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**Metabolism of Sphingosine Bases, XVI**[1]

# Studies on the Stereospecificity of the Introduction of the Hydroxy Group of 4*D*-Hydroxysphinganine (Phytosphingosine)

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**Summary:** 4*D*-Hydroxysphinganine[(2*S*,3*S*,4*R*)-1,3,4-trihydroxy-2-aminooctadecane] has three centers of chirality at carbon atoms 2, 3 and 4. They are introduced sequentially. The first center (2*S*) is introduced by the condensation, with decarboxylation, of palmitoyl-CoA and (*S*)-L-serine to 3-dehydrosphinganine, the second by the stereospecific reduction of (2*S*)-3-dehydrosphinganine to (2*S*,3*R*)-sphinganine.

In this communication we have shown that *Hansenula ciferri* uses 1) [3-$^3$H; 3-$^{14}$C]sphinganine as substrate for the 4-hydroxysphinganine biosynthesis and that the hydroxy group at carbon atom 4 is introduced into sphinganine itself and 2) that the 3-keto derivative is not the substrate. 3) We further studied the utilization of (*R*)- and (*S*)-[2-$^3$H]palmitic acid in the synthesis of sphinganine by this yeast. The hydrogen isotope on carbon atom 4 of the sphinganine molecule, which results from the condensation of (*R*)-[2-$^3$H]palmitic acid and serine with subsequent reduction, is eliminated completely in the transformation of sphinganine to 4-hydroxysphinganine, that of the *S* isomer on the other hand is fully retained. This finding excludes the formation of an intermediate carbonyl group at C-4 of sphinganine.

**Zusammenfassung:** *Stoffwechsel von Sphingosinbasen, XVI: Untersuchungen über die Stereospezifität der Einführung der Hydroxylgruppe an 4D-Hydroxysphinganin (Phytosphingosin).* 4-Hydroxysphinganin[(2*S*,3*S*,4*R*)-1,3,4-Trihydroxy-2-aminooctadecan] besitzt drei chirale Zentren an den Kohlenstoffatomen 2, 3 und 4. Sie werden nacheinander errichtet. Das erste Zentrum (2*S*) entsteht in der decarboxylierenden Kondensation von Palmitoyl-CoA und (*S*)-L-Serin zu 3-Dehydrosphinganin. Das zweite optische Zentrum resultiert aus der stereospezifischen Reduktion der 3-Ketogruppe des 3-Dehydrosphinganins. In dieser Arbeit wird gezeigt, daß *Hansenula ciferri* [3-$^3$H; 3-$^{14}$C]Sphinganin als Substrat für die 4-Hydroxysphinganin-Biosynthese verwendet und daß der Einbau der Hydroxygruppe am Kohlenstoffatom 4 des Sphinganins und nicht des 3-Dehydrosphinganins erfolgt.

(*R*)- und (*S*)-[2-$^3$H]Palmitinsäure werden von dieser Hefe für die Synthese von Sphinganin verwendet. Das Wasserstoffisotop am Kohlenstoffatom 4 des Sphinganins mit *R*-Konfiguration wird bei der Umwandlung von Sphinganin in 4-Hydroxysphinganin vollständig eliminiert, das des *S*-Isomeren bleibt erhalten.

Diese Befunde schließen das Auftreten einer intermediären Carbonylgruppe am Kohlenstoffatom 4 des Sphinganins aus.

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*Enzymes:*

3-Dehydrosphinganine synthase, acyl CoA:serine C-2-acyl transferase (decarboxylating) (EC 2.3.1.?; not yet listed)
3-Dehydrosphinganine reductase, D-sphinganine:NADP oxidoreductase (EC 1.1.1.?; not yet listed)
Sphinganine-1-phosphate aldolase, sphinganine-1-phosphate alkanal-lyase (EC 4.1.2.?; not yet listed)
Sphinganine kinase, ATP:sphinganine phosphotransferase (EC 2.7.1.?; not yet listed).

[1] XV. Commun.: W. STOFFEL and G. ASSMANN, this journal **351**, 1041 [1970].

