$-51.8°$ (c 0.50, 0.1 N sodium hydroxide). The pmr spectrum (d_6-DMSO) exhibited a 3 H singlet at δ 3.48 (2′-O-methyl group). Acid hydrolysis gave the same sugar as 2′-O-methyladenosine.[15]

2′-O-Methylguanosine (IV). 2′-O-Methyl-2-amino-9-β-D-ribofuranosyl-6-purinethione (III, 314 mg) was dissolved in 5 ml of 20% aqueous ammonia. To the cold solution was added 0.8 ml of 30% hydrogen peroxide. The solution was left at room temperature for 4 hr and then evaporated to dryness on a rotary evaporator. The remaining white powder was dissolved in 5 ml of water and placed on an alumina column (40 g, Woelm, neutral, 2.5 cm). The column was eluted with the same solvent system as employed in the purification of VII and 25-ml fractions were collected. Fractions 2–5 were combined and evaporated to dryness. The residue was recrystallized from absolute methyl alcohol to give 150 mg of pure 2′-O-methylguanosine (IX), mp 218–220°.

Anal. Calcd for $C_{10}H_{13}N_5O_5$: C, 44.4; H, 5.1; N, 23.5. Found: C, 44.2; H, 5.2; N, 23.3.

Ultraviolet absorption showed $\lambda_{max}^{pH\ 1}$ 255.5 mμ (ε 10,660), $\lambda_{max}^{pH\ 11}$ 258 mμ (ε 9776), $[\alpha]^{22}D$ $-38.4°$ (c 0.595, water). The pmr spectrum (d_6-DMSO) exhibited a three-proton singlet at δ 3.40 (2′-O-methyl group). Acid hydrolysis showed 2-O-methyl-D-ribose as the only sugar present. The $R_{guanosine}$ values for 2′-O-methylguanosine were identical with those recorded by Hall[5] in the systems A, C, and D for the naturally occurring product.

Chemistry and Metabolism of Sphingolipids. 3-Oxo Derivatives of N-Acetylsphingosine and N-Acetyldihydrosphingosine

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Abstract: N-Acetylsphingosine and N-acetyldihydrosphingosine were prepared in high yields by reaction of the free bases with acetic anhydride in methanol. Selective oxidations of the secondary hydroxyl groups in these N-acetyl derivatives with chromic anhydride in benzene–pyridine yielded the previously undescribed 3-oxo compounds. Reductions of the 3-oxo compounds with sodium borodeuteride yielded monodeuterated *erythro* and *threo* forms of the N-acetyl bases; mass spectra confirmed the location of deuterium on C_3. Some N-acetyldihydrosphingosine-3,5-d_3 was formed by 1,4 addition in the reduction of 3-oxo-1-hydroxy-2-acetamido-4-octadecene.

Sphingosine (I) is representative of a group of related long-chain aliphatic 2-amino-1,3-diols which are basic structural constituents of all sphingolipids found in animal tissues. The predominant base may be sphingosine or the C_{20} homolog, occurring in mixtures with smaller quantities of their saturated forms, dihydrosphingosine and C_{20}-dihydrosphingosine. Sphingolipid bases have the D-*erythro* configuration; the double bond in sphingosine is *trans*.[1] Several methods have been reported for the chemical synthesis of long-chain bases of this type.[2,3]

The biosynthesis of sphingolipid bases in animal tissues is believed to involve condensation of hexadecanal with serine–pyridoxal phosphate and simultaneous or subsequent decarboxylation, yielding dihydrosphingosine.[4] Subsequent dehydrogenation yields sphingosine.[4] Recent studies by Greene, Kaneshiro, and Law[5] have shown that the synthesis of phytosphingosine and other sphingolipid bases in whole yeast cells is similar to the pathway for sphingosine and dihydrosphingosine in animal systems.

(1) Studies on the stereochemistry of sphinogosine and related bases were reported by several investigators. See H. E. Carter, D. S. Galanos, and Y. Fujino, *Can. J. Biochem. Physiol.*, **34**, 320 (1956); M. Prostenik, M. Munk-Weinert, and D. E. Sunko, *J. Org. Chem.*, **21**, 406 (1956).

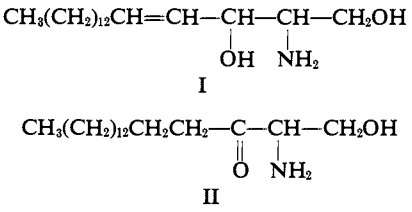
(2) See, for example, D. Shapiro and T. Sheradsky, *ibid.*, **28**, 2157 (1963).

