Synthesis of 4-Oxonorleucine and Comparison with an Amino Acid Isolated from Citrobacter freundii*

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Many new amino acids have been discovered in plants (1-9) and in microorganisms (10-13) which are not normally found in animal tissues. Some of these show little structural similarity to the amino acids found in animal protein whereas others are closely related structural analogues. The isolation of 4-oxonorleucine from acid hydrolysates of a polysaccharide obtained from Citrobacter freundii was recently reported (14). As this amino acid had not previously been found in nature, it was of interest to firmly establish its identity by synthesis and by a comparison of the properties of the isolated material with those of the synthetic product. In addition, a comparison was made of 4-hydroxynorleucine obtained by reduction of the isolated amino acid with synthetic 4-hydroxynorleucine.

EXPERIMENTAL PROCEDURE

Method of Synthesis—The synthesis of 4-oxonorleucine was achieved by condensation of bromomethyl ethyl ketone with the sodio derivative of diethylacetamidomalonate in benzene. The diethylacetylacetamidomalonate was hydrolyzed with acid to remove ester and N-acetyl groups and to effect decarboxylation. The acid anion was removed from the product of hydrolysis by ion exchange chromatography.

The synthesis of 4-hydroxynorleucine was performed by reduction of 4-hydroxynorleucine with hydrogen with platinum oxide as catalyst.

Bromomethyl Ethyl Ketone—Methyl ethyl ketone, 900 g, was dried overnight over 90 g of anhydrous calcium chloride and filtered. The filtrate was fractionally distilled, and the fraction, b.p. 78.5-80.5°, was collected. Bromine, 710 g, was slowly added, with stirring, to a mixture containing 500 g of methyl ethyl ketone, 1110 g of water, and 133 g of potassium chloride at 90°. Once the bromination reaction was initiated, the temperature was maintained at 35-40°. After addition of the bromine, the mixture was allowed to stand for 1 hour. The organic layer was separated and shaken with a mixture of magnesium oxide and water. The organic layer was dried overnight over 90 g of anhydrous calcium chloride and filtered. The filtrate was evaporated to dryness in a vacuum. This process was repeated three more times to remove excess hydrochloric acid. A yield of 34.8 g (61%) with a melting point of 67-68° was obtained.

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collected. A yield of 6.0 g (79%) with a melting point of 142-143° was obtained.

\[
\text{C}_{6}\text{H}_{13}\text{NO}_3
\]

Calculated: C 49.65, H 7.64, N 9.65
Found: C 49.74, H 7.67, N 9.49

4-Hydroxynorleucine—To 50 ml of 95% ethanol were added 50 mg of platinum oxide catalyst and 2 ml of an aqueous solution containing 2 g of 4-oxonorleucine. Hydrogen gas was passed through the solution until the catalyst turned black. The gas pressure was raised to 50 p.s.i. and left for 1 hour at 24° with shaking. The catalyst was removed by filtration, and the filtrate was concentrated to 20 ml at 35° in a vacuum and placed overnight at 4°. The precipitate was collected by filtration and dissolved in 10 ml of ethanol. After standing overnight at 4°, the product was collected. This material was redissolved in ethanol and placed at 4°, and the precipitate was collected. A yield of 1.56 g (78%), m.p. 199-200°, was obtained.

\[
\text{C}_{6}\text{H}_{13}\text{NO}_3
\]

Calculated: C 48.9, H 8.94, N 9.38
Found: C 48.95, H 8.94, N 9.52

4-Oxonorleucine from Citrobacter freundii—The 4-oxonorleucine was obtained from acid hydrolysates of the purified polysaccharide isolated from C. freundii as previously described (14). The 4-hydroxynorleucine was prepared by reduction of 4-oxonorleucine (14).

Elemental analysis were performed by Schwarzkopf Microanalytical Laboratory, New York, and by Galbraith Laboratories, Inc., Knoxville, Tennessee.

