomycin A"), a degradation product common to both isomers. Earlier studies established the formula of neomycins B and C as $C_{23}H_{46-48}N_6O_{12-13}$,[4] while from data presented below (reported briefly earlier)[5] and other recent studies[6-8] this may be narrowed to $C_{23}H_{46}N_6O_{13}$.



Neomycin B is degraded by mild acidic methanolysis[4,9] to neamine (Ia, $C_{12}H_{26}N_4O_6$) and methyl neobiosaminide B (IIB, $C_{11}H_{21}N_2O_7$-O-CH_3),[5] the methyl glycoside of neobiosamine B. Similar degradation of neomycin C yields neamine and the isomeric methyl neobiosaminide C (IIC).[4,5,9] While the complete structure of neamine is in doubt, it has been shown to consist of deoxystreptamine (Ib),[10] probably of all-*trans* stereochemistry,[6] and an acid-unstable moiety $(C_6H_{14}N_2O_4)$[6-8] attached at the C-4 position of deoxystreptamine.[6]



Ia, R = $C_6H_{13}N_2O_3$
b, R = H

In a series of preliminary reports, structures have been assigned to neobiosamines B and C,[1,5,11-13] and a full description of degradation products from methyl neobiosaminide B has recently appeared.[14] The present paper describes the preparation and properties of those compounds obtained from degradation of methyl neobiosaminide C which have allowed the assignment of a structure to neobiosamine C.[12]

Cleavage of neomycin C in dilute methanolic hydrogen chloride[4] afforded crude neamine (Ia) and methyl neobiosaminide C (IIC). The latter was carefully chromatographed over charcoal–Celite; individual eluates were characterized by rotation, papergrams and microanalyses and combined into larger fractions a-e (*cf.* Fig. 1 and Experimental section). These results are summarized in Fig. 1. Although fraction a contained neomycin and neamine (readily differentiated from methyl neobiosaminide by R_f values; *cf.* Table I) fractions b-e contained only methyl neobiosaminides. It is observed in Fig. 1 that the specific rotations of fractions b, c and d differed widely. Since these fractions contained only methyl neobiosaminide, this may be attributed either to mixtures of methyl neobiosaminides B and C or to mixtures of α- and β-glycosidic anomers, or to both. Unfortunately, the systems employed in the paper chromatograms did not distinguish methyl neobiosaminide B from C (*cf.* Table I). However, the crude methyl neobiosaminides B and C differ considerably in specific rotation,[9,14] much as do neomycins B and C,[4] and it was to be anticipated that neobiosamines B and C also differ in rotation. Neobiosamine B has been shown (*cf.* Fig. 2) to have $[\alpha]_D$ +33°.[14] In the present work samples of methyl neobiosaminides (IIC) were hydrolyzed

TABLE I

AVERAGE R_f VALUES OF NEOMYCIN C AND FRAGMENTS

	BAW 221[a]	PAW 10:1:9[a]
Neomycin C	0.0 (0.00)[b,d]	0.33
Neamine	.0–0.51 (0.22–0.49)[b]	.42
Methyl neobiosaminide C	.81, 0.33[e] (0.82, 0.44)[b,d]	.69, 0.51
Neobiosamine C	.19 (0.25)[b,d]	.43
Neosamine C	.17 (0.23)[b,d]	
D-Ribose	.55 (0.60)[b]	

[a] Footnote 23. [b] Ref. 14. [c] Footnote 25. [d] Values for neomycin B and fragments.

(1) Paper VI in this series: K. L. Rinehart, Jr., A. D. Argoudelis, T. P. Culbertson, W. S. Chilton and K. Striegler, *J. Am. Chem. Soc.*, **82**, 2970 (1960).

(2) (a) Robert F. Carr Fellow, 1957–1958; (b) taken from the Ph.D. Thesis of P. W. K. Woo, University of Illinois, August, 1958.

(3) "Neomycin, Its Nature and Practical Application," S. A. Waksman, ed., The Williams and Wilkins Co., Baltimore, Md., 1958.

(4) J. H. Ford, M. E. Bergy, A. A. Brooks, E. R. Garrett, J. Alberti, J. R. Dyer and H. E. Carter, THIS JOURNAL, **77**, 5311 (1955).

(5) K. L. Rinehart, Jr., P. W. K. Woo, A. D. Argoudelis and A. M. Giesbrecht, *ibid.*, **79**, 4567 (1957).

(6) J. R. Dyer, Ph.D. Thesis, University of Illinois, September, 1954.

(7) P. D. Shaw, Ph.D. Thesis, University of Illinois, August, 1956.

(8) M. P. Georgiadis, M.S. Thesis, University of Illinois, February, 1960.

(9) J. D. Dutcher, N. Hosansky, M. N. Donin and O. Wintersteiner, THIS JOURNAL, **73**, 1384 (1951).

(10) F. A. Kuehl, M. N. Bishop and K. Folkers, *ibid.*, **73**, 881 (1951).

(11) K. L. Rinehart, P. W. K. Woo and A. D. Argoudelis, *ibid.*, **79**, 4568 (1957).

(12) K. L. Rinehart, Jr., and P. W. K. Woo, *ibid.*, **80**, 6463 (1958).

(13) K. L. Rinehart, Jr., P. W. K. Woo and A. D. Argoudelis, *ibid.*, **80**, 6461 (1958).

(14) K. L. Rinehart, Jr., A. D. Argoudelis, W. A. Goss, A. Sohler and C. P. Schaffner, *ibid.*, **82**, 3938 (1960).

