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Metabolism of Sphingosine Bases, III[1, 2]

Chemical Syntheses of ^{14}C and ^{3}H labeled *erythro*- and *threo*-Dihydrosphingosines and Sphingosines

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Summary: The chemical syntheses of [3-^{14}C]- and [5-^{3}H]*erythro*-DL-dihydrosphingosine, [5-^{3}H]*threo*-DL-dihydrosphingosine, [1-^{3}H;3-^{14}C]*erythro*-DL-dihydrosphingosine and [7-^{3}H]*erythro*-DL-sphingosine are described.

Zusammenfassung: Es wird die chemische Synthese von [3-^{14}C]- und [5-^{3}H]*erythro*-DL-Dihydrosphingosin, [5-^{3}H]*threo*-DL-Dihydrosphingosin, [1-^{3}H;3-^{14}C]*erythro*-DL-Dihydrosphingosin und [7-^{3}H]-*erythro*-DL-Sphingosin beschrieben.

The results of previous studies on the metabolism of dihydrosphingosine and sphingosine bases in the rat from this laboratory[1–3] were obtained after we succeeded in the preparation of a number of specifically ^{3}H and ^{14}C labeled long chain sphingosine bases. We were able to demonstrate that different enantiomeric forms of dihydrosphingosine and sphingosine labeled in their hydrocarbon chain yielded labeled *palmitic acid* as the one fragment in the degradation, whereas [1-^{3}H] and [1-^{14}C] labeled dihydrosphingosines liberated ^{3}H and ^{14}C labeled *ethanolamine* as two carbon unit. These labeled sphingosine bases proved also to be valuable substrates in our *in vitro* studies on the mechanism of the degradation and of the biosynthetic steps[4].

In this paper we want to describe some of the syntheses of ^{3}H and ^{14}C labeled long chain bases relevant to our results previously described. The following bases were synthesized: [3-^{14}C]*erythro*- and [5-^{3}H]*erythro*-DL-dihydrosphingosine, [5-^{3}H]-*threo*-DL-dihydrosphingosine, [7-^{3}H]*erythro*-DL-sphingosine and [1-^{3}H;3-^{14}C]*erythro*-DL-dihydrosphingosine.

Labeled dihydrosphingosines

^{3}H labeled *N*-acyl-dihydrosphingosine obtained from cerebrosides has been prepared by the WILZBACH technique[5] by BRADY et al.[6]. However the radioactivity is distributed over the whole molecule. [4,5-^{3}H]dihydrosphingosine has been obtained by catalytic reduction of sphingosine in a tritium atmosphere[7, 8]. The introduction of the marker in specific positions of the molecule on the other hand can only be achieved by total synthesis.

Only two of the published syntheses appeared to us to be applicable for the synthesis of labeled dihydrosphingosine, namely that of GROB and

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Abbreviations used: GLC = gas-liquid chromatography; TLC = thin layer chromatography.

[1] I. Commun.: W. STOFFEL and G. STICHT, this journal **348**, 941 [1967].

[2] II. Commun.: W. STOFFEL and G. STICHT, this journal **348**, 1345 [1967].

[3] IV. Commun.: W. STOFFEL, D. LEKIM and G. STICHT, this journal **348**, 1570 [1967].

[4] W. STOFFEL, G. STICHT and D. LEKIM, this journal, in preparation.

[5] K. E. WILZBACH, J. Amer. chem. Soc. **79**, 1013 [1957].

[6] R. O. BRADY, R. M. BRADLEY, O. M. YOUNG and H. KALLER, J. biol. Chemistry **240**, 3693 [1965].

[7] S. GATT, J. biol. Chemistry **238**, 3131 [1963].

[8] S. GATT, J. biol. Chemistry **241**, 1724, 2731 [1966].