It is well established that the initial step in the biosynthesis of sphinganine consists of the condensation of palmitoyl-CoA and (2*S*)-L-serine yielding (2*S*)-3-dehydrosphinganine[2–6] catalyzed by a pyridoxal phosphate dependent synthase. This reaction, which proceeds with retention of the optical center at C-2 of serine, is followed by the stereospecific NADPH reduction of the 3-keto group yielding the second center of chirality at C-3 with 3*R* configuration[3,7]. The hydride ion is transferred from the B-side of the pyridine nucleotide, as demonstrated with A and B-NADP$^3$H using a purified reductase isolated from rat or beef liver microsomal fraction.

With regard to the biosynthesis of 4-hydroxysphinganine, LAW and his collaborators[8] established the precursor function of palmitic acid and serine. Our experiments[9] and those reported by WEISS and STILLER[10] demonstrated that sphinganine is the direct precursor. THORPE and SWEELEY[11] tried to determine the origin of the 4-hydroxy group using $^{18}O_2$ and $H_2{}^{18}O$. However the oxygen seemed to be derived from neither source.

We demonstrated that the degradation of 4-hydroxysphinganine follows the same route as that elaborated for sphinganine and 4*t*-sphingenine. The primary hydroxy group is first phosphorylated. The C-C linkage between C-2 and C-3 is cleaved in an aldolase-type reaction yielding phosphorylethanolamine and 2-hydroxypalmitaldehyde[12]. This may be oxidized to 2-hydroxypalmitic acid and eventually decarboxylated to pentadecanoic acid. Pentadecanoic acid has been described as a degradation product by BARENHOLZ and GATT[13].

In this paper we want to report the results of experiments which we carried out to determine

1) the direct precursor for the introduction of the hydroxy group yielding 4-hydroxysphinganine. We reinvestigated whether the 4-hydroxy group is introduced prior to the condensation to 3-dehydrosphinganine, or into the 3-keto intermediate itself, or whether sphinganine is the direct precursor. The use of [3-$^3$H; 3-$^{14}$C]*erythro*-sphinganine as substrate should answer this question.

2) The stereochemical event in the formation of the center of chirality at C-4 due to the introduction of the hydroxy group into sphinganine was studied. Since C-4 of 4-hydroxysphinganine corresponds to C-2 of palmitoyl-CoA, the substrates in the condensation reaction with serine to 3-dehydrosphinganine, (*R*)-, and (*S*)-[2-$^3$H]palmitic acids should be most suitable for studying the stereochemistry of this process.

### *Sphinganine, the direct precursor of 4-hydroxysphinganine*

The introduction of the 4-hydroxy group of the final 4-hydroxysphinganine could occur at more than one stage of the biosynthesis. We have demonstrated before that the hydroxy group is introduced into the complete $C_{18}$-skeleton of the long chain bases. We now wanted to determine, whether the hydroxylation follows directly the condensation of palmitoyl-CoA and serine to 3-dehydrosphinganine or whether the reduction of the 3-keto group precedes the hydroxylation to sphinganine. The crucial experiment would involve the use of [3-$^3$H; 3-$^{14}$C]-sphinganine as substrate and the determination of the isotope ratio in 4-hydroxysphinganine. We carried out this experiment by supplementing the culture medium of *Hansenula ciferri* with [3-$^3$H; 3-$^{14}$C]-sphinganine with a $^3$H/$^{14}$C-ratio of 52:1, under the usual conditions. *N*-Acetyl-4-hydroxysphinganine and *N*-acetylsphinganine were isolated from the medium and the sedimented yeast as described under "Experimental". Fig. 1 represents the radio thin-layer chromatogram of the *N*-acetylated long chain base fraction. About 60% was recovered as *N*-acetyl-4-hydroxysphinganine (A) and 40% as *N*-acetylsphinganine (B). *N*-Acetyl-4-hydroxysphin-

[2] W. STOFFEL, D. LEKIM and G. STICHT, this journal **349**, 664 [1968].

[3] W. STOFFEL, Chem. Physics Lipids **5**, 139 [1970].