(3) See, for example, D. Shapiro, H. Segal, and H. M. Flowers, *J. Am. Chem. Soc.*, **80**, 2170 (1958), and references cited therein.

(4) R. O. Brady, J. V. Formica, and G. J. Koval, *J. Biol. Chem.*, **233**, 1072 (1958).

(5) M. L. Greene, T. Kaneshiro, and J. H. Law, *Biochim. Biophys. Acta*, **98**, 582 (1965).

The possibility has not been excluded that a 3-oxo compound (II) may be an intermediate in the biosynthetic pathway as proposed by Sprinson and Coulon some years ago.[6] Based on experiments with serine-H,[3]



I

II

Weiss was led to speculate that 3-oxo forms of the bases may be intermediates.[7] Although chemically related to the sphingolipid bases, the ketones are not presently known to be of biological origin. Their possible existence in sphingolipids remains obscure, however, since they do not survive the hydrolytic conditions commonly used to liberate long-chain bases from their parent sphingolipids.

Since the chemical and chromatographic properties of 3-oxo compounds related to sphingosine and dihydrosphingosine had not been described previously, methods for their chemical synthesis have been investigated. The present report describes the synthesis of the stable N-acetyl derivatives of 3-oxo-1-hydroxy-2-amino-octadecane and 3-oxo-1-hydroxy-2-amino-4-octadecene by selective oxidation of secondary OH groups with

(6) D. B. Sprinson and A. Coulon, *J. Biol. Chem.*, 207, 585 (1954).

(7) B. Weiss, *ibid.*, **238**, 1953 (1963).

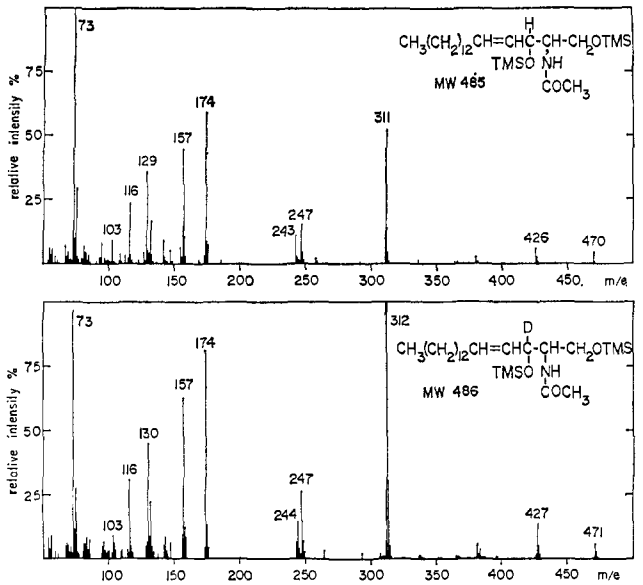


Figure 1. Mass spectra of 1,3-di-O-trimethylsilyl-N-acetylsphingosine (upper) and 1,3-di-O-trimethylsilyl-N-acetylsphingosine-d_3 (lower).

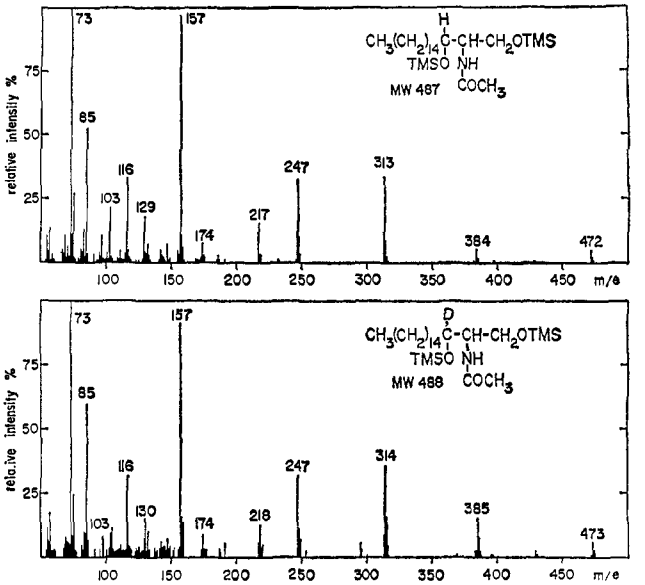


Figure 2. Mass spectra of 1,3-di-O-trimethylsilyl-N-acetyldihydrosphingosine (upper) and 1,3-di-O-trimethylsilyl-N-acetyldihydrosphingosine-d_3 (lower).

chromic anhydride in a mixture of pyridine and benzene. Reduction of N-acyl-3-oxo bases with $NaBD_4$ or $NaBT_4$ affords an excellent route to isotope-labeled ceramides for biochemical studies.