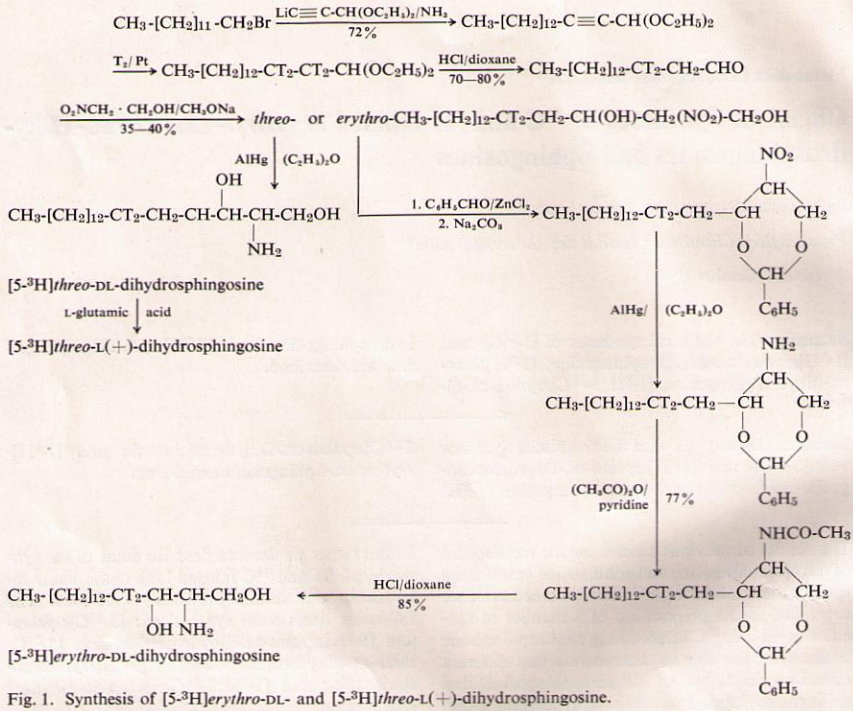

Fig. 1. Synthesis of [5-^{3}H]*erythro*-DL- and [5-^{3}H]*threo*-L(+)-dihydrosphingosine.

JENNY[9,10] and of SHAPIRO et al.[11,12]. ^{3}H labeled palmitaldehyde was required for the synthesis of *threo*- and *erythro*-dihydrosphingosine according to GROB, ^{3}H or ^{14}C labeled palmitoyl chloride for the elegant synthesis of *erythro*-dihydrosphingosine according to SHAPIRO et al.

Fig. 1, 4 and 5 summarize the steps leading to these labeled precursors and also the subsequent reactions to the desired products. ^{3}H labeled palmitaldehyde of high specific activity was prepared by the catalytic reduction of 2-hexadecyne-1-al diethyl acetal in an atmosphere of ^{3}H to [2,3-$^3H_4$]palmitaldehyd diethylacetal. Acid hydrolysis with simultaneous complete exchange of the α-protons yielded [3-^{3}H]-palmitaldehyde. Condensation of this aldehyde with nitroethanol yielded 13—17% of *threo*-2-nitrooctadecan-1,3-diol in form of crystals and in the mother liquid a mixture of the *threo*- and of the *erythro*-nitrodiol. The *threo*-form was reduced to the respective aminodiol with aluminium amalgam in ether, whereas the *erythro*-nitrodiol was first transformed into the *erythro*-nitrodiol-benzylidene derivative. Quantitative reduction with Al/Hg to the amino compound and acid hydrolysis yielded *erythro*-dihydrosphingosine. The two diastereomeric dihydrosphingosines were chromatographi-

[9] C. A. GROB, E. F. JENNY and H. UTZINGER, Helv. chim. Acta **34**, 2249 [1951].

[10] C. A. GROB and E. F. JENNY, Helv. chim. Acta **35**, 2106 [1952].

[11] D. SHAPIRO, K. H. SEGAL and H. M. FLOWERS, J. Amer. chem. Soc. **80**, 2170 [1958].

[12] D. SHAPIRO and T. SHERADSKY, J. org. Chemistry **28**, 2157 [1963].

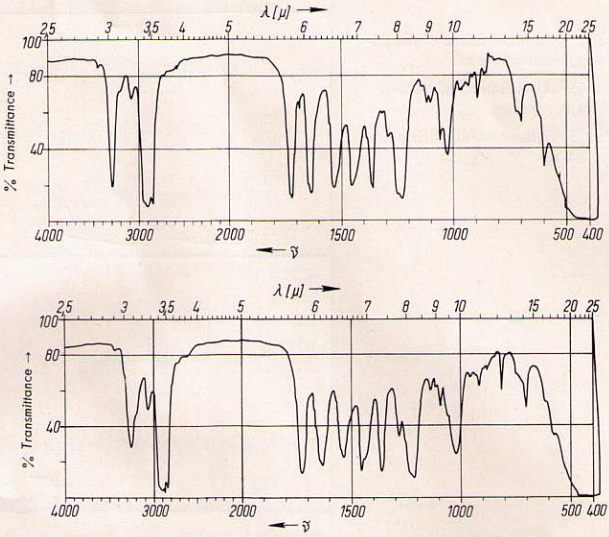

Fig. 2. i.r.-Spectra of the triacetyl-derivatives of

a) [5-^{3}H]*erythro*-DL-dihydrosphingosine;

b) [5-^{3}H]*threo*-DL-dihydrosphingosine.

cally pure (TLC: system: chloroform/methanol/2N NH_3 40:10:1)[13] and their triacetyl-derivatives were identical with those reported in the literature for the inactive compounds. The i.r.- and n.m.r.-spectra are given in fig. 2 and 3. The i.r.-spectra of the triacetyl derivatives of *threo*- and *erythro*-dihydrosphingosine differ only by a medium absorption band at 815 cm^{-1}; also their n.m.r.-spectra are almost identical.

The [^{3}H] label of the two dihydrosphingosines at C-5 was not exchangeable. Their specific activity (2,5 μC/μmole) remained constant under the chromatographic procedures used (TLC, SiO_2-chromatography, GLC on SE-30 stationary phases) and after acid and alkaline treatment. The racemate of *threo*-dihydrosphingosine was separated into the optical antipodes via the L-glutamate, thus yielding *threo*-L(+)-dihydrosphingosine.