**RESULTS**

Properties of 4-Oxonorleucine and of Amino Acid Isolated from C. freundii—Elemental analyses of synthetic 4-oxonorleucine and of the amino acid isolated from hydrolysates of C. freundii polysaccharide are recorded in Table I. From the data it can be seen that the formula \(\text{C}_{6}\text{H}_{13}\text{NO}_3\) represents most closely the composition of each material. Titration of either substance in formol solution (17) with standard alkali gave neutral equivalent values which are in agreement with the formula \(\text{C}_{6}\text{H}_{13}\text{NO}_3\). The melting point of 4-oxonorleucine is identical with that found for the isolated amino acid. Moreover, the melting point of the mixture showed no depression.

Partition paper chromatography of 4-oxonorleucine and of the isolated amino acid gave identical mobilities in acidic and basic solvent systems. It is of interest to note that 4-oxonorleucine and the amino acid yield a yellow color with the ninhydrin reagent (18).

An infrared absorption spectrum of 4-oxonorleucine and of the amino acid taken in a potassium bromide pellet is given in Fig. 1. It is apparent that the spectra of both samples are identical at every major point of reference. The strong absorption bands between 6.0 and 6.4 μ are typical of amino acids, and the band at 6.28 μ can be assigned to an ionic carboxyl absorption. The strong absorption band at 5.82 μ is typical of that given by an undisturbed stretching vibration of a carbonyl situated between two methylene groups.

Nuclear magnetic resonance spectra of 4-oxonorleucine and of the isolated amino acid taken in deuterium oxide are shown in Fig. 2. It is obvious that the spectra of both samples are similar, and that the number of protons on each of the carbon atoms in each material is identical. Thus, the groupings, \(-\text{C}_2\text{H}_5\), \(-\text{CH}_3\), and \(-\text{CH}_2\), previously identified in the isolated amino acid (14) are also present in synthetic 4-oxonorleucine.

From the data which have been presented, it seems justifiable to conclude that the amino acid isolated from hydrolysates of the polysaccharide obtained from C. freundii is identical with 4-oxonorleucine.

**Properties of 4-Hydroxy norleucine and of Amino Acid Isolated from C. freundii after Reduction**—Elemental analyses of synthetic 4-hydroxy norleucine and of the product obtained after reduction of the amino acid isolated from hydrolysate of C. freundii are recorded in Table II. From the data it can be seen that the formula \(\text{C}_6\text{H}_{15}\text{NO}_2\) represents closely the composition of each material. The melting point of 4-hydroxy norleucine is slightly below that found for the reduced amino acid. However, the melting point of the mixture showed no depression of the value obtained for 4-hydroxy norleucine.

Partition paper chromatography of 4-hydroxy norleucine and of the reduced amino acid gave identical mobilities in acidic and basic solvent systems. It is of interest to note that 4-hydroxy norleucine and the reduced amino acid yield a purple color with the ninhydrin reagent (18).

An infrared absorption spectrum of 4-hydroxy norleucine and of the reduced amino acid taken in a potassium bromide pellet is given in Fig. 3. It is apparent that the spectra of both samples are similar at every major point of reference. The
FIG. 2. Nuclear magnetic resonance spectrograms taken on a 2% solution of substance in deuterium oxide at a sweep width of 500 c.p.s. at 60 megacycles and sweep time of 500 seconds; spectrum amplification 25, integral amplification, 80; gain cut by one-tenth at 4.63 p.p.m. Curve A, amino acid isolated from hydrolysate of C. freundii polysaccharide; Curve B, 4-oxonorleucine.

Table II

Comparison of properties of reduced isolated amino acid and of 4-hydroxynorleucine