Results

The reaction of sphingosine or dihydrosphingosine with acetic anhydride in methanol gave N-acetyl derivatives in high yields. Gas chromatography of the crude products, after first converting free hydroxyl groups to trimethylsilyl ethers,[8] indicated that O-acetylation had not occurred to a significant extent under these conditions. Infrared spectra of the N-acetyl derivatives confirmed the absence of ester groups in the products. Bands at 1310, 1555–1565, and 1655–1660 cm^{-1} were indicative of an amide group in each compound; the spectrum of N-acetylsphingosine had a band at 970 cm^{-1} for a *trans* olefinic bond, as expected. On thin layer chromatography in chloroform–methanol (93:7) both products exhibited single spots with the same R_f value of 0.13 ± 0.05.

For mass spectrometric studies of these compounds, using a combined instrument for recording spectra of substances as they are eluted from a gas chromatograph, N-acetylsphingosine and N-acetyldihydrosphingosine were converted to 1,3-di-O-trimethylsilyl derivatives. The mass spectrum of 1,3-disilyloxy-2-acetamido-4-octadecene is shown in Figure 1. The molecular weight (485) is clearly indicated by the presence of peaks at *m/e* 470 (M − 15) for loss of a methyl group from one of the trimethylsilyl residues, *m/e* 426 (M − 59) for loss of the acetamido group plus a neighboring hydrogen, and *m/e* 395 (M − 90) for loss of trimethylsilanol. Cleavage of the molecule between carbon atoms 2 and 3 with charge retention on the larger fragment gave a major peak at *m/e* 311 (M − 174); a large peak was also observed at *m/e* 174. The small peak at *m/e* 103 may be due to loss of the terminal $CH_2OSi(CH_3)_3$.

(8) R. C. Gaver and C. C. Sweeley, *J. Am. Oil Chemists' Soc.*, 42, 294 (1965).

The mass spectrum of the saturated compound, 1,3-disilyloxy-2-acetamidooctadecane, from dihydrosphingosine, showed a series of fragment ions that were analogous to those obtained with the sphingosine derivative. As shown in Figure 2, peaks were observed at *m/e* 472, 428, 397, and 384 for loss of 15, 59, 90, and 103 mass units, respectively, from the molecular ion (487). It is interesting to note that cleavage of the molecule between carbons 2 and 3 to give M − 174 at *m/e* 313 is less pronounced in the saturated derivative. On the other hand, a peak at *m/e* 157 is stronger in the saturated derivative.

Exploratory studies of various conditions for the selective oxidation of the secondary, allylic hydroxyl group in N-acetylsphingosine were monitored by examining ultraviolet spectra of the crude products, since formation of the expected conjugated ketone would be accompanied by the appearance of strong absorption near 230 mμ.[9]

When N-acetylsphingosine was treated for prolonged periods with manganese dioxide[10] in neutral solvents such as chloroform, no reaction occurred. On the other hand, exposure of N-acetylsphingosine to three oxidizing equivalents of chromic anhydride in dry pyridine, for 5 hr at room temperature, gave a product with strong ultraviolet absorption at λ_{max} 230 mμ. The crude product was examined by thin layer chromatography. Two major components were found; one cochromatographed with starting material while the other, a less polar substance, was the desired product. Some oxidation of N-acetyldihydrosphingosine also occurred under these conditions, as judged by thin layer chromatography.

Additional experiments were undertaken with chromic anhydride in a variety of solvents to determine optimal conditions for the oxidation. Reactions con-

(9) R. B. Woodward, *J. Am. Chem. Soc.*, 64, 76 (1942); L. F. Fieser and M. Fieser, "Steroids," Reinhold Publishing Corp., New York, N. Y., 1959, p 19.