3[-^{14}C] and [1-^{3}H;3-^{14}C]erythro-DL-dihydrosphingosine

A very suitable way to synthesize ^{14}C labeled dihydrosphingosine was to follow the procedure of SHAPIRO et al.[11] but starting with [1-^{14}C]palmitic acid. The latter was prepared by a GRIGNARD reaction with pentadecyl bromide and $^{14}CO_2$ and then reacted with thionylchloride to [1-^{14}C]palmitoyl chloride. The C-acylation of sodium acetoacetate yielded ethyl [3-^{14}C]2-acetyl-3-oxooctadecanoate (90% yield), which was used for the JAPP-KLINGEMANN-reaction. The 2-phenylhydrazone of ethyl [3-^{14}C]2,3-dioxooctadecanoate was obtained in a 90% yield. Reductive acetylation, $NaBH_4$-reduction of the 3-oxo group and acid hydrolysis of the *N*-acetyl group yielded the crystalline ethyl [3-^{14}C]-2-amino-3-hydroxyoctadecanoate hydrochloride. This then was either reduced with $LiAlH_4$ to [3-^{14}C]dihydrosphingosine or with $LiAlT_4$ to [1-^{3}H;3-^{14}C]dihydrosphingosine. The pure *erythro*-form was isolated from both via their *N*-dichloroacetyl derivative and the free base recovered after mild alkaline hydrolysis of the dichloroacetyl-group.

[7-^{3}H]erythro-DL-sphingosine

erythro-Sphingosine labeled in the hydrocarbon chain, was required for our biochemical studies. Also for this radioactive synthesis we applied the

[13] K. SAMBASIVARAO and R. H. MCCLUER, J. Lipid Res. **4**, 106 [1963].

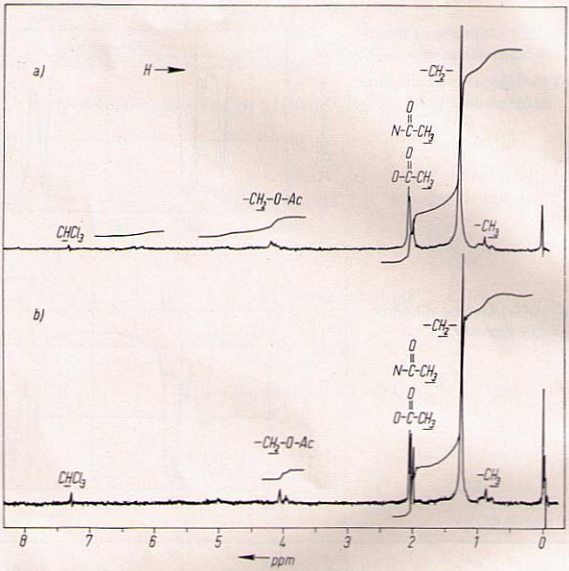

Fig. 3. n.m.r.-Spectra of the triacetyl-derivatives of

a) [5-^{3}H]*erythro*-DL-dihydrosphingosine;

b) [5-^{3}H]*threo*-DL-dihydrosphingosine.

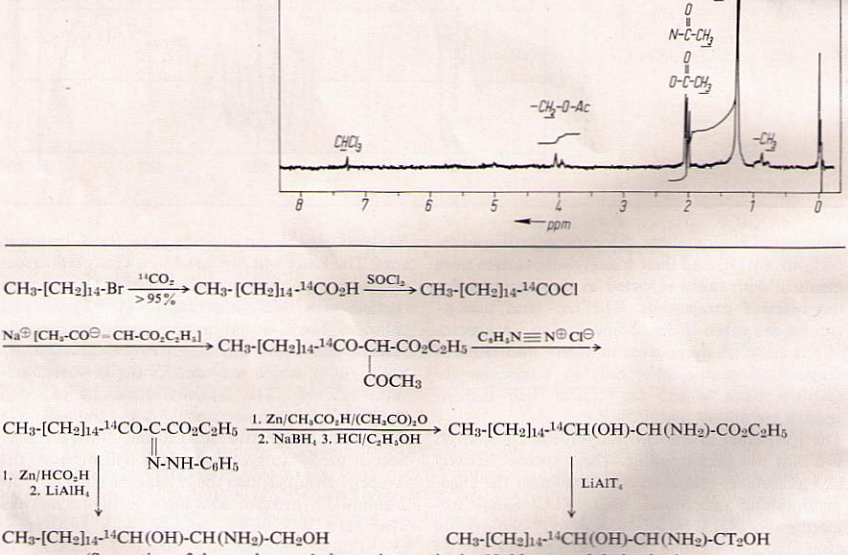

Fig. 4. Synthesis of [3-^{14}C] and [1-^{3}H;3-^{14}C]*erythro*-DL-dihydrosphingosine.

synthetic sequence of SHAPIRO et al.[14] successfully, which under strictly controlled conditions proved in our hands to be the most valuable and simple method. As summarized in fig. 5 this procedure consists of the same reaction sequence as outlined for dihydrosphingosine except that 2*t*-hexadecenoyl chloride was used for the C-acylation of ethyl acetoacetate. We synthesized [5-^{3}H]2*t*-hexadecenoic acid in the following way: condensation of 1-

[14] D. SHAPIRO, K. H. SEGAL and H. M. FLOWERS, J. Amer. chem. Soc. **80**, 1194 [1958].