[4] P. E. BRAUN and E. E. SNELL, J. biol. Chemistry **243**, 3775 [1968].

[5] R. N. BRADY, S. J. DIMARI and E. E. SNELL, J. biol. Chemistry **244**, 491 [1969].

[6] E. E. SNELL, S. J. DIMARI and R. N. BRADY, Chem. Physics Lipids **5**, 116 [1970].

[7] W. STOFFEL, D. LEKIM and G. STICHT, this journal **349**, 1637 [1968].

[8] M. L. GREENE, T. KANESHIRO and J. H. LAW, Biochim. biophysica Acta [Amsterdam] **98**, 582 [1965].

[9] W. STOFFEL, G. STICHT and D. LEKIM, this journal **349**, 1149 [1968].

[10] B. WEISS and R. L. STILLER, J. biol. Chemistry **242**, 2903 [1967].

[11] S. R. THORPE and C. C. SWEELEY, Biochemistry [Washington] **6**, 887 [1967].

[12] W. STOFFEL and G. ASSMANN, this journal, in preparation.

[13] Y. BARENHOLZ and S. GATT, Biochem. biophysic. Res. Commun. **27**, 319 [1967].

ganine and *N*-acetylsphinganine, isolated in pure form as judged by thin-layer and gas chromatography (Fig. 2), had identical and unchanged isotope ratios.

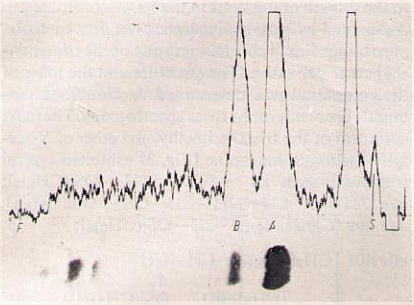



Fig. 1. Radio thin-layer chromatogram of *N*-acetylated long chain base fraction.
A: *N*-Acetyl-4-hydroxysphinganine, B: *N*-acetylsphinganine.
Silica gel H; solvent system: chloroform/methanol 8:1.

*Stereospecificity of the introduction of the 4-hydroxy group into sphinganine*

In separate experiments *Hansenula ciferri* was grown in 1 *l* cultures, the medium of which was supplemented with 40 μmol of (*R*)-[2-$^3$H; 1-$^{14}$C]-palmitic acid with a $^3$H/$^{14}$C-isotope ratio of 73:1 and (*S*)-[2-$^3$H; 1-$^{14}$C]palmitic acid with an isotope ratio of 58:1. After four days, when the stationary phase was reached, *N*-acetyl-4-hydroxysphinganine and *N*-acetylsphinganine were obtained after mild alkaline hydrolysis of the *O*-esters[14]. They were separated by preparative thin-layer chromatography on silica gel H (solvent system: chloroform/methanol 8:1) and proved to be pure by radio thin-layer chromatography. The two compounds were further characterized by combined gas-liquid chromatography-mass spectroscopy of their trimethylsilyl derivatives. The mass spectrum of the fully acetylated 4-hydroxysphinganine exhibited typical fragments[11] at *m/e* 84, *m/e* 144, *m/e* 485.

*Hansenula ciferri* grown in the medium supplemented with (*S*)-[2-$^3$H; 1-$^{14}$C]palmitic acid yielded a chromatographically pure *N*-acetyl-4-hydroxy-

[14] H. E. Carter and D. Shapiro, J. Amer. chem. Soc. 75, 5131 [1953].