(10) J. Attenburrow, A. F. B. Cameron, J. H. Chapman, R. M. Evans, B. A. Hems, A. B. A. Jansen, and T. Walker, *J. Chem. Soc.*, 1094 (1952).

ducted in pyridine, 86.5% acetic acid, and glacial acetic acid gave some of the presumed product, but several other compounds were also produced in significant amounts. With diethyl ether, benzene, or water as solvent, chromic anhydride gave little or no oxidation of either the allylic or saturated secondary alcohols. A mixture of dry benzene and pyridine (1:1) appeared to be the solvent of choice for the oxidation, since crude extracts of these reaction mixtures consisted almost exclusively of starting material and a single oxidation product.

Silicic acid chromatography was used to purify the major oxidation products and to recover substantial amounts of unreacted material. Although the final yields ranged from 30 to 50%, recovered starting material could be reoxidized to improve the over-all yield. Infrared and ultraviolet spectra of the products, circular dichroism curves of the olefinic 3-oxo compound related to sphingosine,[11] and sodium borohydride reductions established the preferential oxidation of the secondary hydroxyl group. The ultraviolet spectrum of 3-oxo-1-hydroxy-2-acetamido-4-octadecene is shown in Figure 3; it supported the presence of an α,β-unsaturated ketone, with the predicted λ_{max} 230 mμ (ϵ 14,000). On addition of sodium borohydride the peak rapidly disappeared. The infrared spectrum of the oxidation product had bands at 1265, 1630, and 1700 cm^{-1} for aliphatic ketone, 980 cm^{-1} for *trans* C=C, 1300, 1565, and 1650 cm^{-1} for an amide, and an intensified band at 1070 cm^{-1} for a primary alcohol. The band at 1100 cm^{-1} in N-acetylsphingosine, indicative of a secondary alcohol, was absent in the spectrum of the oxidation product, but reappeared in the spectrum of the product recovered after sodium borohydride reduction. Since α,β-unsaturated ketones adjacent to an asymmetric carbon atom exhibit multiple Cotton effects,[12] a circular dichroism curve was examined. The presence of two small peaks at 340 and 290 mμ, and definite indication of a large peak below the limit of resolution of the instrument (250 mμ), provided additional evidence for the postulated structure of the oxidation product from N-acetylsphingosine.

Reduction of the oxidation product of N-acetyl sphingosine with sodium borodeuteride gave a mixture of four compounds. which could be separated as 1,3-di-O-trimethylsilyl derivatives by gas chromatography on 3% SE-30 at 220°. Two of the compounds representing about 75% of the mixture were the *erythro* and *threo* forms of 1,3-di-O-trimethylsilyl-N-acetylsphingosine, each of which contained one deuterium atom. Their mass spectra (Figure 1) were virtually identical and the location of the deuterium atom in the fragment at *m/e* 312, corresponding to *m/e* 311 in the parent compound (Figure 1), provided conclusive evidence for the 3-oxo structure. Had oxidation of the primary OH group occurred with chromic anhydride, a shift of *m/e* 174 to 175 would have been observed in this spectrum. If both hydroxyl groups had been oxidized, appropriately higher *m/e* values would have been observed for M − 15, M − 59, and M − 90 ions. The finding of a new peak at *m/e* 104 in the deuterium-substituted N-acetyl

(11) The authors are grateful to Mr. Roland Haines, Chemistry Department, University of Pittsburgh, for running circular dichroism curves with a Dichrographic.

(12) C. Djerassi, R. Riniker, and B. Rinker, *J. Am. Chem. Soc.*, 78, 6477 (1956).

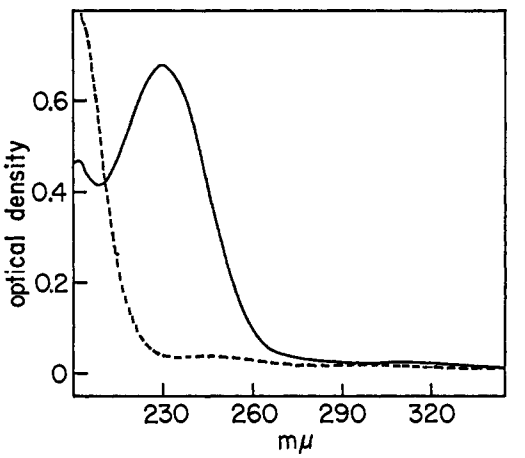
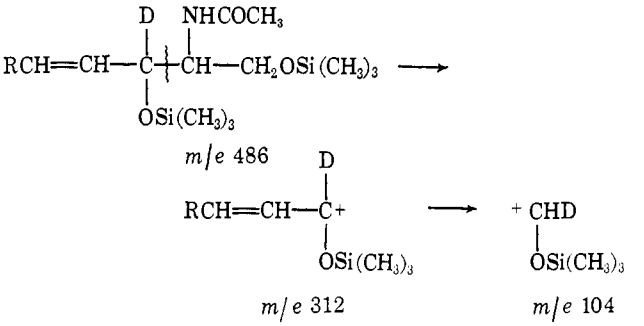


