BIOCHEMISTRY OF THE SPHINGOLIPIDES

V. THE STRUCTURE OF SPHINGINE*

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In 1916 Levene and West (2, 3) reported that chemical reduction of dihydrosphingosine yielded an optically active base sphingine which they considered to be an amino alcohol, but which they did not characterize further. Recently Carter et al. (4) discovered that catalytic reduction of triacetylsphingosine produced acetic acid, presumably as a result of the hydrogenolysis of the allylic acetoxy group. A further study of this reaction seemed desirable in order to verify the structure proposed for the hydrogenolysis product and to compare its properties with those reported for sphingine.

Two competing reactions (see the accompanying equations) occur in the reduction of triacetylsphingosine.

\[
\begin{align*}
R\text{CH} &= \text{CH} - \text{CH} - \text{CH} - \text{CH}_2 & & \text{Pt} \rightarrow & & R\text{CH} &= \text{CH} - \text{CH}_2 \text{CH} - \text{CH}_2 \\
& & \text{O} & & & & \text{O} \\
& & \text{NH} & & & & \text{NH} \\
& & \text{Ac} & & & & \text{Ac} \\
\text{H}_2 \text{(Pt)} & & \text{H}_2 \text{(Pt)} \& & \text{H}_2 \text{(Pt)} & & \text{H}_2 \text{(Pt)}
\end{align*}
\]

Direct hydrogenation of the double bond produces triacetyldihydrosphingosine, which is stable toward further reduction. Hydrogenolysis of the acetoxy group gives an unsaturated intermediate which then undergoes hydrogenation of the double bond. The product thus obtained was an amorphous solid which could not be fractionated or obtained in a crystal-

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line form from the usual solvents. In attempting to acetylate this material, however, it was discovered that a mixture of pyridine and acetic anhydride gave a nicely crystalline product which was essentially free of triacetylhydrosphingosine. The properties of this material (m.p. 108–109°; \( \alpha \righthook{27}^\circ = +22.5^\circ \) (chloroform solution)), together with its method of preparation, leave no doubt as to its identity with diacetylsphingine.\(^1\)

Hydrolysis of diacetylsphingine gave the crystalline free base. This was further characterized by preparation of the N-benzoyl and O,N-di-benzoyl derivatives. Analytical data for these substances, together with previous work on the structure of sphingosine, established the structure of sphingine as 1-hydroxy-2-aminooctadecane. This structure was confirmed by oxidation of N-benzoylsphingine with chromic acid. The product was an optically active substance giving correct analytical data for benzyolaminostearic acid. Racemization of this acid was readily effected through the azlactone. The product was identical with synthetic benzoyldL-\(\alpha\)-aminostearic acid.

\[
\begin{align*}
CH_2(CH_2)_{14}CH(CH=CH)OH & \xrightarrow{\text{CrO}_3} CH_2(CH_2)_{14}CHCO_2H \\
| & |
\text{NH} & \text{NH} \\
| & |
\text{CO} & \text{CO} \\
\text{C}_6\text{H}_5 & \text{C}_6\text{H}_5
\end{align*}
\]

\(N\)-Benzoylsphingine \((-\)-Benzoyl-\(\alpha\)-aminostearic acid

These results afford final proof of the structure of sphingine and of sphingosine.

The conversion of sphingosine to an optically active \(\alpha\)-aminostearic acid afforded an approach to the stereochemistry of the sphingosine molecule. Determination of the configuration of the aminostearic acid by the usual physical and enzymatic methods did not seem promising, due to the insolubility of the amino acid in water, dilute acid, and dilute alkali. Therefore, a study was undertaken of the rotation of acyl derivatives of \(\alpha\)-amino acids in aqueous organic solvents, based on the preliminary observations of Stevens (5) that various derivatives of \(p\)-methoxyphenyl-L-alanine showed consistent levo shifts in optical activity on diluting their alcohol solutions with water. Alcohol was not a suitable solvent for benzyolaminostearic acid but dioxane and glacial acetic acid proved satisfactory. The specific rotations of a series of acyl derivatives of L- and D-amino acids were determined in the anhydrous solvents and in solutions

\(^1\) In later work it was discovered that acetonitrile is a very satisfactory solvent for purification of diacetylsphingine and other sphingosine derivatives.
containing 10 and 20 per cent water. The results of these studies are shown in Table I.