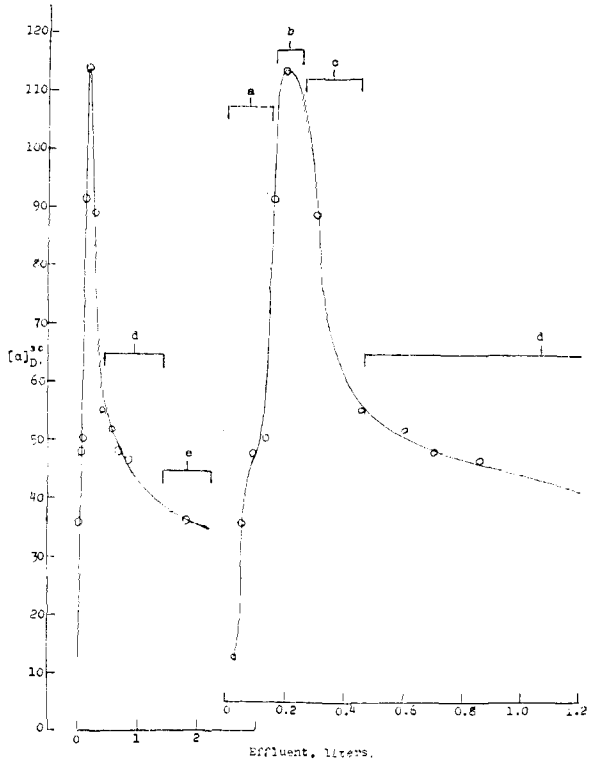


Fig. 1.—Specific rotation (degrees) of fractions a-e from chromatography of crude methyl neobiosaminide C.

under mild acidic conditions to the constant value $[\alpha]_D$ +104°(*cf.* Fig. 2). Since this end rotation was approached from both higher and lower values it is considered to be the mutarotation value of neobiosamine C (III). This was confirmed by paper chromatography (Table I) which showed that the product was a single compound, giving positive amine (ninhydrin) and aldehyde (aniline hydrogen phthalate) tests, different from the starting material methyl neobiosaminide C.[15]

These results allowed the identification of all fractions giving this end rotation value as anomeric methyl neobiosaminides C. The lowest rotating methyl neobiosaminide C (fraction d-2, *cf.* Experimental section) was designated methyl β-neobiosaminide C, in accord with Hudson's nomenclature.[16] For most degradative experiments the anomeric configuration was of no importance and mixtures of the methyl α- and β-glycosides were employed.

Neobiosamine C was first suggested to be a disaccharide by Dutcher, *et al.*,[9] who reported the formation of furfural (from a pentose) and a crystalline, reducing diamine hydrochloride from vigorous acid hydrolysis of crude methyl neobiosaminide C. The earlier authors assigned the formula $C_6H_{14}N_2O_3 \cdot 2HCl$ (that of a trideoxydiaminohexose)[17] to the diamine salt and the formula $C_{12}H_{24}N_2O_7$ to methyl neobiosaminide C. In the present work the formula for methyl neobiosaminide C has been established to be $C_{12}H_{24}N_2O_8$, that of neobiosamine C to be $C_{11}H_{22}N_2O_8$, that of the reducing diamine (neosamine C) to be $C_6H_{14}N_2O_4$, each containing one oxygen atom more than its corresponding earlier formula. The present formulations are based on analyses of methyl neobiosaminide C (IIC), its monohydrate, its N,N′-dibenzoyl (VII) and N,N′-di-(*p*-nitrobenzoyl) derivatives, and of the N,N′-dibenzoyl derivative (VIII) of neobiosaminol C (IV, the sodium borohydride reduction product of neobiosamine C).

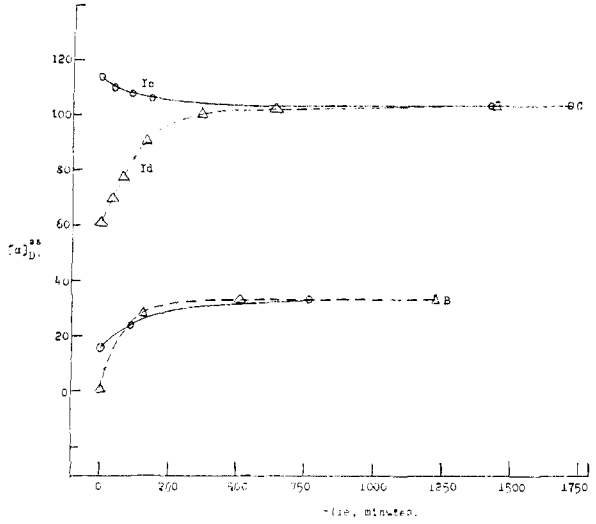


Fig. 2.—Specific rotation of hydrolyzates: C, methyl neobiosaminide C; Ic, fraction c; Id, fraction d-2; B, methyl neobiosaminide B, ref. 14.

Conclusive evidence for the formulas was provided by the isolation of neosamine C (V) from vigorous acid hydrolysis of methyl neobiosaminide C (IIC). Microanalytical data for this compound and for N,N′-dibenzoylneosaminol C (IX, from N-benzoylation of the sodium borohydride reduction product, VI) are in excellent agreement with its formulation as a dideoxydiaminohexose $C_6H_7O(NH_2)_2(OH)_3$, but exclude a trideoxydiaminohexose. Finally, the structure of neosamine C has been established.