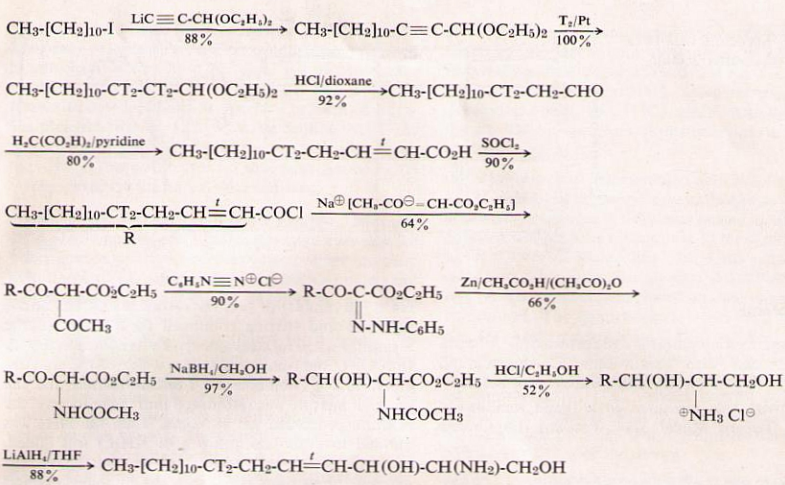

Fig. 5. Synthesis of [7-^{3}H]*erythro*-DL-sphingosine.

Iodoundecane with propiolaldehyde diethyl acetal in liquid NH_3, catalytical reduction of this acetal in an atmosphere of ^3H to myristaldehyde diethyl acetal, acid hydrolysis of the acetal, which is accompanied by a simultaneous exchange of the two α-protons, thus yielding [3-^{3}H] myristaldehyde. The aldehyde was condensed with malonic acid. After decarboxylation [5-^{3}H]2*t*-hexadecenoic acid was obtained in 80% yield. Its chloride was prepared by the reaction with $SOCl_2$. For the subsequent C-acylation it is very important to use re-distilled acid chloride. Otherwise the *O*-acylation product is the main reaction product. This product (a dihydro-α-pyrone derivative) and its reactions have been extensively studied[15]. All steps leading to [7-^{3}H]-*erythro*-sphingosine were carried out with high yields. Fig. 6 gives the i.r.-spectrum of [7-^{3}H]triacetyl-*erythro*-DL-sphingosine. Furthermore the structure was confirmed by oxidative ozonolysis of the triacetyl derivative. Radioactive myristic acid, identified as its methylester, was the only labeled degradation product.

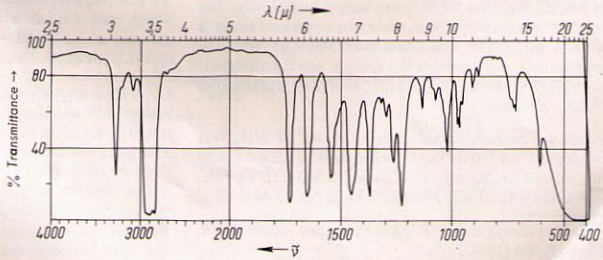

Fig. 6. i.r.-Spectrum of [7-^{3}H]triacetyl-*erythro*-DL-sphingosine.

[15] G. Sticht, doctoral thesis, University of Cologne 1966.

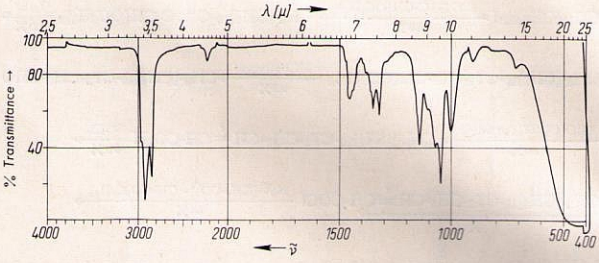

Fig. 7. i.r.-Spectrum of 2-hexadecyne-1-al diethyl acetal.

Experimental

Boiling and melting points are uncorrected; i.r.-spectra were recorded with Perkin-Elmer i.r.-spectrograph Model 125, n.m.r.-spectra with a Varian A-60 model.

Radioactivity was measured in a liquid scintillation counter, Tricarb Model 3214, Packard, La Grange (USA).

[5-^3H]threo- and erythro-dihydrosphingosine

We synthesized *nitroethanol* according to the procedure of GORSKI et al.[16] modified by CONTROULIS et al.[17] from nitromethane and formaldehyde in the presence of K_2CO_3 and obtained the product in a yield of 30% of theory, b.p.$_{0.075}$ 53°C; n_D^{21}: 1.4406.