In every case there was a consistent shift in rotation from the anhydrous to the aqueous solvents, derivatives of L-amino acids becoming more levo-rotatory and those of D-amino acids more dextrorotatory. It is interesting to note that free amino acids show corresponding shifts in rotation as the pH of the solution increases from a low value to the isoelectric region. These changes are ascribed to the conversion of a unionized carboxyl group to the carboxylate anion. At first glance it seemed possible that the shifts in rotation of the acyl derivatives also might result from ionization of the carboxyl groups, since increases in dielectric constant due to dilution of an organic solvent with water might be expected to increase the extent of ionization. However, the behavior of benzoyl-L-alanine in aqueous solutions does not support this view, since the rotation shifts in the dextro direction in going from pH 2 to pH 5. Extension of such data in aqueous solution is hampered by the low solubility of acyl derivatives of amino acids in water at a low pH. Regardless of the explanation, the consistent behavior of the acyl derivatives studied affords an additional physical basis for relating optical properties of amino acids to configura-

Table I

Rotation of Acyl Derivatives of Amino Acids

<table>
<thead>
<tr>
<th>Acyl derivative</th>
<th>Solvent</th>
<th>Specific rotation $[\alpha]^2D$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anhydrous</td>
<td>10 per cent water</td>
</tr>
<tr>
<td></td>
<td>degrees</td>
<td>degrees</td>
</tr>
<tr>
<td>Benzoyl-L-alanine</td>
<td>Acetic acid</td>
<td>+24.2</td>
</tr>
<tr>
<td></td>
<td>Dioxane</td>
<td>+19.6</td>
</tr>
<tr>
<td></td>
<td>Acetic acid</td>
<td>-23.8</td>
</tr>
<tr>
<td></td>
<td>Dioxane</td>
<td>-19.3</td>
</tr>
<tr>
<td></td>
<td>Acetic acid</td>
<td>-31.2</td>
</tr>
<tr>
<td></td>
<td>Dioxane</td>
<td>-106.0</td>
</tr>
<tr>
<td></td>
<td>Acetic acid</td>
<td>-30.0</td>
</tr>
<tr>
<td></td>
<td>Dioxane</td>
<td>-26.2</td>
</tr>
<tr>
<td></td>
<td>Acetic acid</td>
<td>+33.6</td>
</tr>
<tr>
<td></td>
<td>Dioxane</td>
<td>+41.9</td>
</tr>
<tr>
<td></td>
<td>Acetic acid</td>
<td>-29.6</td>
</tr>
<tr>
<td></td>
<td>Dioxane</td>
<td>-43.4</td>
</tr>
<tr>
<td></td>
<td>Acetic acid</td>
<td>-42.5</td>
</tr>
<tr>
<td></td>
<td>Dioxane</td>
<td>-72.0</td>
</tr>
<tr>
<td></td>
<td>Acetic acid</td>
<td>+57.5</td>
</tr>
<tr>
<td></td>
<td>Dioxane</td>
<td>-4.3</td>
</tr>
<tr>
<td></td>
<td>(-)-Benzoyl-(\alpha)-aminostearic acid</td>
<td>-12.8</td>
</tr>
</tbody>
</table>

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tion. On this basis it is suggested tentatively that the sphingine oxidation product is benzoyl-d-α-aminostearic acid. This result raises the possibility that serine may serve as the precursor of the terminal 3 carbons of the sphingosine molecule. If, in the biosynthesis, a condensation occurs involving the carboxyl group of serine, a D configuration of the amino carbon atom would be expected in the derived α-aminostearic acid. This possibility is being investigated.

EXPERIMENTAL

Preparation of Diacetylsphingine—Triacetylsphingosine (2.12 gm., 5.0 mm) was dissolved in 100 ml. of warm 95 per cent ethanol and reduced in the Adams hydrogenation apparatus with 100 mg. of freshly prepared platinum oxide (6). When reduction was complete, the reaction mixture was warmed to dissolve the precipitate which had formed and the catalyst was collected on a filter. The clear filtrate was concentrated to dryness in vacuo, giving 1.97 gm. of a white amorphous residue which melted at 88–106°. This crude product was dissolved by warming in a mixture of 10 ml. of pyridine and 10 ml. of acetic anhydride and allowed to stand 2 hours at room temperature. A white crystalline precipitate formed. It was removed by filtration, washed free of solvent with water, and dried in a vacuum desiccator. The product weighed 1.02 gm. and melted at 90–108°. Recrystallization from 15 ml. of 95 per cent ethanol gave white needles melting at 107–109°. In a second run 5.0 gm. of triacetylsphingosine in 100 ml. of warm ethanol were reduced with 200 mg. of platinum oxide, giving 2.47 gm. of crude diacetylsphingine. The products from similar runs were combined (6.29 gm.) and recrystallized from 125 ml. of methanol, giving 5.6 gm. of pure diacetylsphingine (m.p. 108–109°; [α]_D^25 = +22.5° (0.107 gm. in 10 ml. of chloroform)).