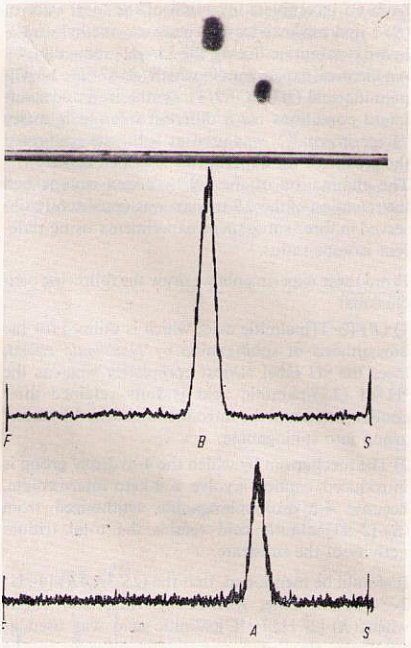



Fig. 2. Radio thin-layer chromatogram of purified A) *N*-acetyl-4-hydroxysphinganine, B) *N*-acetylsphinganine.
Silica gel H; solvent system: chloroform/methanol 8:1.

sphinganine fraction with a total radioactivity of $3.98 \times 10^6$ dpm $^3$H and $7.37 \times 10^4$ dpm $^{14}$C and therefore with an isotope ratio of 54:1, nearly identical with that of the substrate (*S*)-[2-$^3$H; 1-$^{14}$C]palmitic acid ($^3$H/$^{14}$C-ratio 58:1). On the other hand *N*-acetyl-4-hydroxysphinganine recovered from the *Hansenula* culture grown in a medium containing (*R*)-[2-$^3$H; 1-$^{14}$C]palmitic acid (isotope ratio 73:1) contained very little tritium $2.96 \times 10^5$ dpm $^3$H, but almost the same amount of $^{14}$C radioactivity $6.57 \times 10^4$ dpm $^{14}$C as in the parallel experiment with the 2*S* isomer. The isotope ratio had dropped from 73:1 in the substrate palmitic acid to 4.5:1 in the product 4-hydroxysphinganine. The small residual $^3$H radioactivity (6.1%) may be explained by arises

from an incomplete inversion of the tosyl ester of (*S*)-2-hydroxyhexadecyl acetate or methyl (*S*)-2-hydroxypalmitate during the $LiAlH_4$ reduction.

An identical experiment in which (*R*)-[2-$^3$H; 1-$^{14}$C]-palmitic acid ($^3H/^{14}C$ 49:1), synthesized under different conditions *via* a different intermediate (see "Experimental"), was used as substrate confirmed the results of the experiment described earlier.

The elimination of the 2*R* hydrogen isotope and the retention of the 2*S* tritium was consistently observed in three subsequent experiments using different isotope ratios.

From these experiments we draw the following conclusions:

1) (*R*)-[2-$^3$H]palmitic acid, which is utilized for the biosynthesis of sphinganine by *Hansenula ciferri*, loses the $^3$H label almost completely whereas the $^3$H of (2*S*)-palmitic acid is fully retained after condensation and the introduction of the 4-hydroxy group into sphinganine.

2) The mechanism by which the 4-hydroxy group is introduced cannot involve a 4-keto intermediate, because 4-hydroxysphinganine synthesized from (*S*)-[2-$^3$H]palmitic acid retains the total tritium activity of the substrate.

It should be mentioned, that the (2*S*,3*R*,4*R*)-[4-$^3$H; 3-$^{14}$C]sphinganine, formed by *Hansenula ciferri* when (*R*)-[2-$^3$H; 1-$^{14}$C]palmitic acid was used as substrate, showed a remarkable increase in the $^3H/^{14}C$-ratio. Whereas the substrate had a ratio of $^3H/^{14}C$ 57:1 that of *N*-acetylsphinganine increased to 280:1. This isotope ratio also remained constant in hexadecanal, obtained from sphinganine after hydrolysis of *N*-acetylsphinganine and periodate oxidation of the free base. On the other hand (*S*)-[2-$^3$H; 1-$^{14}$C]palmitic acid was incorporated into sphinganine and 4-hydroxysphinganine without any change in the isotope ratio. The strong isotope effect in the displacement of the *R*-tritium by a hydroxy group may provide guidance for further studies of the mechanism of this reaction.

### $^{18}O_2$ studies

The source of the oxygen being introduced at C-4 of sphinganine, thus forming 4*D*-hydroxysphinganine, was reinvestigated by growing *Hansenula ciferri* in an atmosphere of 98% $^{18}O_2$ for a period of four days. The yeast was harvested by centrifugation. The acetylated long chain bases were extracted from the supernatant and from the sediment with hexane. The *N*-acetylated bases were isolated and separated as outlined before and described under "Experimental". The isolation and identification of the two products were facilitated by the addition of [1-$^{14}$C]palmitic acid to the growth medium prior to the growth of the yeast in the $^{18}O_2$-atmosphere.