Isolation of the pentose moiety inferred from furfural formation was complicated by the stability of the glycosidic bond in neobiosamine. Under conditions sufficiently vigorous to cleave the disaccharide linkage, the pentose was destroyed. Resistance to hydrolysis is characteristic of glycosides of 2-amino sugars and is due to the preferred protonation of the amino group and consequent shielding by the positively charged ammonium group of the glycosidic bond from proton attack.[18,19] This difficulty is most readily circumvented by conversion of the basic amino groups to

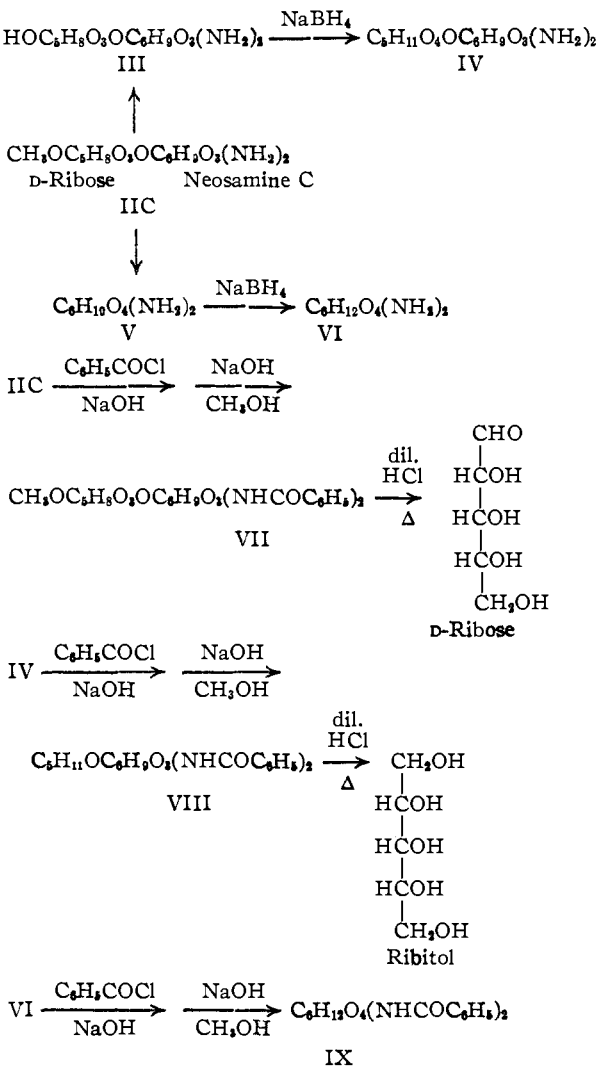
(15) Although R_f values (Table I) do not distinguish neobiosamine C from its further hydrolysis product neosamine C (*cf.* below), the second expected hydrolysis product, D-ribose (*cf.* below), is readily differentiated. Furthermore, D-ribose was shown by separate experiments to be stable under the hydrolytic conditions employed; *i.e.*, if formed it would have been detected. This stability implies, moreover, the stability under these conditions of ribose in the disaccharide neobiosamine C.

(16) C. S. Hudson, THIS JOURNAL, **31**, 66 (1909).

(17) All nitrogen atoms in the neomycins were shown early to be in primary amino groups.[9]

(18) P. W. Kent and M. W. Whitehouse, "Biochemistry of the Amino-sugars," Butterworths, London, 1955, p. 233.

(19) A. B. Foster and D. Horton, *Adv. in Carbohydrate Chem.*, **14**, 213 (1959).



neutral amide groups. The glycosides then may be cleaved readily, though amide hydrolysis is a competing reaction.[18,19] In the present study methyl N,N′-dibenzoylneobiosaminide C (VII) was hydrolyzed under relatively mild optimum conditions, established in preliminary runs, to a mixture of the pentose, neosamine C and neobiosamine C and their benzoylated derivatives.

The pentose was established by color tests (red to aniline acid phthalate,[20] brown to *p*-dimethylaminoaniline–trichloroacetic acid,[21] colorless to orcinol[22]) to be an aldopentose (rather than a ketopentose), by R_f values (Table II) to be ribose, and by its negative rotation to be D-ribose.

The glycosidic linkage between neobiosamine C and neamine is cleaved easily in 0.4 *N* methanolic hydrogen chloride and methyl neobiosaminide C is hydrolyzed in 1 *N* hydrochloric acid at 89° to neobiosamine C without cleavage of the disaccharide glycosidic bond (*cf.* above). From these observations it may be inferred that the external glycosidic bond is much less shielded than the internal glycosidic link; hence, that neobiosamine C is probably a neosaminidoribose (III), with the amino groups protecting the internal bond, rather than a ribosido-neosamine [$HOC_6H_8O_2(NH_2)_2OC_5H_9O_4$], where the external bond should be preferentially shielded. This inference was confirmed by borohydride reduction of neobiosamine C (III) to neobiosaminol C (IV), a step in which the external glycosidic moiety is reduced. Hydrolysis of this distinguishes a neosaminidoribitol from a ribosido-neosaminol. In point of fact, hydrolysis of N,N′-dibenzoylneobiosaminol C (VIII) gave ribitol (identified by R_f value and negative reaction to aniline acid phthalate), rather than ribose, thus confirming the structure inferred above.

The evidence of the present paper establishes the partial structure of neobiosamine C to be III. The remainder of the structural determination, largely effected by periodate oxidation and already reported in brief,[12] will be described in a forthcoming report.