Propynal diethyl acetal: Acrolein was brominated in ether according to GRARD to *2,3-dibromopropanal*, the diethyl acetal of which was formed with a mixture of triethyl orthoformiate and ethanol, b.p.$_{13}$ 108—112°C, n_D^{20}: 1.4969; yield: 80% of theory.

Dehalogenation was performed with $NaNH_2$ in liquid ammonia in the following manner: 34.5 g (1.5 moles) of sodium was dissolved over a period of 2 hours in 1.5 *l* of liquid NH_3 after the addition of 500 mg of $Fe(NO_3)_3$. 130 g (0.45 moles) of *2,3-dibromopropanal diethyl acetal* was added dropwise at the boiling point of NH_3. After the evaporation of NH_3 overnight 50 g of NH_4Cl and then 1 *l* of water were added slowly. The product was extracted with ether, the combined extracts washed with water and dried over Na_2SO_4. After the evaporation of the solvent the residue was fractionated: b.p.$_{760}$ 138—140°C; n_D^{20}: 1.4141; yield 28 g (0.218 mole) 48.5% of theory.

2-hexadecyne-1-al diethyl acetal: 150 m*l* of NH_3 was condensed in a 500 m*l* three necked flask under exclusion of air-moisture, 500 mg $Fe(NO_3)_3$ and 0.700 g (100 mmole) of lithium was added. After an 1 hour period of stirring 6.4 g (50 mmole) of *propyne diethyl acetal* was added dropwise and stirring continued for 2 hours. In the meantime a 250 m*l* steel autoclave was cooled in dry ice to −70°C and rinsed three times with −70°C cold dry ether. 13.1 g (50mmole) of *1-bromotridecane* and the reaction mixture was introduced into the autoclave, the reaction proceeded for 48 hours. Then the NH_3 was allowed to evaporate and 6 g of NH_4Cl was added, water and ether were added. The aqueous phase was extracted three times with ether, the combined extracts washed with water, dried over Na_2SO_4, concentrated and the residue fractionated by high vacuum distillation. The product was distilled at b.p.$_{0.01}$ 125°C, n_D^{20}: 1.4490; yield 11.14 g (36 mmole), 72% of theory. The i.r.-spectrum is given in fig. 7. The product proved to be pure in GLC.

[3-^3H]palmitaldehyde: 2 g of *2-hexadecyne-1-al diethyl acetal* was dissolved in ethyl acetate and hydrogenated over PtO_2 in a *tritium* atmosphere. After filtration the solvent was evaporated, the residue dissolved in a mixture of 7 m*l* of water, 1.5 m*l* 2N HCl and a sufficient amount of dioxane to obtain one phase at the reflux temperature. The mixture was refluxed under N_2 for 8 hours. The dioxane and water were evaporated largely under reduced pressure and the aldehyde extracted with petroleum ether. The petroleum ether extracts were washed with 10 m*l* of a 20% Na_2CO_3 solution and water, dried over Na_2SO_4 and concentrated. The residue, which was diluted with inactive palmitaldehyde was distilled at b.p.$_{0.1}$ 125—130°C; yield: 78% of theory; specif. activity 31.2 μC/μmole.

[5-^3H]2-nitrooctadecane-1,3-diol: 1.90 g (21 mmole) of *nitroethanol* was dissolved in a solution of 11 m*l* methanol, 0.35 m*l* of water and 50 mg (2.4 mmole) of sodium. 3.74 g (15.6 mmole) of *[3-^3H]palmitaldehyde* dissolved in 23 m*l* of methanol was added and the clear solution left at 18°C in the dark for 4 days. The yellow reaction mixture was carefully acidified with 2 m*l* of 2N HCl and concentrated to dryness at 40°C under reduced pressure. The waxy residue was distributed between ether and water. The washed ethereal solution was dried over Na_2SO_4 and concentrated. 4.6 g of the solid residue was

[16] I. M. GORSKI and S. P. MAKAROV, Ber. dtsch. chem. Ges. **67**, 996 [1934].

[17] I. CONTROULIS, H. C. REBSTOCK and H. M. CROOKS jr., J. Amer. chem. Soc. **71**, 2463 [1949].

diluted with 3.73 g of the mixture of isomers, obtained from cold palmitaldehyde. This product was dissolved in petroleum ether (30—60°C) and left at room temperature. After 8 hours at 18°C 1.8 g (5.44 mmoles) of *threo*-nitrooctadecadiol was collected in form of colorless platelets, m.p. 81—82.5°C. After cooling to 0°C the mother liquid yielded 5.9 g of the mixture of isomers, m.p. 38°C. These were used for the isomerisation to the *erythro*-form via the benzylidene derivate:

erythro-5-nitro-4-[2-^3H]pentadecyl-2-phenyl-1,3-dioxane: 4.8 g (14.5 mmole) of *[5-^3H]2-nitrooctadecane-1,3-diol* was vigorously stirred under exclusion of air moisture with 10 ml of *benzaldehyde* and 2.5 g freshly molten and pulverized $ZnCl_2$ under N_2 for 24 hours at room temperature. 100 ml of dry ether and 5.8 g of K_2CO_3 were added and the agitation continued for 5 hours. The ether was evaporated. 200 ml of 2N Na_2CO_3 was added and the excess of benzaldehyde removed by steam distillation. The residue was extracted with ether, the combined extracts washed twice with 2N Na_2CO_3 and water and dried over Na_2SO_4. The solvent was evaporated and the residue chromatographed over a 100 g column of Al_2O_3. The product was eluted with petroleum ether-benzene (1:1); m.p. 49°C; yield 2.12 g (5.1 mmoles), 35% of theory.