<table>
<thead>
<tr>
<th></th>
<th>Reduced amino acid</th>
<th>4-Hydroxynorleucine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon, %</td>
<td>48.72</td>
<td>48.85</td>
</tr>
<tr>
<td>Hydrogen, %</td>
<td>9.14</td>
<td>8.94</td>
</tr>
<tr>
<td>Nitrogen, %</td>
<td>9.41</td>
<td>9.43</td>
</tr>
<tr>
<td>Empirical formula</td>
<td>C₆H₁₂NO₃</td>
<td>C₆H₁₂NO₄</td>
</tr>
<tr>
<td>Color with ninhydrin</td>
<td>Purple</td>
<td>Purple</td>
</tr>
<tr>
<td>Melting point</td>
<td>201°</td>
<td>199°</td>
</tr>
<tr>
<td>Mixed melting point (50%-50%)</td>
<td>199</td>
<td>199</td>
</tr>
<tr>
<td>RF at 24° relative to proline</td>
<td>1.34</td>
<td>1.35</td>
</tr>
<tr>
<td>Pyridine-water, 4:1</td>
<td>1.60</td>
<td>1.60</td>
</tr>
<tr>
<td>1-Butanol-water-acetic acid, 4:5:1</td>
<td>1.60</td>
<td>1.60</td>
</tr>
</tbody>
</table>

FIG. 3. Infrared spectrograms taken on 2.0 mg of substance in 800 mg of potassium bromide pellet. Curve A, amino acid isolated from hydrolysate of C. freundii polysaccharide after reduction; Curve B, 4-hydroxynorleucine.

Strong absorption bands between 6.0 and 6.4 μ, typical of amino acids, are present in both materials. However, the strong absorption band at 5.82 μ present in the spectra of 4-oxonorleucine and the amino acid is absent in the spectra of 4-hydroxynorleucine and the reduced amino acid. Moreover, a strong band appears at 2.90 μ in the spectra of the latter two materials, which is typical for the O—H valence stretching vibrations of an unbonded hydroxyl. The strong 2.90 μ absorption band is absent in the spectra of 4-oxonorleucine and the amino acid.

From the data it seems justifiable to conclude that the product obtained upon reduction of the amino acid isolated from C. freundii is identical with 4-hydroxynorleucine.

Discussion

Proline and hydroxyproline are the only amino acids found in animal proteins which give a yellow color in the ninhydrin reaction. A few amino and cyclic imino acids found in plants, which include derivatives of pipicolic acid (1), proline (2), cyclopropane (3), azetidine (4), γ-methyleneglutamic acid (3), and γ-amino-γ-methylenebutyric acid (6) among others, may yield yellow color complexes with the ninhydrin reagent. In contrast, most amino acids which have a primary amino group yield a purple color in the ninhydrin reaction. As 4-oxonorleucine contains a primary amino group, it might at first seem unexpected that this compound gives a yellow rather than a purple color complex with ninhydrin. However, it is of interest to note that one of the enol forms (II, Scheme 1) of 4-oxonorleucine (1) bears a striking resemblance to the structure of γ-methyleneglutamic acid (III) and to γ-amino-γ-methylenebutyric acid (IV).

It appears, therefore, that a strong attraction may exist in...
these compounds between the electropositive nitrogen atoms and the electronegative ethylene carbons to form pyrrolidine-like structures (V to VII). This bonding is strong enough, under conditions of the ninhydrin reaction, to give the primary amino nitrogen properties of a secondary amino. Reduction of the oxo group to 4-hydroxynorleucine in 4-oxonorleucine or of the methylene group to \( \gamma \)-methylglutamic acid in \( \gamma \)-methylene-glutamic acid removes the restriction for the amino group; thus, purple color complexes are formed with ninhydrin.

The results of the present investigations clearly verify that 4-oxonorleucine is present in acid hydrolysates of a polysaccharide obtained from *C. freundii*. The biological importance or distribution of this new and hitherto undescribed amino acid in products of bacterial origin is unknown. However, the detection of 4-oxonorleucine in hydrolysates of another polysaccharide isolated from *Salmonella dahlem* (14) indicates that this amino acid can be found in products obtained from bacteria of different genera.

**Summary**

The synthesis of 4-oxonorleucine and of 4-hydroxynorleucine is described. A comparison was made of the elemental analyses, spectral data, and physical and chemical properties of 4-oxonorleucine with an amino acid isolated from hydrolysates of a polysaccharide obtained from *C. freundii*. The 4-hydroxynorleucine was compared with the reduced amino acid.

Synthetic 4-oxonorleucine is identical with the isolated amino acid and synthetic 4-hydroxynorleucine is identical with the reduced isolated amino acid.

**References**

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