Experimental[23]

Preparation of methyl neobiosaminide C (IIC) was effected by methanolysis of neomycin C according to previous procedures.[4,14] In six runs employing 30.0 g. of dried neomycin C sulfate, $[\alpha]^{25}D$ +78°,[4] the weight of neamine hydrochloride[4] isolated was 12.7–14.4 g. (80–90%), and that of methyl neobiosaminide C hydrochloride, $[\alpha]^{30}D$ +59 to +65° (*c* 0.8–1.7, water), was 10.2–12.6 g. (77–94%). A small intermediate fraction (neamine and methyl neobiosaminide C) weighed 2.1–2.6 g.

Chromatography of Methyl Neobiosaminide C.—Crude methyl neobiosaminide C hydrochloride from the above methanolyses was converted by passage over Dowex 2[24] (hydroxyl phase) to the free base and chromatographed according to a previously described procedure.[14] Results of a chromatogram of 18.0 g. of the free base, $[\alpha]^{30}D$ +71° (*c* 0.84, water), employing a Darco–Celite column (5 cm.

(20) F. Cramer, "Paplerchromatographie," 4th ed., Verlag Chemie, Weinheim/Bergstr., 1958.

(21) R. B. Koch, W. F. Geddes and F. Smith, *Cereal Chem.*, **28**, 424 (1951).

(22) A. Bevenue and K. T. Williams, *Arch. Biochem. Biophys.*, **34**, 225 (1951).

(23) Melting points were determined in a Hershberg melting point bath and are uncorrected. Microanalyses were by Mr. Josef Nemeth, Mrs. Ruby Ju, Mrs. Maria Benassi, Mrs. H. A. Stingl and Miss Claire Higham of the Microanalytical Laboratory, University of Illinois, and by the Clark Microanalytical Laboratory. Infrared spectra were obtained as mulls with a Perkin–Elmer double beam spectrophotometer, model 21, by Mr. James Brader, Mr. Sy Portnow, Mr. Paul McMahon and Mrs. Betty Verkade. Those referred to in the text are available in the Ph.D. Thesis of P. W. K. Woo, University of Illinois, 1958, *Univ. Microfilms* (Ann Arbor, Mich.), L. C. Card No. Mic 59-603; *C. A.*, **53**, 14920e (1959).

Solvent systems and spray reagents employed for paper chromatography (ascending) are abbreviated in the text. The systems BAW 221, PAW 10:1:9 and BAW 415, and the sprays NIN, AHP and Orcinol have been described previously[14]; others are: PhenNC: an equilibrated two-phase system, from phenol and water (9:1, by weight), consisting of an organic mobile phase and an aqueous stationary phase to which have been added a few crystals of potassiun cyanide and enough aqueous ammonia to bring the ammonia concentration to 1% (w./v.). PhenW: a one-phase system, consisting of 100 g. of phenol saturated with an aqueous solution (*ca.* 20 ml.) containing 3.7% sodium dihydrogen phosphate and 6.3% sodium citrate. PWN: a one-phase system, consisting of 1 ml. of 30% ammonia and 14 ml. of water diluted to 50 ml. with isopropyl alcohol. PyW: a one-phase system, consisting of pyridine and water (39:21 by volume). PBW 141: a one-phase system, consisting of *n*-propyl alcohol, *n*-butyl alcohol and water (1:4:1 by volume). AMA: a solution of 0.2 g. of *p*-dimethylaminoaniline monohydrate and 1.0 g. of trichloroacetic acid in 50 ml. of water. EM: the Elson–Morgan reagents (*cf.*, *e.g.*, ref. 20). Phlor: a freshly prepared solution consisting of 25 ml. of glacial acetic acid, 1 ml. of concentrated hydrochloric acid and 2.5 ml. of a 5% solution of phloroglucinol in ethanol. PP: solutions of 1% aqueous sodium periodate and 1% aqueous potassium permanganate, applied successively with a 3- to 4-minute interval.

(24) A strongly basic anion-exchange resin obtained from the Dow Chemical Co.

TABLE II

R_f VALUES OF PENTOSES

Compound	R_f ± av. dev. (no. detns.) BAW 415[a]	BAW 221[a]	PhenNC[a]	PhenW[a]
Ribose	0.365 ± 0.005 (6)	0.553 ± 0.012 (2)	0.630 ± 0.006 (3)	0.598
Lyxose	.341 ± .007 (4)	.500 ± .010 (2)	.555 ± .003 (2)	.513
Xylose	.333 ± .002 (4)	.490 ± .007 (2)	.470 (1)	.456
Arabinose	.317 ± .006 (4)	.479 ± .002 (2)	.545 (1)	.514
Xylulose	.387 ± .009 (3)	.585 (1)		
Ribulose	.382 ± .005 (3)	.605 (1)		
Hy (hydrolysate)	.366 ± .008 (4)[b]	.547 ± .006 (2)[c]	.631 ± .010 (2)[b]	.583[d]
Hy + ribose				.578[d]
Hy + lyxose				.583. 0.466[d]
Hy + xylose				.578, .430[d]
Hy + arabinose				.598, .500[d]

[a] *Cf.* footnote 23. [b] A 4% solution of methyl N,N'-dibenzoylneobiosaminide C in 2 *N* hydrochloric acid, heated under reflux for 2 hr., then extracted with ether to remove benzoic acid and lyophilized; applied to papergrams as an aqueous solution. [c] A 2% solution of the dibenzoyl derivative in 1.5 *N* hydrochloric acid, kept at 90° for 10 hr., then neutralized to *p*H 2 with aqueous sodium hydroxide and applied to papergrams. [d] A 2% solution of the dibenzoyl derivative in 1.5 *N* hydrochloric acid, heated under reflux for 1.25 hr., then deionized with Amberlite IR 4B[26] (carbonate phase) and Dowex 1[27] (hydrogen phase) and lyophilized; applied as an aqueous solution.