Figure 3. Ultraviolet spectrum (ethanol) of 3-oxo-1-hydroxy-2-acetamido-4-octadecene (———) and a repeated recording after addition of sodium borohydride (- - - - - -).

sphingosine derivative is inexplicable since it cannot be related to loss of $CHDOSi(CH_3)_3$ from the terminal group including carbon atom one. It may be, however, that initial fragmentation of M to yield M − 174 continues with rearrangement loss of $CHOSi(CH_3)_3$ or $CDOSi(CH_3)_3$ and neighboring hydrogen. Retention

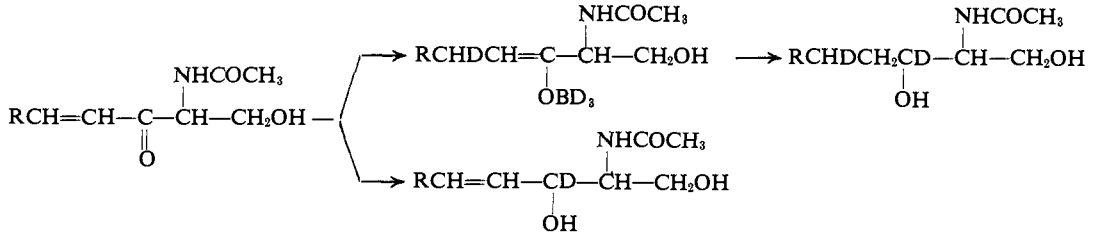


of deuterium in the fragment ion at *m/e* 427 (M − 59) indicates that loss of the acetamido group and a hydrogen occurs by a selective rearrangement process which does not involve the hydrogen (or deuterium) atom on C_3.

Mass spectra of the other two compounds in this mixture were also identical, suggesting a second *erythro–threo* pair. One of these compounds had the same retention time as that for 1,3-di-O-trimethylsilyl-N-acetyldihydrosphingosine, and mass spectrometry confirmed this identification. Substitution with two deuterium atoms was indicated by the spectrum, and from their location in the M − 174 fragment ion at *m/e* 315, we have inferred that 1,4 addition occurred partially in the reduction of the conjugated ketone with sodium borodeuteride. Thus, nucleophilic attack of borodeuteride ions at the carbonyl carbon atom as well as at the β-carbon atom of the double bond led to the formation of a mixture of saturated and unsaturated alcohols, each as an *erythro–threo* pair, as shown in Chart I. These results are consistent with previous studies on 1,4 additions with metal hydride ions, as summarized recently by Jackson and Zurqiyah.[13] Some control of the proportion of saturated alcohol presumably can be exerted through choice of solvent and molar ratios of reactants.[13]

(13) W. R. Jackson and A. Zurqiyah, *J. Chem. Soc.*, 5280 (1965).

Chart I



The specific rotation of N-acetylsphingosine was $-10°$ while that of 3-oxo-1-hydroxy-2-acetamido-4-octadecene was $+11.8°$. It is of interest that N-acetylsphingine (2-acetamidooctadecan-1-ol), lacking a hydroxyl group and therefore asymmetry at C_3 (as in the ketone), has a reported specific rotation of $+12°$.[14]

Oxidation of N-acetyldihydrosphingosine gave 3-oxo-1-hydroxy-2-acetamidooctadecane in 40 to 50% yield. Although ultraviolet spectra could not be used, in this case, to confirm the oxidation of the secondary OH group, infrared spectra of the saturated ketone and the conjugated ketone were nearly identical. The 1090-cm^{-1} band for a secondary alcohol was absent in the oxidation product and the area under the band at 3300 cm^{-1} was reduced. Characteristic absorption for ketones appeared at 1275 and 1710 cm^{-1}; these bands were absent in the product recovered after sodium borohydride reduction of the saturated 3-oxo derivative. The mass spectrum of *erythro*-1,3-di-O-trimethylsilyl-N-acetyldihydrosphingosine recovered after sodium borodeuteride reduction of the 3-oxo compound is shown in Figure 2. The product contained one deuterium atom; its location in the M − 174 fragment ion at *m/e* 314 agrees with the results obtained with the allylic system in sphingosine and provides conclusive evidence for preferential oxidation of the secondary hydroxyl group in dihydrosphingosine.