*N*-Acetyl-4-hydroxysphinganine was proved to be pure by gas-liquid chromatography of the trimethylsilyl ether derivative. The structure and the mass of the compound was determined by combined gas-liquid chromatography mass spectroscopy. The mass spectrum of the tris-*O*-trimethylsilyl ether of *N*-acetyl-4-hydroxysphinganine (Fig. 3) exhibited typical fragments such as *m/e* 103 [$CH_2$—$OSi(CH_3)_3$]; *m/e* 174 [$H_3C$—CO—NH—CH—$CH_2$—$OSi(CH_3)_3$]; *m/e* 299 [$CH_3(CH_2)_{13}$—CH—$OSi(CH_3)_3$];

*m/e* 401 $\left[\begin{array}{l} CH_3(CH_2)_{13}-CH-CH \\ \qquad\qquad\quad | \qquad\quad | \\ \qquad (CH_3)_3SiO \quad OSi(CH_3)_3 \end{array}\right]$.

Also the mass spectrum of tetraacetyl-4-hydroxysphinganine was recorded (Fig. 4). There was no fragment containing the $^{18}$O-atom at C-4 such as *m/e* 144 [$CH_3CO$—O—CH—CH—O—CO—$CH_3$] which would indicate incorporation of $^{18}O_2$. Also the molecule peak *m/e* 485 was identical with that of tetraacetyl-4-hydroxysphinganine isolated from *Hansenula ciferri* grown under normal aeration. These results confirm the previous finding of THORPE and SWEELEY[11], which ruled out molecular $^{18}O_2$ as the source of oxygen.

## Discussion

The present paper describes the results of experiments which were concerned with a) the nature of the immediate precursor of 4-hydroxysphinganine and b) the stereospecific introduction of the center of chirality at C-4 of sphinganine, which arises from the hydroxylation to 4-hydroxysphinganine.

Our results obtained from repeated experiments with [3-$^3$H; 3-$^{14}$C]*erythro*-sphinganine prove that *Hansenula ciferri* transforms the double labelled sphinganine to 4-hydroxysphinganine without the loss of the $^3$H isotope. These results prove that sphinganine, and not 3-dehydrosphinganine, is the direct precursor of 4-hydroxysphinganine. WEISS and STILLER[10] also demonstrated that *Hansenula ciferri* utilizes sphinganine present in the medium for the synthesis of 4-hydroxysphinganine. How-

ever, their substrate, [4,5-$^3H_2$]sphinganine, did not allow a conclusion to be drawn regarding the immediate precursor of 4-hydroxysphinganine.

It should be possible to study the stereospecificity of the introduction of the hydroxy group in position 4 of sphinganine with the 4*R*- and 4*S*-antipodes of (2*S*,3*R*)-[4-$^3$H]sphinganine as substrates. A chemical synthesis of these two enantiomers could be achieved, in principle, starting with (*S*)- and (*R*)-[2-$^3$H]palmitic acids. However it appeared a more feasible approach to supplement growing cultures of *Hansenula cif.* with (*R*)- and (*S*)-[1-$^{14}$C; 2-$^3$H]palmitic acids and then to isolate sphinganine and 4-hydroxysphinganine formed from these precursors. The $^3H/^{14}C$-ratio of the 4-hydroxysphinganine would then indicate conclusively whether the introduction of the hydroxy group leads to the elimination or retention of the $^3$H isotope of the enantiomeric forms. The results of these experiments were unambigous. The