× 40 cm.) containing 280 g. of Darco–Celite (2:1, by weight), are summarized in Figs. 1 and 3. Effluent was collected in 10-ml. fractions and eluates before no. 55 (forerun, *cf.* Fig. 3) were discarded. Subsequent eluates were combined and lyophilized in larger fractions a to e on the basis of their rotations and papergrams (developed with NIN).[23]

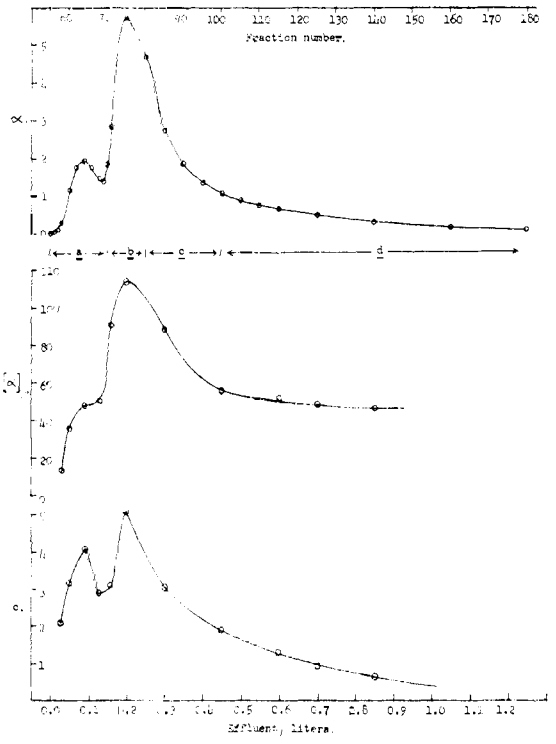


Fig. 3.—Properties of eluates from chromatography of crude methyl neobiosaminide C: α, observed rotation (degrees); $[\alpha]_D$, specific rotation (degrees); *c*, concentration (g./100 ml.).

Fraction a (from eluates 55 to 70, 0.0 to 0.15 l.) was estimated by graphical integration to contain 3.7 g. of material. Paper chromatography indicated the presence of neomycin, R_f (BAW 221)[28] 0.0, (PAW 10:1:9)[23] 0.33; and neamine, R_f (BAW 221) 0.0–0.51, (PAW 10:1:9) 0.42; and, probably, a third component, R_f (BAW 221) 0.23, (PAW 10:1:9) 0.25.

Fraction b (eluates 71 to 80, 0.15 to 0.25 l.) weighed 3.4 g., $[\alpha]^{30}D$ +115° (*c* 1.18, water). Papergrams and microanalyses indicated it to be principally methyl neobiosaminide C sulfate, R_f (BAW 221) 0.81, 0.32, (PAW 10:1:9) 0.69, 0.51.[25]

Anal. Calcd. for $C_{12}H_{24}N_2O_8 \cdot 1.2H_2SO_4 \cdot H_2O$: C, 31.32; H, 6.14; N, 6.09; S, 8.35. Found: C, 31.50; H, 6.44; N, 6.39; S, 8.24.

Fraction c (eluates 81 to 100, 0.25 to 0.45 l.) weighed 4.5 g., $[\alpha]^{30}D$ +78° (*c* 1.55, water). The sulfate was passed through a Dowex 2[24] column and lyophilized to the pale yellow, amorphous, free base, $[\alpha]^{26}D$ +111° (*c* 2.0, 1 *N* aq. HCl). Papergrams and the mutarotation value of its hydrolysate (*cf.* below) indicated it to be pure methyl neobiosaminide C, R_f (BAW 221) 0.80, 0.31, (PAW 10:1:9) 0.69, 0.51.

Fraction d (eluates 101 to 200, 0.45 to 1.45 l.) weighed 7.8 g., $[\alpha]^{33}D$ +46.2° (*c* 0.4, water).

Anal. Calcd. for $C_{12}H_{24}N_2O_8 \cdot 1.35H_2SO_4 \cdot H_2O$: C, 30.36; H, 6.10; N, 5.90; S, 8.83. Found: C, 30.01; H, 6.20; N, 5.79; S, 8.94.

The sulfate was dissolved in carbonate-free water and passed over Dowex 2[24] (hydroxyl phase). The column was washed with carbonate-free water and the basic eluate was collected in two portions. The first portion was lyophilized to give white methyl neobiosaminide C (fraction d-1), $[\alpha]^{31}D$ +68° (*c* 0.6, water).

Anal. Calcd. for $C_{12}H_{24}N_2O_8 \cdot H_2O$: C, 42.10; H, 7.66; N, 8.19. Found: C, 42.12; H, 7.84; N, 8.26.

A portion of this material was finely ground and dried over phosphorus pentoxide. The loss in weight after 13 hr. at room temperature (0.7 mm.) and 22 hr. at 56° (1 mm.) corresponded to 1.1 moles of water. The dried sample melted 98–130°.

Anal. Calcd. for $C_{12}H_{24}N_2O_8$: C, 44.44; H, 7.46; N, 8.64. Found: C, 44.60; H, 7.72; N, 8.55.

The second portion of the eluate from the Dowex 2[24] column was freeze-dried to give slightly yellow methyl β-neobiosaminide C (fraction d-2), $[\alpha]^{31}D$ +60.1° (*c* 2.0, water). The material gave a negative Fehling test and was identified by the mutarotation value of its hydrolysate (*cf.* below) and papergrams, R_f (BAW 221) 0.81, (PAW 10:1:9) 0.69.