Experimental Section

Thin layer chromatographic plates (2 × 8 in.) were coated with silica gel G (Brinkmann Instruments Co.) by dipping clean glass plates into a suspension of silica gel–chloroform–methanol (1:2:1, w/v/v).[15] After thorough air drying, solutions were applied to the plates with a microliter syringe and chromatography was carried out in tanks lined with solvent-saturated Whatman No. 3M paper. Compounds on the developed plates were detected by exposure to iodine vapor or by charring with strong sulfuric acid.

Purification of the 3-oxo derivatives was achieved by silicic acid column chromatography. The silicic acid (Unisil, 100–200 or 200–325 mesh, from Clarkson Chemical Co.) was dried overnight at 125–130° before use, and packed as a slurry in anhydrous, reagent grade chloroform.

Gas chromatography of N-acetyl-O-trimethylsilyl derivatives of sphingolipid bases, on 2.5% SE-30 columns at 180–200°, has been described.[8]

Microanalyses were performed by Dr. Alfred Bernhardt, Max Planck Institut, Mulheim (Ruhr), Germany. Infrared spectra were obtained with potassium bromide disks, using a Beckman IR-4 spectrophotometer, and ultraviolet spectra were recorded in ethanol solutions with a Cary Model 14 recording ultraviolet spectrophotometer. Melting points were determined with a Fisher–Johns apparatus or a Kofler-type hot stage.

Mass spectra were obtained with an LKB 9000, consisting of a gas chromatograph and single focusing mass spectrometer coupled directly through Becker–Ryhage molecule separators.[16] N-Acetyl-1,3-ditrimethylsilyl derivatives were chromatographed at 220° on 3% SE-30; spectra were recorded at 70 ev and 3.5 kv accelerating voltage, with an ion source temperature of 250°.

erythro-**Dihydrosphingosine.** Chemical synthesis of the free base was carried out by the method of Shapiro, *et al.*, for the synthesis of sphingosine.[17] Gas chromatographically pure palmitic acid[18] was used as starting material. Yields of various intermediates generally agreed with those reported. One of the intermediates, ethyl 2-acetamido-3-oxooctadecanoate, was impure when crystallized from methanol as recommended;[17] satisfactory purity was obtained, however, by crystallization from petroleum ether (bp 67–75°). The identity and purity of each intermediate (except palmitoyl chloride) were determined by thin layer chromatography in petroleum ether–diethyl ether–glacial acetic acid (80:20:1.5) or chloroform–methanol (95:5), melting point, and infrared spectrum. The final product melted at 86–88°; its R_f in chloroform–methanol–water (100:42:6) was identical with that of an authentic sample.

trans-D-*erythro*-**1,3-Dihydroxy-2-amino-4-octadecene (Sphingosine, I).** Natural sphingosine was isolated from ceramides of a commercial beef lung lipid fraction by the method of Tipton.[19] Its chromatographic behavior on thin layer chromatography was the same as that of synthetic base.

N-Acetyldihydrosphingosine. Synthetic dihydrosphingosine (100 mg, 0.33 mmole) was dissolved in 10 ml of methanol with warming, 2.3 ml of acetic anhydride was added, and the solution was allowed to stand at room temperature overnight. The reaction mixture was then transferred to 20 ml of ice water, an additional 10 ml of cold water was added, and the finely divided precipitate was collected on a medium porosity sintered-glass funnel. Washing with water and drying on the funnel *in vacuo* over calcium chloride yielded 130 mg (114%) of white powder, mp 122–127°. The infrared spectum of the product was consistent with the ceramide structure; no carbonyl ester or ketone bands were present. Analysis for nitrogen by the micro-Kjeldahl technique gave an average for duplicates of 3.94% (calcd 4.08%). Gas chromatography of the 1,3-di-O-trimethylsilyl derivative on 3% SE-30 at 220° showed a single peak. The mass spectrum of this derivative is shown in Figure 2.