907.5 mg (2.16 mmoles) of *erythro-5-nitro-4-[2-^3H]pentadecyl-2-phenyl-1,3-dioxane* was dissolved in 25 ml of ether saturated with water, 1.6 ml of water and aluminium amalgam (prepared from 1 g of Al-powder) were added and the reaction mixture was stirred at 0°C for 2 hours and at room temperature for an additional 20 hours. The product was thoroughly extracted with ether, the extracts were dried over Na_2SO_4 and concentrated to dryness. The waxy residue melted at 47—49°C; yield: 950 mg of crude product.

950 mg of the crude *benzylidene derivative of erythro-2-aminooctadecane-1,3-diol* was acetylated in 12 ml of dry pyridine with 8 ml of acetic anhydride over a period of 24 hours at room temperature. The mixture was concentrated below 40°C under vacuum and the residue dissolved in ethyl acetate. The solution was washed with 2N HCl, 2N Na_2CO_3 and water, dried over Na_2SO_4 and concentrated. The residue (970 mg, m.p. 120°C) was recrystallized from acetone. Yield: 744 mg (1.73 mmoles) 80% of theory; m.p. 127°C. Three recrystallizations elevated the m.p. to 131°C; specif. activity 2.5 mC/mmole. The i.r.-spectrum of this benzylidene derivative is given in fig. 8.

[5-^3H]erythro-DL-2-aminooctadecane-1,3-diol: 300 mg (0.695 mmole) of the *benzylidene derivative of erythro-2-acetamido-octadecane-1,3-diol* was heated at 100°C with constant stirring with a mixture of 12 ml of dioxane and 12 ml of 4N HCl. The acidic solution was extracted with ether and then made alkaline by the addition of solid K_2CO_3. The product precipitated and was extracted with chloroform. The chloroform solution was dried over K_2CO_3 and concentrated. The residue (217 mg, 0.72 mmole) was contaminated slightly. Chromatographically pure *erythro-dihydrosphingosine* was obtained by preparative TLC, solvent system: chloroform/methanol/2N NH_4OH 40:10:1. The product was eluted from the silica gel with chloroform/methanol 1:1, m.p. 83°C. Yield: 72% of the theory.

[5-^3H]threo-2-aminooctadecane-1,3-diol was obtained by reduction of the [5-^{3}H]*threo*-2-nitrooctadecane-1,3-diol with aluminium amalgam under the same condition as described for the *erythro*-derivative with a 67% yield, m.p. 93—95°C.

The *triacetyl derivatives of the threo- and erythro-dihydrosphingosine* were prepared with acetic anhydride in pyridine, isolated in the usual manner and recrystallized from hexane. [5-^{3}H]*threo*-triacetyldihydrosphingosine had a m.p. 66.5°C, of [5-^{3}H]*erythro*-triacetyldihydrosphingosine 91—92°C.

[3-^{14}C] and [1-^3H;3-^{14}C] erythro-DL-dihydrosphingosine [1-^{14}C]palmitic acid was prepared by reacting 20 mmoles (5.82 g) of *1-bromopentadecane* in 15 ml of dry ether with 23 mmoles of magnesium powder in 5 ml of ether and 9.2 mmoles of CO_2 liberated from 9.2 mmoles (1.815 g) of $Ba^{14}CO_3$ (10 mC) under standard conditions. Yield 8.3 mmoles (2.13 g) of *[1-^{14}C]palmitic acid*

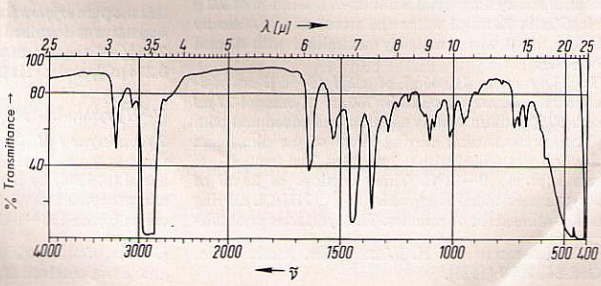

Fig. 8. i.r.-Spectrum of *erythro*-5-acetamido-4-[2-^{3}H]-pentadecyl-2-phenyl-1,3-dioxane.

