Chemistry and Metabolism of Sphingolipids. Chemical Synthesis of 2-Amino-1-hydroxyoctadecan-3-one (3-Ketosphinganine)*

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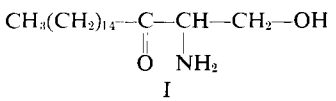
ABSTRACT: A method is reported for the chemical synthesis of 3-ketosphinganine from commercially available sphinganine. The amino group of sphinganine was protected with a carbobenzyloxy group, the secondary alcohol was selectively oxidized to the ketone with chromic anhydride, and the product was converted by hydrogenolysis into 3-ketosphinganine.

Mass spectra of the carbobenzyloxy derivatives of sphinganine and 3-ketosphinganine are presented. The structure of free 3-ketosphinganine was confirmed by reduction with sodium borodeuteride, N acetylation, and gas chromatography–mass spectrometry of the trimethylsilyl derivative.

A ketone (I) was first speculated to be an intermediate in sphingolipid biosynthesis by Sprinson and Coulon (1954) on the basis of experiments with rats, in which it was shown that



I

subcutaneously injected labeled serine was incorporated into sphingosine of brain lipids. Brady and Koval (1958) subsequently demonstrated requirements for palmitoyl-CoA, pyridoxal phosphate, serine, Mn(II), and NADPH in an *in vitro* microsomal system from rat brain. Definitive evidence has been obtained recently by Stoffel *et al.* (1968) and Braun and Snell (1968) for the intermediate role of I in the synthesis of sphinganine with a cell-free fraction from yeast (*Hansenula ciferrii*).

We wish to report details of a simple and efficient method for the chemical synthesis of I from sphinganine, fashioned after a previously described oxidation of *N*-acetylsphinganine to the *N*-acetyl derivative of I with chromic anhydride (Gaver and Sweeley, 1966).

Experimental Section

N-Carbobenzyloxysphinganine. This compound was first made by Weiss (1957), according to a procedure different from the following one. Carbobenzyloxychloride was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and sphinganine was purchased from Miles Laboratories, Elkhart, Ind. Sphinganine (50 mg, 166 μmoles) was dissolved with warming in 50 ml of ethyl acetate in a 250-ml glass-stoppered erlenmeyer flask. An equal volume of 0.2 M sodium carbonate (pH 9.5) was added, followed by 31.2 mg (183 μmoles) of carbobenzyloxychloride. The biphasic system was stirred vigorously for 5 min at room temperature, after which it was transferred to a small separatory funnel, the lower aqueous phase was removed, and the ethyl acetate layer was washed to neutrality with water. After removing ethyl acetate *in vacuo*, the residue was dried by azeotropization with absolute ethanol and the product was transferred in a small volume of chloroform to 3 g of silicic acid (200–325 mesh Unisil from Clarkson Chemical Co., Williamsport, Pa., heat activated at 110° for 24 hr prior to use) previously equilibrated in a small column with chloroform. The column was eluted with chloroform and gave a fraction, collected from 40 to 80 ml, which contained 54.3 mg (75% yield) of *N*-carbobenzyloxysphinganine that was chromatographically homogeneous in thin-layer chromatography solvent systems A–C (channel 3, Figure 1). The product was dissolved in 65 ml of hot 70% ethanol (Weiss, 1957) to give a water-clear solution which was filtered through glass wool, slowly cooled to 5°, and then left to stand over a period of 24 hr. After careful removal of the supernatant solvent with a pipet, the bright white crystalline lath clusters (42 mg) were recovered on a porous porcelain plate and dried *in vacuo* over P_2O_5 and paraffin chips. The product was stable at room temperature: mp (Kofler) 113–114°, net yield 58%.

N-Carbobenzyloxy-3-ketosphinganine. *N*-Carbobenzyloxysphinganine (20 mg, 46 μmoles) was weighed into a 50-ml glass-stoppered round-bottom flask and dissolved in 10 ml of dry benzene (percolated through anhydrous magnesium sulfate in a column). A mixture of chromic anhydride (310 mg, 3.1 mmoles) and 10 ml of pyridine (freshly redistilled from solid barium oxide) was added to the benzene solution, and the mixture was agitated with a stream of nitrogen bubbles for 5 min. The flask was then stoppered and shaken gently for 20 hr, after which the slurry was transferred to a 250-ml separatory funnel containing equal volumes of diethyl ether and water. After shaking, the isolated ether layer was washed five times with water, concentrated to a small volume *in vacuo*, and taken to dryness by azeotropization with absolute ethanol. The residue was dissolved in a small volume of chloro-

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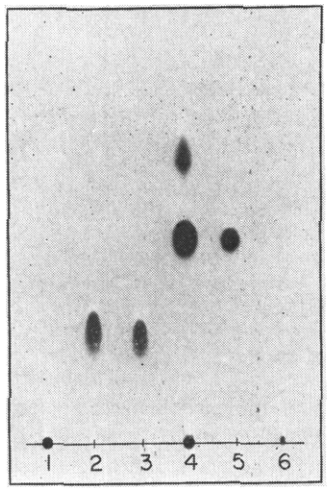

FIGURE 1: Blueprint of a thin-layer chromatographic plate, solvent system chloroform–methanol (25:1, v/v). (1) Sphinganine, (2) crude *N*-carbobenzyloxysphinganine before silicic acid chromatography (unreacted noniodine staining carbobenzyloxychloride is present at R_F 0.54), (3) pure *N*-carbobenzyloxysphinganine, (4) crude *N*-carbobenzyloxy-3-ketosphinganine, (5) pure *N*-carbobenzyloxy-3-ketosphinganine, and (6) 3-ketosphinganine.