Anal. Calcd. for $C_{12}H_{24}N_2O_8 \cdot H_2O$: C, 42.10; H, 7.66; N, 8.19. Found: C, 42.06; H, 7.66; N, 8.01.

Fraction e.—Approximately 800 ml. of effluent after eluate 200 was collected in one portion and lyophilized to about 1 g. of white solid, probably a mixture of methyl neobiosaminides B and C, $[\alpha]^{30}D$ +37.3° (*c* 1.93, water), m.p. 140–200° dec.; R_f (BAW 221) 0.81, 0.35, (PAW 10:1:9) 0.69, 0.51.

Anal. Calcd. for $C_{12}H_{24}N_2O_8 \cdot 1.38\ H_2SO_4 \cdot H_2O$: C, 30.18; H, 6.07; N, 5.87. Found: C, 30.20; H, 6.20; N, 6.11.

(25) Multiple R_f values frequently observed for the methyl neobiosaminides probably are due to differing ionic species of the compound (*cf.* ref. 14).

TABLE III

ROTATION OF METHYL NEOBIOSAMINIDE HYDROLYSATES

Run	Methyl neobiosaminide Concn., g./100 ml.	Acid Concn., N	Time, min. $[\alpha]^{25}D$						
I[a]	1.004	1	0	40	80	160	363	640	1448
			+61°	69.9	77.7	90.3	100.1	102.1	104.0
II[b]	1.060	0.5	0	45	100	183		1428	1713
			+113.5°	109.8	107.3	106		104	104.2

[a] Fraction d-2 in text. [b] Fraction c in text.

The total methyl neobiosaminide C in fractions b to d was 15.7 g. (estimated 15.3 g. by graphical integration of Fig. 1), and the total amount of material isolated from the column, including *ca.* 1 g. of low-rotating solid obtained by stripping with acetone, was 20.4 g. (*ca.* 79% recovery).

Methyl β-N,N'-Di-(*p*-nitrobenzoyl)-neobiosaminide C.—A mixture of 0.160 g. of methyl β-neobiosaminide C (Fraction d-2 above), 10 ml. of 0.5 *N* sodium hydroxide and 4.0 g. of *p*-nitrobenzoyl chloride powder was shaken for 5 min. at ice-bath temperature. One milliliter of 25% aqueous sodium hydroxide was added and the mixture was shaken for an additional 5 min.; this step was repeated four times. The mixture stood an additional hour in the ice-bath and was then shaken for 18 hr. at room temperature. The solid product was filtered and triturated several times, first with 1 *N* sodium hydroxide solution, then with water. The impure product, 0.4 g. (133% of theoretical), was recrystallized twice from methanol–water to give colorless, silky needles, which were dried for 7 hr. at 56° (1 mm.); these darkened 235° and melted 266–268° dec.

Anal. Calcd. for $C_{12}H_{22}N_2O_8(C_7H_4NO_3)_2 \cdot H_2O$: C, 48.75; H, 5.04; N, 8.75. Found: C, 48.88; H, 4.82; N, 8.51.

Methyl β-N,N'-Dibenzoylneobiosaminide C (VII).—A mixture of 0.304 g. of methyl β-neobiosaminide C (fraction d-2 above), 9.5 ml. of 1 *N* sodium hydroxide and 4.0 ml. of benzoyl chloride was shaken in an ice-bath (<20°) while 8 ml. of 25% sodium hydroxide was added in portions. The mixture was shaken for 6 hr. and finally stood for 20 hr. at 30°. The gummy organic residue was washed with water and heated under reflux for 4 hr. with 50 ml. of 0.25 *N* 80% methanolic sodium hydroxide solution, then cooled. The solution was adjusted to *p*H 7 with hydrochloric acid and evaporated to dryness *in vacuo*. The residue (VII) crystallized from methanol–water as white needles; weight 0.200 g. (42%), m.p. 254°. In a second run the yield of VII was 64%, m.p. 255–257°. A sample from the second run dried *in vacuo* for 4.5 hr. at 100° proved to be the monohydrate.

Anal. Calcd. for $C_{12}H_{22}N_2O_8 \cdot (C_6H_5CO)_2 \cdot H_2O$: C, 56.72; H, 6.23; N, 5.09. Found: C, 56.68; H, 6.25; N, 5.44.

The same sample, recrystallized from methanol–ether and dried for 22 hr. at 78° (0.5 mm.), had m.p. 254–256.5°.

Anal. Calcd. for $C_{12}H_{22}N_2O_8(C_6H_5CO)_2$: C, 58.63; H, 6.06; N, 5.27. Found: C, 58.58; H, 6.02; N, 5.50.

Neobiosamine C (III). Mutarotation Value.—Two solutions of methyl neobiosaminide C (runs I and II, from chromatographic fractions d-2 and c) were divided into small sealed tubes. The tubes were immersed in a constant temperature bath at 89° and removed at intervals for rotation determinations. Results are given in Table III and Fig. 2. An aliquot from run II (183-min.) gave a positive Fehling test. After hydrolysis was complete (640 and 1428 min.) papergrams showed only one spot from each run, corresponding to neobiosamine C (blue to NIN, brown to AHP,[23] R_f (BAW 221) 0.194, (PAW 10:1:9) 0.43.