(90% of theory). After the dilution with inactive palmitic acid to 26.8 mmoles (6.88 g) of palmitic acid 20 ml $SOCl_2$ were added and the mixture refluxed for 2 hours. Excess of $SOCl_2$ was evaporated under vacuum and the acyl chloride distilled at b.p.$_{0.1}$ 141°C. 3 g of inactive palmitoyl chloride was distilled from the same distillation flask; yield: 10.16 g (36 mmoles) of *[1-^{14}C]palmitoyl chloride*.

ethyl [3-^{14}C]2-acetyl-3-oxooctadecanoate: The following condensation was carried out in analogy to the procedure of HELFERICH et al.[18]. 39.1 mmoles (900 mg) of sodium was pulverized under xylene, washed with dry ether and then suspended in 150 ml of dry ether. 44.5 mmoles (5.85 g) of *ethyl acetoacetate* was added to the mixture and stirred overnight. The suspension of sodium ethyl acetoacetate was cooled to 5°C. Then 36.4 mmoles (10.0 g) of *[1-^{14}C]palmitoyl chloride* was added dropwise. The reaction mixture became clear and then again turbid due to the insoluble sodium chloride. After refluxing for 1 hour it was poured on ice water and the mixture acidified with 10 ml of 20% H_2SO_4. The reaction product was extracted with ether, the ether extracts washed with water, dried over Na_2SO_4 and concentrated. The residue was recrystallized from 95% ethanol; yield: 28.4 mmoles (10.46 g) of *ethyl [3-^{14}C]2-acetyl-3-oxooctadecanoate* (78% of theory). The mother liquids were concentrated and dissolved in 20 ml ethyl acetate and refluxed with 160 mg (1 mmole) ethyl acetoacetate and 2 g of dry K_2CO_3 for 4 hours. K_2CO_3 was filtered off, washed with ethyl acetate and the combined solutions washed with cold 2N HCl and water, dried over Na_2SO_4, concentrated and chromatographed on 20 g of silicid acid. Ether/petroleum ether 1:7 eluted 2.25 g of addition product. The total yield therefore amounted to 12.71 g (34.5 mmoles), 95% of theory.
In addition 394 mg of [1-^{14}C]palmitic acid were recovered.

ethyl [3-^{14}C]2,3-dioxooctadecanoate 2-phenylhydrazone: 5.6 g of freshly destilled *aniline* was dissolved in 66 ml of water and 20 ml of conc. HCl. A solution of 4.3 g $NaNO_2$ in 8 ml of water was added dropwise to the cooled solution (0—5°C). Then the solution was partially neutralized by the careful addition of a solution of 7.2 g Na_2CO_3 in 72 ml of water and stored at 0°C. Shortly before use it was completely neutralized with diluted Na_2CO_3.
To 28.4 mmoles (10.46 g) of *ethyl [3-^{14}C]2-acetyl-3-oxooctadecanoate* dissolved in 700 ml of ethanol 27 ml of a 50% sodium acetate solution was added and with vigorous mechanical stirring 88 ml of the diazonium solution was added within 3 minutes. The temperature was kept at 10—12°C. Three portions of 25 ml of acetone were added followed by 15 g of NH_4Cl. Stirring was continued for 45 minutes. The hydrazone crystallized at 15°C overnight. Yield: 10.93 g (25.1 mmoles) 89% of theory; m.p. 47—47.5°C.

ethyl [3-^{14}C]2-amino-3-oxooctadecanoate hydrochloride: 11.5 mmoles (4.95 g) of the *phenylhydrazone* was added in small portions to a suspension of 6 g of Zn-powder in 60 ml of 98% formic acid, the temperature adjusted to 45—50°C. The reduction was continued for an additional 20 minutes, the mixture filtered, the Zn-powder washed with formic acid and the filtrate poured into 50 ml of 2N HCl. The crystalline product was filtered, washed with water and dried. Recrystallisation from 10 parts of tetrahydrofuran yielded 9.7 mmoles (3.66 g) of the hydrochloride, 84% of theory; m.p. 122—124°C.

*[3-^{14}C]erythro- and threo-*DL*-dihydrosphingosine:* A suspension of 3.4 mmoles (1.286 g) of *ethyl 2-amino-3-oxooctadecanoate hydrochloride* was added dropwise to a suspension of 500 mg of $LiAlH_4$ in 20 ml of absolute tetrahydrofuran. The reaction was continued at 40°C for 1 hour. The mixture was cooled to 0°C and excess $LiAlH_4$ destroyed by the addition of ethylacetate. 25 ml of 10% sodium-potassium tartrate, 5 ml of 2N NaOH and 5 ml of a saturated NaCl solution were added and the product extracted with ether. The combined ether extracts were dried over Na_2SO_4 and concentrated under vacuum. Yield 0.995 g (3.16 mmoles). 93% of theory.

*[3-^{14}C]erythro-*DL*-dihydrosphingosine:* The diastereomeric mixture was transformed into the *N*-dichloroacetyl derivative and the crystalline *erythro* form (m.p. 138°C), hydrolyzed to free *[3-^{14}C]erythro-*DL*-dihydrosphingosine*, according to SHAPIRO et al. Yield 250 mg (0.83 mmoles), 26% of theory; m.p. 83—84°C, with a specif. activity of 0.214 μC/μmole. It proved to be chemically and radiochemically pure in TLC and radio-GLC.