N-Acetylsphingosine. A sample of sphingosine (167 mg, 0.56 mmole) was dissolved in 10 ml of methanol and the solution was mixed with 1.0 ml of acetic anhydride. After standing overnight at room temperature, 20 ml of water was added and the mixture was chilled in ice for several hours. Centrifugation gave a gummy white solid which was resuspended in water and filtered. Washing with water and drying *in vacuo* over calcium chloride gave a white powder, mp 85–87°, in 93% yield. The product was reprecipitated from a methanol solution by addition of water, $[\alpha]^{26}D$ −10.0° (*c* 1.7, methanol). Gas chromatography of the 1,3-di-O-trimethylsilyl derivative gave a single peak. The product was identified by infrared spectroscopy and mass spectrometry (Figure 1).

Anal. Calcd for $C_{20}H_{39}NO_3$: C, 70.33; H, 11.51; N, 4.10. Found: C, 71.05; H, 11.65; N, 6.32.[20]

3-Oxo-1-hydroxy-2-acetamidooctadecane. A solution of N-acetyldihydrosphingosine (24.2 mg, 71 μmoles) in 1.5 ml of dry benzene was mixed with 1.5 ml of a solution containing 65 mg of dry chromic anhydride in 10 ml of pyridine (distilled over barium oxide). After filling the glass-stoppered centrifuge tube with nitrogen,

(14) H. E. Carter and C. G. Humiston, *J. Biol. Chem.*, **191**, 727 (1951); D. E. Sunko and M. Prostenik, *J. Org. Chem.*, **18**, 1523 (1953), reported a specific rotation of +11.74°.

(15) C. C. Sweeley, *J. Lipid Res.*, **4**, 402 (1963).

(16) R. Ryhage, *Anal. Chem.*, **36**, 759 (1964).

(17) D. Shapiro, H. Segal, and H. M. Flowers, *J. Am. Chem. Soc.*, 80, 1194 (1958); D. Shapiro, H. M. Flowers, and S. Spectro-Shefer, *ibid.*, **81**, 4360 (1959).

(18) Applied Science Corp., State College, Pa.

(19) C. L. Tipton, *Biochem. Prepn.*, **9**, 127 (1963).

(20) The high nitrogen value is attributed to formation of some methane instead of carbon dioxide in micro-Dumas analysis of long-chain, aliphatic amines. See A. Steyermark, "Quantitative Organic Microanalysis," Academic Press Inc., New York, N. Y., 1961, p 152.

the mixture was shaken thoroughly and left at room temperature for 2 hr with occasional shaking. During this time the reaction mixture became progressively more turbid with a brown precipitate. The reaction mixture was added to 6.5 ml of cold 5 *N* hydrochloric acid, 6.5 ml of cold, distilled water, and 25 ml of diethyl ether. After shaking, the ether layer was removed and the lower layer was extracted several times with small volumes of ether. The combined extracts were washed to neutrality, dried over anhydrous sodium sulfate, and evaporated *in vacuo* to give 22 mg of a white-yellow solid.

The crude product was dissolved in chloroform and applied to an 8-g column of silicic acid. Elution with 100 ml of chloroform removed 2 mg of a mixture of unidentified products. The desired product (12 mg) was recovered by elution with 150 ml of 1% methanol in chloroform, and unreacted starting material (19 mg) was eluted with 5% methanol in chloroform. Lyophilization of an acetic acid solution of the product yielded a white powder, mp 104–105°. On thin layer chromatography in chloroform–methanol (95:5) the product gave a single spot with R_f 2.5 relative to that of N-acetyldihydrosphingosine.

Anal. Calcd for $C_{20}H_{39}NO_3$: C, 70.33; H, 11.51; N, 4.10. Found: C, 70.09; H, 10.99; N, 5.75[20] (Kjeldahl: N, 4.00).

3-Oxo-1-hydroxy-2-acetamido-4-octadecene. N-Acetylsphingosine (65.5 mg, 192 μmoles) was oxidized by chromic anhydride in benzene–pyridine as described above. Silicic acid column chromatography of the crude product (37.6 mg) gave 16.3 mg of the 3-oxo compound. The product was crystallized from pentane, mp 69–70°, $[\alpha]^{25}D$ +11.8° (*c* 11.3, ethanol), λ_{max}^{EtOH} 230 mμ (ε 14,300).

Anal. Calcd for $C_{20}H_{37}NO_3$: C, 70.75; H, 10.98; N, 4.13. Found: C, 70.47; H, 10.66; N, 4.39.