Preparation of N,N'-Dibenzoylneobiosaminol C (VIII).—A solution of 1.020 g. of methyl neobiosaminide C (fraction d-2 above) in 48.5 ml. of 1 *N* aqueous hydrochloric acid stood at 92° for 19 hr., then was decolorized with Darco, filtered and concentrated *in vacuo*. The concentrate was neutralized with aqueous sodium hydroxide to *p*H 2, then cooled in an ice-bath and 0.7 g. of sodium borohydride in 14 ml. of water was added to the 24 ml. of solution. The reaction mixture stood for 30 min. at 10° and for one day at room temperature; it was then acidified to *p*H 2.5. One-half of the solution, which gave a negative Fehling test, was neutralized with aqueous sodium hydroxide and lyophilized, then dissolved in 0.1 *N* methanolic hydrogen chloride. Inorganic salt was filtered and the filtrate was flash distilled to remove borate. Water was added, the solution was concentrated and the product was benzoylated during 66 hr. according to the Schotten–Baumann procedure described above for methyl N,N'-dibenzoylneobiosaminide C. The gummy product was dissolved in aqueous methanolic (1:5, volume) 0.9 *N* sodium hydroxide solution and heated under reflux for 1 hour. Since the isolated product still contained ester (infrared band) the saponification was repeated with aqueous methanolic 0.09 *N* sodium hydroxide. N,N'-Dibenzoylneobiosaminol C, obtained by neutralization with hydrochloric acid and lyophilization, was triturated several times with water, filtered and washed with ether. It was ester-free by infrared, insoluble in water and ethyl acetate, soluble in methanol and hot ethanol; weight 386 mg. (59%), sintered 187° and melted 210–215°. For further purification it was treated with Darco in methanol, triturated with water, filtered, dried *in vacuo* over calcium chloride; weight 0.293 g. (45%), m.p. 217–227° dec. The analytical sample was dried for 9 hr. at 56° (0.04 mm.).

Anal. Calcd. for $C_{25}H_{32}N_2O_{10} \cdot 1/2\ H_2O$: C, 56.70; H, 6.28; N, 5.29. Found: C, 56.12; H, 6.31; N, 5.10.

Neosamine C (V).—A solution of 0.191 g. of methyl neobiosaminide C (fraction b above) in 5 ml. of 6 *N* aqueous hydrochloric acid was heated for 1.5 hr. under reflux. The black solution was decolorized with Norit and concentrated *in vacuo*. Addition of acetone precipitated a gum, which was separated by decantation, dissolved in water and lyophilized to yield 0.158 g. of a green glass. This was dried for 9 hr. over potassium hydroxide at room temperature (0.1 mm.), dissolved in hot methanol and precipitated by anhydrous ether, filtered and dried *in vacuo*. The yield of white, flocculent granules of neosamine C dihydrochloride was 0.119 g. (93%). An analytical sample was dried for 6.5 hr. at 56° (0.02 mm.) over anhydrous calcium sulfate and paraffin flakes.

Anal. Calcd. for $C_6H_{14}N_2O_4 \cdot 2\ HCl$: C, 28.70; H, 6.42; N, 11.16. Found: C, 28.64; H, 6.40; N, 10.75.

The very hygroscopic neosamine C dihydrochloride sintered at 140° and gradually darkened, but did not melt below 230°; it had $[\alpha]^{23}D$ +69° (17 min. to 14 hr., *c* 0.87, water) and R_f (BAW 221) 0.162–0.175 (purple to NIN, brown to AHP).

N,N'-Dibenzoylneosaminol C (IX).—A suspension of 1.97 g. of methyl N,N'-dibenzoylneobiosaminide C in 98 ml. of 1.5 *N* hydrochloric acid was heated for 10 hr. at 93°. The black heterogeneous mixture was extracted with ether to yield 0.58 g. of benzoic acid (82% of theoretical), decolorized with charcoal, filtered, neutralized dropwise with 2 *N* sodium hydroxide solution and concentrated to 33 ml. The hydrolysate was reduced with sodium borohydride and benzoylated, approximately according to the procedure described above for the preparation of N,N'-dibenzoylneobiosaminol C. The polybenzoylated product was heated for 175 min. in 80% methanolic sodium hydroxide (*p*H maintained 7 to 11), cooled, neutralized to *p*H 7.2 with hydrochloric acid and lyophilized to a largely inorganic solid. This was triturated with water and the insoluble crude N,N'-dibenzoylneosaminol C was filtered and dried *in vacuo* at room temperature; weight 0.369 g. (26%). This was further purified by washing with water and recrystallization from methanol, then methanol–ether. The pure compound weighed 0.161 g. (11%), m.p. 186–188.5°; an analytical sample was dried over calcium sulfate and paraffin flakes for 11.5 hr. at 62° (0.05 mm.). The infrared spectrum showed no ester carbonyl absorption.

Anal. Calcd. for $C_6H_{14}N_2O_4(C_6H_5CO)_2$: C, 61.84; H, 6.23; N, 7.21. Found: C, 61.80; H, 6.23; N, 7.28.

From the mother liquors was obtained an additional 28 mg. (2%) of product, m.p. 183–184°.

In a second run, employing 1.123 g. of methyl N,N'-dibenzoylneobiosaminide C, the product IX weighed 68 mg. (8%), m.p. 164–168°, $[\alpha]_D$ +25° (*c* 0.49, methanol). Recrystallization from methanol–ether yielded a white powder, m.p. 173–178°.

Anal. Found: C, 61.82; H, 6.14; N, 6.97.

The infrared spectra of both the analytical sample and the sample of m.p. 164–168° from the second run are very similar to that of the analytical sample obtained in the first run.