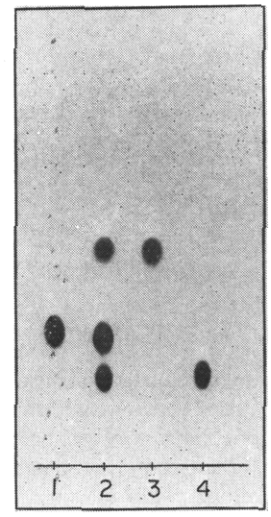

FIGURE 2: Blueprint of a thin-layer chromatographic plate, solvent system A. (1) Sphingosine; (2) sphinganine, sphingosine, and 3-ketosphinganine; (3) 3-ketosphinganine; and (4) sphinganine.

form and distributed across the origin of a thin-layer plate, which was developed in chloroform–methanol (25:1, v/v). Staining with iodine vapor (channel 4, Figure 1) revealed at least two major bands at R_F 0.47 (product) and 0.67 (an unknown by-product of the reaction). An additional band at R_F 0.25 was sometimes observed in reaction mixtures; this unidentified component was not residual starting material. A residue at the origin was entirely due to chromic anhydride.

After iodine had evaporated from the thin-layer chromatography plate, the silica gel area corresponding to the band with R_F 0.47 was removed and eluted with chloroform–methanol (2:1, v/v), from which 12 mg of an off-white, semicrystalline product was obtained. The product was recrystallized from 9 ml of warm 57% ethanol by slow cooling to 5° and standing for 24 hr, yielding 9 mg of bright white needles that were recovered on a porcelain plate and dried *in vacuo* over P_2O_5 and paraffin chips. The compound was stable at room temperature and was homogeneous in thin-layer chromatography solvents A–C (channel 5, Figure 1): mp (Kofler) 70–71°, net yield 45%.

Reduction of the *N*-carbobenzyloxy-3-ketosphinganine with sodium borohydride (Gaver and Sweeley, 1966) resulted in complete conversion into *N*-carbobenzyloxysphinganine, determined by cochromatography with authentic material in thin-layer chromatography solvents A–C.

3-Ketosphinganine (*I*). Crystalline *N*-carbobenzyloxy-3-ketosphinganine (5.2 mg, 12 μmoles) was dissolved in 5 ml of absolute ethanol in a 3-dram vial containing 5 mg of palladium black (Nutritional Biochemical Corp.) and a small magnetic stirring bar. Hydrogen was bubbled gently through the slowly stirred mixture for 10 min, after which the solution was centrifuged, and the supernatant was evaporated to dryness under a stream of nitrogen, yielding 3.4 mg (95% yield) of an unstable white amorphous material that gave only one thin-layer chromatography spot in solvents A–C (channel 6, Figure 1, and channel 3, Figure 2).

Reduction of I with sodium borodeuteride in a dilute methanol solution gave a material that was chromatographically identical with sphinganine in solvent system A. For further identification by mass spectrometry, the product was converted into the *N*-acetyl-*O*-trimethylsilyl derivative as previously described (Gaver and Sweeley, 1966).

Methods

Analytical and preparative thin-layer chromatography analyses were made on Merck precoated silica gel G plates (8 × 8 in. from Brinkman Instruments, Westbury, N. Y.) in three solvent systems: (A) chloroform–methanol–2 N ammonium hydroxide (40:10:1, v/v), (B) chloroform–methanol–water (65:25:4, v/v), and (C) chloroform–methanol (10:1, v/v). The glass tanks were lined with Whatman No. 3MM filter paper.

The spots on the thin-layer chromatography plates were visualized by exposure to iodine vapor, and the blueprints were prepared according to the method of Eisenberg (1962) using No. 1919-A Diazo Projection Paper (B. K. Elliot Co., Pittsburgh, Pa.).

Mass spectra were recorded by direct probe analysis with an LKB 9000 mass spectrometer; the ion source and probe temperatures were 270° and 100°, respectively, an electron energy of 70 eV was used (60-μA trap current), and the accelerating voltage was 3500 V. Elemental compositions of selected ions were determined by high-resolution mass spectrometry with a modified MS 902 mass spectrometer on-line with an S.D.S. Sigma 7 computer. All measurements were within 2 ppm of calculated masses.