Analysis—C_{37}H_{54}O_2N (369.6). Calculated. C 71.49, H 11.73, N 3.79
Found. “ 71.49, “ 11.73, “ 3.85

N-Acetylsphingine—Diacetylsphingine (4.9 gm.) was dissolved with warming in a solution of 1.0 gm. of potassium hydroxide in 130 ml. of 90 per cent methanol. The reaction mixture was allowed to stand at room temperature for 8 hours. The solution was cooled and the crystalline precipitate was filtered, washed thoroughly with water, and dried in vacuo over phosphorus pentoxide. The crude residue (3.87 gm., 89 per cent of the theoretical yield) was recrystallized twice from 40 ml. of methanol, giving 3.26 gm. of N-acetylsphingine melting at 101–103° ([α]_D^25 = +12.0° (0.101 gm. in 10 ml. of chloroform)).

Analysis—C_{32}H_{44}O_2N (327.6). Calculated. C 73.34, H 12.62, N 4.28
Found. “ 73.37, “ 12.69, “ 4.49
Sphingine—Pure N-acetylphosphingine (4.64 gm.) was refluxed for 8 hours with 150 ml. of 90 per cent methanol containing 10 gm. of potassium hydroxide. The reaction mixture was cooled and diluted with 400 ml. of water, forming a heavy white precipitate. The mixture was transferred to a separatory funnel and extracted with two 300 ml. portions of ether which dissolved the precipitate. The ether extract was washed twice with 250 ml. portions of water and dried over anhydrous sodium sulfate. The ether solution was concentrated to dryness, giving 4.43 gm. of rectangular plates melting at 72-88°. The crude cream-colored reaction product was washed with 25 ml. of anhydrous ether which removed the color, leaving 4.04 gm. of white plates which melted at 82-88°. Three recrystallizations from 100 ml. portions of hexane and one recrystallization from 100 ml. of acetonitrile gave 3.05 gm. of sphingine (m.p. 84-89°; \([\alpha]_D^{27} = -5.5°\) (0.100 gm. in 10 ml. of chloroform)).

Analysis—C_{14}H_{23}ON (285.5). Calculated. C 75.72, H 13.77, N 4.91

Found. " 75.74, " 13.69, " 4.97

N-Benzoylsphingine—3 gm. of sphingine were emulsified in 10 ml. of 2 N sodium hydroxide and 50 ml. of ether by vigorous shaking. 2 ml. of benzoyl chloride and 20 ml. of 2 N sodium hydroxide were added to the mixture in portions. The reaction mixture was cooled under the tap and shaken until the odor of benzoyl chloride had disappeared. The mixture was transferred to a separatory funnel and the white precipitate which formed at the interface of the water and ether layers was brought into solution by the addition of methanol. The ether-methanol layer was separated, washed with water until neutral to litmus, and concentrated to dryness in vacuo, giving 4.12 gm. of crystalline residue. This crude reaction product was recrystallized from 55 ml. of 95 per cent ethanol, giving 3.33 gm. of needles melting at 112-114°. A second recrystallization from 100 ml. of 90 per cent ethanol gave 2.95 gm. of crystals melting at 112-114°. 1 gm. of this product was recrystallized from 50 ml. of acetonitrile, giving 0.50 gm. of pure N-benzoylsphingine (m.p. 112-113° with transition in crystalline form at 92-93°; \([\alpha]_D^{27} = +21.8°\) (0.100 gm. in 10 ml. of chloroform)).