D-Ribose. I. Identification from Hydrolysis of Methyl N,N'-Dibenzoylneobiosaminide C (VII).—Suitable conditions were established for the hydrolysis of methyl N,N'-dibenzoylneobiosaminide C by paper chromatographic analysis of the hydrolysates. A hydrolytic run is described in detail to illustrate the procedure.

A mixture of 20 mg. of methyl N,N'-dibenzoylneobiosaminide C (m.p. 250–253°) and 1 ml. of 1.5 *N* hydrochloric acid was heated under reflux. The reaction was quenched at different intervals and aliquots were applied to papergrams (1-λ spots for development with AHP or NIN, 2-λ spots for EM). The pentose spot, R_f (BAW 221) 0.493 ± 0.003 (7 values), red to AHP, appeared after 0.5 hr., reached maximum intensity between 0.75 and 1.75 hr., weakened after 3 hr. and was barely visible after 6 hr. On the same papergram the R_f value of ribose was 0.505 (*cf.* below).

Complete solution of the benzoyl compound occurred after 0.5 hr., and the color of the hydrolysate continually darkened. Solid matter (benzoic acid and decomposition products) were obtained on cooling the mixture. In addition to the pentose spot, three other AHP-positive spots appeared at various times during a 24-hr. hydrolysis, at R_f (BAW 221) 0.80, 0.67 and 0.20; the latter spot gave a red color with EM. These are tentatively attributed to partially benzoylated, benzoylated and free neosamine C and neobiosamine C.

The pentose spots were compared by color tests and R_f values to known pentoses run simultaneously on the same papergrams. The four aldopentoses and the hydrolysate pentose gave positive reactions with AHP (red or pink), Phlor (purple) and AMA (brown) and negative reactions with orcinol, EM and NIN.[23] The two ketopentoses gave different color reactions with AHP (brown), AMA (pink) and orcinol (dark green), though they gave the same purple color with Phlor and no color with NIN and EM. The results of these color tests and the R_f values in several solvent systems (*cf.* Table II) clearly indicated that ribose was a hydrolytic product.

II. **Isolation.**—A mixture of 0.531 g. of methyl N,N'-dibenzoylneobiosaminide C in 25 ml. of 1.5 *N* hydrochloric acid was heated under reflux for 1.25 hr. The light brown hydrolysate, α_D +0.40°, positive to NIN and AHP, was neutralized to *p*H 5 by the addition of Amberlite IR-4B[26] (carbonate phase), then passed over a Dowex-50[27] column (hydrogen phase). Resin treatments were repeated until the effluent was negative to NIN but still positive to AHP. The effluent was freeze-dried and the residue was extracted with water. Lyophilization of the extract gave 17 mg. of white solid, which was chromatographed over a cellulose column, 1 cm. × 30 cm., packed with a slurry of 9 g. of cellulose powder (Whatman) in 25 ml. of water-saturated *n*-butyl alcohol. The sample was dissolved in a few milliliters of PBW 141[23] and applied to the column. The column was eluted with the latter solvent in one hundred 0.5-ml. fractions. A single AHP-positive band was obtained. The most concentrated fractions were combined to yield 1.6 mg. of solid, $[\alpha]^{27}_D$ −21.4 ± 7.5° (*c* 0.13, PBW 141). Authentic D-ribose had $[\alpha]^{27}_D$ −23.3° (*c* 1, PBW 141), The solid obtained from the column gave only one AHP-positive spot, R_f (BAW 221) 0.628, while ribose had R_f (BAW 221) 0.626.

III. **Acid Stability.**—A sample of 59.2 mg. of D-ribose in 5 ml. of 0.4 *N* hydrochloric acid was heated in a constant temperature bath at 89°. The initial rotation, $[\alpha]^{26}_D$, was −16°, while that after 310 min. was −15°.

A sample of ribose (0.5%) in 1.5 *N* hydrochloric acid was heated for 7 hr. at 90°. Samples (1-λ) were withdrawn at intervals and applied to a papergram. The intensity of the red AHP spot, R_f (BAW 221) 0.538 ± 0.008 (8 values), decreased only slightly after 6 to 7 hr. of heating.

Hydrolysis of N,N'-Dibenzoylneobiosaminol C (VIII). Identification of Ribitol.—Hydrolysis was effected by heating a solution containing 8.7 mg. of N,N'-dibenzoylneobiosaminol C in 0.5 ml. of 1 *N* hydrochloric acid for 1 hour under reflux. A papergram of the solution showed the absence of ribose but the presence of ribitol (brown to PP,[28] colorless to AHP and NIN), R_f (BAW 221) 0.616. On the same papergram authentic ribitol had R_f 0.612 (brown to PP, colorless to AHP and NIN), while ribose had R_f 0.584 (brown to PP, red to AHP ,colorless to NIN).

On the same papergram the hydrolysate showed an additional major spot with R_f 0.515–0.526 (positive to NIN and AHP), probably an N-benzoylneosamine salt, together with additional minor spots at 0.836–0.867, 0.354 and 0.18. On the same papergram neosamine C had R_f 0.17, 0.083, neobiosamine C R_f 0.179, 0.055.[25]

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(26) A weakly basic anion exchange resin obtained from the Rohm and Haas Co.

(27) A strongly acidic cation exchange resin obtained from the Dow Chemical Co.

(28) For this reagent the solution was first neutralized with sodium bicarbonate solution to *p*H 5, since in a preliminary run acid was found to decompose the paper to give products, detected as large brown spots, in the same region with ribose and ribitol.