Reduction of N-Acetyl-3-oxo Bases with Sodium Borohydride. About 3 mg of sample was dissolved in 1 ml of methanol and the solution was cooled to 10–12°. One drop of 1 *N* sodium hydroxide and 0.2 ml of a solution of 20 mg of sodium borohydride in 10 ml of methanol were added. The solution was left at 10–12° for 15 min. Another 0.2 ml of sodium borohydride solution was added and the mixture was kept at 10–12° for an additional 15 min. Cold, saturated sodium chloride (1 ml) was added and the product was extracted into diethyl ether. The emulsion which resulted was broken by the addition of 1 ml of water. The aqueous layer was extracted twice more with ether, and the combined total extracts were washed with water and evaporated to dryness. Vacuum drying over calcium chloride and lyophilization from acetic acid solution gave about 3 mg of a white-yellow solid. The products were examined by thin layer chromatography, and infrared and ultraviolet spectra were recorded.

Samples of about 1 mg were reduced with excess sodium borodeuteride in methanol. After standing overnight at room temperature, excess water was added and the mixture was extracted twice with equal volumes of diethyl ether. The combined ether layers were washed once with water, dried over anhydrous sodium sulfate, and evaporated to dryness *in vacuo*. The crude product was prepared for gas chromatography and mass spectrometry by addition of 0.1 ml of a mixture consisting of pyridine, hexamethyldisilazane, and trimethylchlorosilane in the proportions 10:2:1.[8]

Acknowledgments. This investigation was supported by Public Health Service Research Grant AM-04307 from the National Institute of Arthritis and Metabolic Diseases. The experimental studies herein reported constituted a portion of the Ph.D. Thesis of Robert C. Gaver, University of Pittsburgh, 1964. We are indebted to Mr. John Naworal and Mrs. Thelma Hamilton for assistance in recording mass spectra and in preparation of the mass spectral tables.

Communications to the Editor

5-Hydroxy-7-chlortetracycline

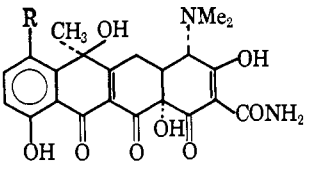
Sir:

The outstanding antibacterial properties of the tetracycline antibiotics, coupled with their remarkable clinical effectiveness, have provided the stimulus for an intensive effort to find new members of the class with superior properties. The chemical mating of oxytetracycline (IIa) and chlortetracycline (IIb) has been recognized as a desirable, but elusive, goal. We wish to report the preparation of the desired 5-hydroxy-7-chlortetracycline (IIc) and present some of its chemical and biological properties.

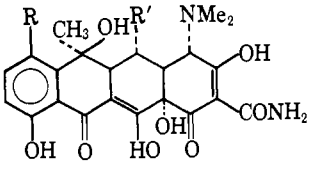
Recently the terminal stages of the biosynthesis of oxytetracycline (IIa) and tetracycline (IId) have been reported and these antibiotics were shown to arise through a common intermediate (Ia).[1] By logical extension 5a,11a-dehydrochlortetracycline (Ib) would be the biological parent of 5-hydroxy-7-chlortetracycline (IIc). Despite severe difficulties occasioned by the unusual lability of 5-hydroxy-7-chlortetracycline in neutral or alkaline solutions, we have succeeded in obtaining the antibiotic as its crystalline hydrochloride after incubation of 5a,11a-dehydrochlortetracycline hydrochloride (Ib) with a washed cell preparation of *Streptomyces rimosus*, strain BE514 (ATCC 13,224).[2]

(1) P. A. Miller, J. H. Hash, M. Lincks, and N. Bohonos, *Biochem. Biophys. Res. Commun.*, 18, 325 (1965).

The culture was grown under conditions generally suitable for other *S. aureofaciens* and *S. rimosus* strains.



Ia, R = H
b, R = Cl



IIa, R = H; R′ = OH
b, R = Cl; R′ = H
c, R = Cl; R′ = OH
d, R = R′ = H

The cells were removed by centrifugation, washed with dilute buffer, and resuspended in one-fourth of the original volume, using pH 6.6 buffer; 500 μg/ml of substrate (Ib) was added, and, after about 6 hr, a transformation yield of approximately 200 μg/ml was obtained. The product was isolated by a process consisting of acidification, filtration, butanol extraction of the filtrate, pH gradient partition chromatography on cellulose powder at low temperatures, countercurrent distribution, and crystallization from acid–water. By working quickly and avoiding pH's above 3 and temperatures above 25° as far as possible, satisfactory isolation yields of crystalline antibiotic were obtained.

(2) The same transformation (Ib to IIc) has been observed independently by Dr. J. R. D. McCormick of these laboratories working with whole cell systems.