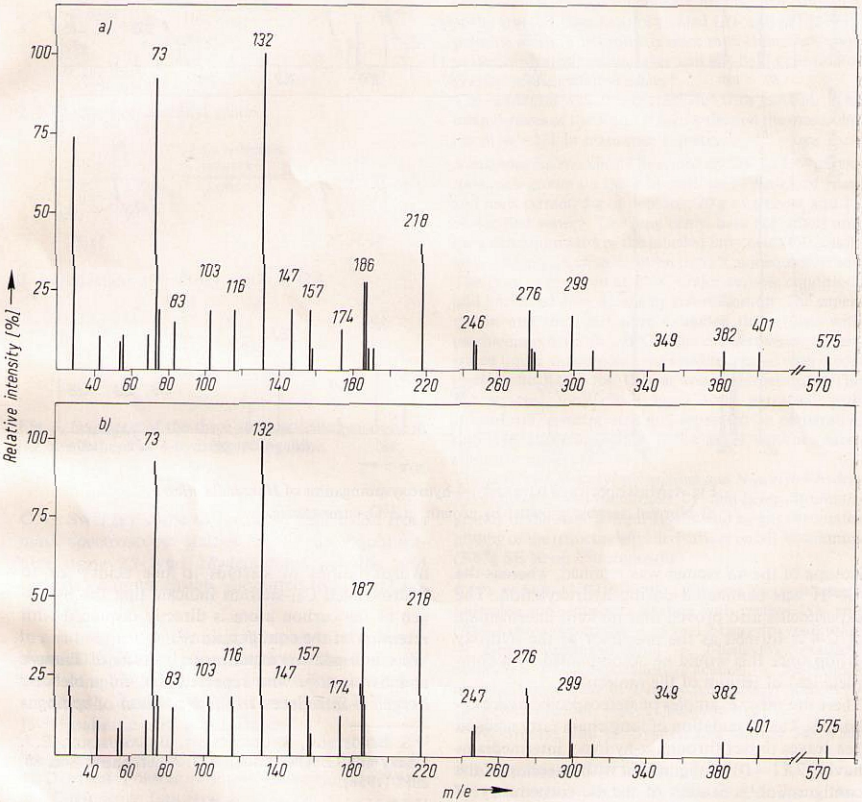



Fig. 3. Mass-spectra of tris-*O*-trimethylsilyl ether of *N*-acetyl-4-hydroxysphinganine of *Hansenula cif.*
a) Normal aerobic growth; b) growth in $^{18}O_2$-atmosphere.

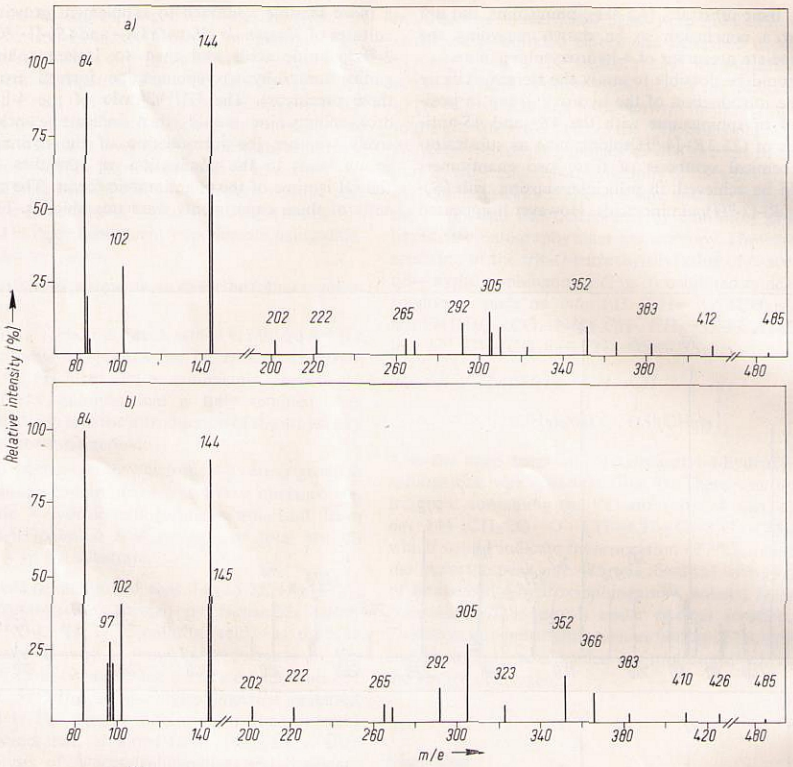



Fig. 4. Mass-spectra of tetraacetyl-4-hydroxysphinganine of *Hansenula ciferri*
a) Normal aerobic growth; b) growth in $^{18}O_2$-atmosphere.