Analysis—C_{26}H_{24}O_{2}N (389.6). Calculated. C 77.07, H 11.13, N 3.60

Found. " 77.17, " 11.36, " 3.67

Oxidation of N-Benzoylsphingine to (—)-Benzoyl-α-aminostearic Acid—3 gm. of N-benzoylsphingine were warmed with 150 ml. of glacial acetic acid until all of the solid dissolved. To the solution were added 30 ml. of 50 per cent acetic acid containing 1.8 gm. of chromium trioxide. The mixture was heated under a reflux for 30 minutes. The reaction mixture was then cooled under the tap, diluted with 180 ml. of water, and allowed
to stand at room temperature for 30 minutes. A green precipitate formed in the dark solution. The mixture was transferred to a separatory funnel and extracted with two 250 ml. portions of ether. The greenish yellow ether extract was separated and washed with 200 ml. of 5 per cent hydrochloric acid. It was then washed repeatedly with water until the washings were neutral to litmus. A small amount of white precipitate that formed was removed by filtration. The ether filtrate was concentrated to dryness in vacuo, giving 3.22 gm. of gray-green chalky residue. This material was dissolved in 15 ml. of methanol with warming to form a dark green solution. A few drops of phenolphthalein were added and the solution made slightly alkaline with 0.2 N potassium hydroxide in methanol. The solution was transferred to a separatory funnel and extracted three times with 100 ml. portions of low boiling petroleum ether (30-60°). The methanol layer was then acidified with 5 per cent aqueous hydrochloric acid until the pH was less than 2. Water was added and the crystalline precipitate was extracted with 100 ml. of low boiling petroleum ether. The solution was evaporated under reduced pressure and the crude residue was dried in a vacuum desiccator over phosphorus pentoxide, giving 1.39 gm. of crystalline product melting at 90-93°. Recrystallization of the crude product from 5 ml. of benzene gave 1.13 gm. of (-)-α-benzoyl-α-aminostearic acid (m.p. 93-94°; \([a]_D^27 = -25.0°\) (0.107 gm. in 10 ml. of chloroform)). Two more recrystallizations of this product from 25 ml. portions of benzene did not change the melting point.

**Analysis**—C_{28}H_{41}O_{4}N (403.6). Calculated. C 74.40, H 10.24, N 3.47. Found. C 74.18, H 10.52, N 3.61

**Neutral equivalent**—Calculated, 403.6; found, 411.0

(-)-Benzoyl-α-aminostearic acid is soluble in acetone, ethyl acetate, methanol, chloroform, hot benzene, and hot high boiling petroleum ether. It is only slightly soluble in 95 per cent ethanol or ether.

**Racemization of (-)-Benzoyl-α-aminostearic Acid**—50 mg. of (-)-benzoyl-α-aminostearic acid were heated on the steam bath with 0.4 ml. of acetic anhydride for 1 hour. Water (0.1 ml.) was then added and a crystalline precipitate formed. The reaction mixture was warmed to solution and allowed to cool slowly, a crystalline precipitate again forming. The liquid was removed by a capillary pipette, and the residue was washed twice with 1 ml. portions of water and dried in a vacuum desiccator over phosphorus pentoxide. The product melted over a wide range (65-107°). This crude product was refluxed for 2 hours with 25 ml. of 0.12 N potassium hydroxide in 90 per cent methanol. The reaction mixture was made strongly acid and the crystalline precipitate which formed was collected on a filter, washed with water, and dried in a vacuum desiccator over
phosphorus pentoxide. The product weighed 40 mg. and melted at 106–116°. Recrystallization from 0.5 ml. of methanol gave 30 mg. of benzoyl-
DL-α-aminostearic acid (m.p. 113–116°), which showed no depression when mixed with an authentic sample.

Preparation of Synthetic Benzoyl-DL-α-aminostearic Acid—Since DL-α-
aminostearic acid is insoluble in aqueous sodium or potassium hydroxide, the benzoylation was carried out by emulsifying the amino acid in a solu-
tion of Triton B. 1 gm. of DL-α-aminostearic acid was dissolved in 14
ml. of 38 per cent Triton B solution and 6 ml. of water. 1 ml. of benzoyl
chloride was added and the mixture was cooled and shaken vigorously
until the reaction was complete. The reaction mixture was made strongly
acid with 10 per cent hydrochloric acid and the gelatinous precipitate
was filtered, washed well with water, and dried. The crude product
melted at 70–90°. Attempted recrystallization from methanol resulted
in a colloidal suspension which could not be filtered. Recrystallization
from 20 ml. of acetonitrile gave 0.79 gm. of impure material melting at
65–105°. Concentration of the filtrate gave 0.88 gm. of a light amber
oil. The crude solid was recrystallized from 2 ml. of methanol, giving
0.47 gm. of crystalline material melting at 109–116°. A second recrystal-
lization from 0.8 ml. of methanol gave 0.45 gm. of material melting at
112–116°. A final recrystallization from 1 ml. of methanol gave 0.45 gm.
of benzoyl-DL-α-aminostearic acid melting at 115–116°.

SUMMARY

A convenient method has been developed for the preparation of diace-
tylsphingine by catalytic reduction of triacetylsphingosine. The structure
of sphingine has been established as 1-hydroxy-2-aminooctadecane by ox-
idation of the N-benzoyl derivative to (-)-benzoyl-α-aminostearic acid.
It has been discovered that acyl derivatives of D- and L-amino acids
show characteristic changes in optical rotation on dilution of their dioxane
or acetic acid solutions with water. These data appear to afford a new
method of correlating the configuration of amino acids with their optical
properties. On this basis, the (-)-benzoyl-α-aminostearic acid obtained
from sphingine has been assigned the D configuration.

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