Results and Discussion

Attempts to carry out direct oxidations of sphinganine or its hydrochloride salt to the 3-keto derivative (I) with chromic anhydride were not successful, probably because the product was not stable in the reaction mixture with pyridine and oxidizing agent. An approach through the *N*-carbobenzyloxy

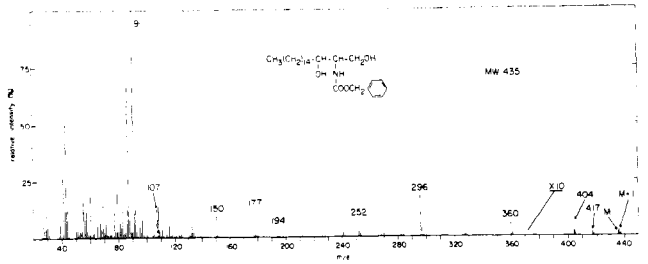

FIGURE 3: Mass spectrum of *N*-carbobenzyloxysphinganine.

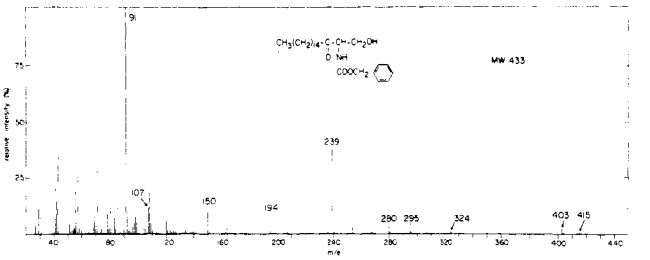

FIGURE 4: Mass spectrum of *N*-carbobenzyloxy-3-ketosphinganine.

derivative of sphinganine was therefore used to protect the product from further reaction in the pyridine–chromic anhydride, and to provide a stable compound from which the 3-ketosphinganine could be generated under mild conditions.

The mass spectrum of *N*-carbobenzyloxysphinganine is shown in Figure 3. Confirmation of the structure shown above the bar graph was afforded by the presence of a molecular ion at *m/e* 435, fragment ions at *m/e* 194 ($C_{10}H_{12}NO_3$) and 241 ($C_{16}H_{33}O$) for cleavage of the C-2–C-3 bond, a rearrangement ion at *m/e* 417 ($C_{26}H_{43}NO_3$) for loss of water, and a large tropylium ion at *m/e* 91. Peaks at *m/e* 107 (C_7H_7O) and 108 (C_7H_8O) are specific for benzyloxy and benzyl alcohol ions. A prominent pathway of decomposition resulted from loss of the terminal CH_2OH group from molecular ion to give a fragment ion at *m/e* 404 ($C_{25}H_{42}NO_3$), with subsequent expulsion of carbon dioxide to give *m/e* 360 ($C_{24}H_{42}NO$), a postulated transition that was supported by a metastable ion at *m/e* 321.5 (calcd 320.8). Ions at *m/e* 296 ($C_{18}H_{34}NO_2$) and 252 ($C_{17}H_{34}N$) are similarly related by a metastable peak at *m/e* 215 (calcd 214.5) for loss of carbon dioxide from *m/e* 296. It is difficult to formulate a mechanism for the formation of *m/e* 177 ($C_{10}H_{11}NO_2$), but the composition of this ion suggests formation by loss of an OH group from *m/e* 194.

The mass spectrum of *N*-carbobenzyloxy-3-ketosphinganine is shown in Figure 4. A molecular ion was not detected but the molecular weight could be deduced from ions at *m/e* 415 ($C_{26}H_{41}NO_3$) and *m/e* 403 ($C_{25}H_{41}NO_3$), attributed to the loss of water and formaldehyde, respectively. The latter decomposition is presumed to result from a McLafferty rearrangement of the molecular ion involving the terminal CH_2OH and the ketone group at C-3. This unusual rearrangement is analogous to one previously described with β-hydroxy esters (Etemadi, 1964) and a sesquiterpenol (Burlingame *et al.*, 1967). Cleavage of the C-2–C-3 bond with charge retention on the carbonyl group gave a prominent ion at *m/e* 239 ($C_{16}H_{31}O$), accompanied by a smaller ion at *m/e* 194 ($C_{10}H_{12}NO_3$) for charge retention on the other fragment from this cleavage. The latter ion loses carbon dioxide to form *m/e* 150 ($C_9H_{12}NO$). A tropylium ion (*m/e* 91) was the most intense ion in the mass spectrum.

Hydrogenolysis of the *N*-carbobenzyloxy-3-ketosphinganine was a very smooth reaction which proceeded to completion in about 5 min. The structure of the product was proved by further reduction to sphinganine with sodium borodeuteride. A mass spectrum of the *N*-acetyl-1,3-di-*O*-trimethylsilyl derivative of the sphinganine, recorded by combined gas chromatography–mass spectrometry, indicated the presence of a single deuterium atom at C-3.

Stability studies with I indicated that it could be stored as a solid or in a dilute solution of chloroform–methanol (2:1, v/v) at −15° for periods up to 3 days, but the compound is unstable at room temperature. After a week at room temperature in an ethanol solution, thin-layer chromatography indicated that I was converted completely into two relatively less polar products. When I was prepared by this method from ^3H-labeled sphinganine of specific activity 2.4 μCi/μmole, much larger amounts of breakdown product were found within a short time after hydrogenolysis of the carbobenzyloxy ketone, indicating an increased lability in the presence of intense β radiation.

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