isotope of the 4*S* isomer was retained, whereas the 4*R*-$^3$H was eliminated during hydroxylation. The experiments also proved that no keto intermediate at C-4 is formed as the precursor of the hydroxy group since this would be accompanied by a complete loss of tritium of the molecule.

There are other examples of stereospecific hydroxylations. The α-oxidation of long chain fatty acids in *pea leaves passes through α-hydroxy intermediates* having 2*R* (=*D*) configuration with retention of the configuration[15]. Studies of the stereochemistry of hydroxylations of steroids to bile acids[16] or to hydroxylated $C_{21}$-steroids indicate that the hydrogen at the carbon atom is directly displaced with retention of the configuration[17].

We confirmed the experimental results of Thorpe and Sweeley[11], who reported that no molecular oxygen is introduced in the 4-position of sphinga-

[15] L. J. Morris and C. Hitchcock, Europ. J. Biochem. [Berlin] **4**, 146 [1968].

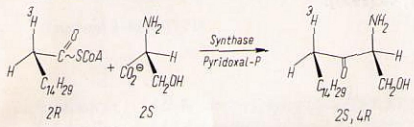
[16] S. Bergström, S. Lindstredt, B. Samuelson, E. J. Corey and G. A. Gregoriou, J. Amer. chem. Soc. **80**, 2337 [1958].

[17] M. Hayano, M. Gut, R. I. Dorfman, A. Schubert and R. Siebert, Biochim. biophysica Acta [Amsterdam] **32**, 269 [1959].

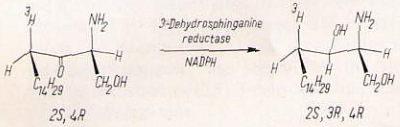
nine. The source of oxygen and the mechanism of the hydroxylation are still unknown.

Regardless of the answer to this question our experiments complete our knowledge regarding the stereochemistry of the sequential introduction of the centers of chirality at C-2, C-3 and C-4 leading to the 2*S*,3*S*,4*R* or the *ribo*-form of 4-hydroxysphinganine. Fig. 5 summarizes these results.

1. Condensation reaction




2. Reduction of carbonyl group




3. Introduction of hydroxy group at C-4

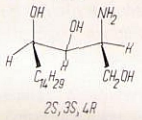



Fig. 5. Sequence of the three stereospecific reactions in the biosynthesis of 4-hydroxysphinganine.

C. C. SWEELEY came to the same conclusion from mass spectroscopic studies of 4-hydroxysphinganine using 2*S*- and 2*R*-deuterated palmitic acid (private communication, submitted to J. biol. Chemistry).

In a subsequent paper we will report on the stereochemical aspect of the introduction of the 4*t*-double bond of sphingenine in rat brain using the stereospecifically double labelled forms of [2-$^3$H]- and [3-$^3$H]palmitic acid as substrates[18].

We gratefully acknowledge the support of this work by the DEUTSCHE FORSCHUNGSGEMEINSCHAFT and the FONDS DER CHEMISCHEN INDUSTRIE.

[18] W. STOFFEL, G. ASSMANN and K. BISTER, this journal, in preparation.

## Experimental

*Chemical syntheses:* [3-$^3$H; 3-$^{14}$C]DL-*erythro*-sphinganine has been synthesized in this laboratory[19]. (*R*)- and (*S*)-[2-$^3$H]palmitic acids were synthesized according to the following reaction sequence[20]: ethyl hydrogen (*S*)- and (*R*)-2-acetoxy succinate were condensed with myristic acid by anodic coupling according to HORN *et al.*[21]. Alkaline hydrolysis yielded (*S*)-and (*R*)-2-hydroxy palmitic acids, which were separated by silicic acid chromatography from the hydrocarbon by-product of the anodic coupling reaction. The methyl esters ($[\alpha]_D^{20}$: $+6.2^0$ and $-6.2^0$) were transformed into the brosylates. Reduction with $LiAl^3H_4$ gave the (*R*)- and (*S*)-[1,2-$^3H_2$]-hexadecanols, with inversion[22]. Chromic acid oxidation of the labelled hexadecanol yielded (*R*)- and (*S*)-[2-$^3$H]-palmitic acids. The products were radiochemically pure as demonstrated by thin-layer and gas-liquid chromatography of their methyl esters*.

The reduction was also carried out with $LiAl^2H_4$. The methyl esters of the *S* and *R* forms showed the molecular ion at *m/e* 271 in mass spectrometry.

*Hansenula ciferri*, kindly provided by Dr. L. J. WICKERHAM, was grown on the YM medium (3 g each of yeast and malt extract, 5 g of peptone, 20 g of glucose and 1 *l* of distilled water). The long chain base (25 μmol) and the ammonium salts of the labelled fatty acids (50 μmol) were added as a clear solution in 50% aqueous ethanol. The yeast was grown at 25°C under aerobic conditions and harvested after 4 days by centrifugation. The supernatant and the yeast were extracted three times with petroleum ether (30–60°C). The extracts were concentrated under vacuum and the residue treated with 0.05N methanolic KOH for 12 h at room temperature. The *N*-acetylated long chain bases were extracted with chloroform, concentrated and separated by preparative thin-layer chromatography (silica gel H, solvent system chloroform/methanol 8:1).

The *purity of N-acetylsphinganine and N-acetyl-4-hydroxysphinganine* was monitored by thin-layer chromatography in the same solvent system and by gas chromatography of the trimethylsilyl derivatives on SE 30 columns (3.8% SE 30 on Chromosorb).

* (*S*)- and (*R*)-[2-$^3$H]palmitic acids were also synthesized following the sequence: starting with methyl (*S*)- and (*R*)-2-hydroxypalmitate → methyl 2-benzyloxypalmitate → 2-benzyloxyhexadecanol → 2-benzyloxyhexadecyl acetate → 2-hydroxyhexadecyl acetate → 2-*p*-bromophenylsulfonyl ester of 2-hydroxyhexadecyl acetate → [2-$^3$H]hexadecanol → [2-$^3$H]palmitic acid[20].

[19] W. STOFFEL and G. STICHT, this journal **348**, 1561 [1967].

[20] W. STOFFEL and K. BISTER, unpubl.

[21] D. H. S. HORN and Y. Y. PRETORIUS, J. chem. Soc [London] **1954**, 1460.

[22] G. J. SCHROEPFER and K. BLOCH, J. biol. Chemistry **240**, 54 [1965].

For the $^{18}O_2$ *experiment* a 250 m*l* culture was grown in an incubation flask (400 m*l*) as described by HAYAISHI[23]. 98% $^{18}O_2$ was purchased from Miles-Yeda Laboratories.

The separated *N*-acetylated bases were also characterized by *degradation:* hydrolysis of the *N*-acetyl group being carried out according to GAVER and SWEELEY[24]. The free bases were oxidized with periodate[25] and the resulting aldehydes analysed by gas-liquid chromatography (15% ethylenglycol succinate polyester on Chromosorb).

*Thin-layer chromatograms* were scanned in a radio chromatogram scanner Packard, model 7201, or a Berthold scanner model LB 2722. A Tricarb liquid scintillation counter, Packard model 3214, was used. Combined gas-liquid chromatography-mass spectroscopy was carried out with the Varian GLC-MS system, model CH 5.

[23] O. HAYAISHI, Oxygenases, p. 7, Acad. Press, NewYork 1962.

[24] R. C. GAVER and C. C. SWEELEY, J. Amer. Oil Chemists' Soc. **42**, 294 [1965].

[25] C. C. SWEELEY and E. A. MOSCATELLI, J. Lipid Res. **1**, 40 [1959].