*[1-^{3}H;3-^{14}C]erythro-*DL*-dihydrosphingosine:* 1.16 mmoles (0.400 g) of *ethyl [3-^{14}C]2-amino-3-hydroxyoctadecanoate hydrochloride* was reduced under the conditions described before with 10 mg $LiAlT_4$ (20 mC). After 15 minutes additional 100 mg $LiAlH_4$ were added. The diastereomeric reaction product was isolated and from this the pure *erythro* form isolated via the dichloroacetyl derivative as described before. Yield: 75 mg (0.25 mmole) 21.5% of theory; m.p. 84.5°C. Specif. activity: ^{14}C: 0.214 μC/μmole; ^{3}H: 2.57 μC/μmole.

*[7-^{3}H]erythro-*DL*-sphingosine*

2-tetradecyne-1-al diethyl acetal was prepared in the same way as described for 2-hexadecyne-1-al diethyl acetal (p. 1566) by the condensation of *1-iodoundecane* and propynal diethyl acetal in liquid NH_3 in an autoclave; b.p.$_{0.1}$ 138—145°C; yield: 92% of theory.

[3-^{3}H]myristaldehyde with a specif. activity of 0.87 μC/μmole was obtained after catalytic hydrogenation of the

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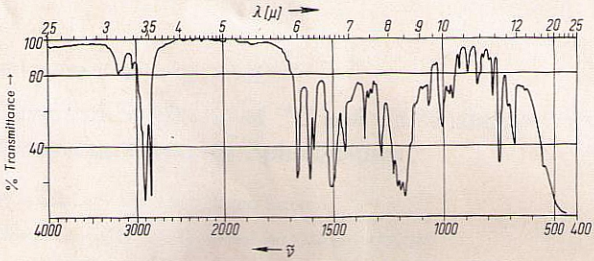

Fig. 9. i.r.-Spectrum of the phenylhydrazone of ethyl [7-^{3}H]2-acetyl-3-oxo-4*t*-octadecenoate.

diethyl acetal in a tritium atmosphere and acid hydrolysis as described for [3-^{3}H]palmitaldehyde. Yield: 92% of theory.

[5-^{3}H]2t-hexadecenoic acid was synthesized by the condensation of *[3-^{3}H]myristaldehyde* and *malonic acid* in a 79% yield according to the procedure of SHAPIRO et al.[15], m.p. 49°C, colorless platelets, specif. activity 0.87 μC/μmole.

[5-^{3}H]2t-hexadecenoyl chloride: To 10.25 g (40.25 mmoles) of *[5-^{3}H]2t-hexadecenoic* acid dissolved in 16 m*l* petroleum ether (60—80°C) 6 m*l* freshly distilled $SOCl_2$ was added over five minutes. The mixture was refluxed for 4 hours. The solvent and excess $SOCl_2$ were evaporated under vacuum and last traces of $SOCl_2$ removed by three flash evaporation of the petroleum ether solution of the acyl chloride. High vacuum distillation yielded the [5-^{3}H]2*t*-hexadecenoyl chloride, b.p.$_{0.005}$ 135°C, n_D^{25}: 1.4644, yield 90% of theory. The product was redistilled three times. The subsequent reactions were carried out as described by SHAPIRO et al.[14].

ethyl [7-^{3}H]4t-2-acetyl-3-oxooctadecenoate (m.p. 35°C) was obtained in 64% yield, the *phenylhydrazone of ethyl [7-^{3}H]2,3-dioxo-4t-octadecenoate* in a 90% yield. The phenylhydrazone was obtained in excellent yield (90% of theory) and in a crystalline form. It had a m.p. of 54—56°C. The high purity of this phenylhadrazone is a prerequisite for the high yield in the step of the following reductive acetylation. The i.r.-spectrum of this unsaturated phenylhydrazone is shown in fig. 9.

The reductive acetylation yielded *ethyl [7-^{3}H]2-acetamido-3-oxo-4t-octadecenoate* in 66% of theory, after recrystallization from methanol m.p. 65°C. N: found 3.62%; calc. 3.68%.

ethyl [7-^{3}H]2-acetamido-3-hydroxy-4t-octadecenoate was obtained from $NaBH_4$ reduction of the respective 3-oxo-ethylester in 97% of theory, m.p. 56°C.

Ethyl [7-^{3}H]2-amino-3-hydroxy-4t-octadecenoate hydrochloride: 3.2 g (8.35 mmoles) of *ethyl [7-^{3}H]2-acetamido-3-hydroxy-4t-octadecenoate* was refluxed for 90 minutes in 30 m*l* of 15% ethanolic HCl. The solvent was evaporated under reduced pressure and the residue (3.7 g) suspended in dry ether, filtered at 0°C with suction and washed with cold dry ether. The colorless hydrochloride (2.32 g) was recrystallized from ethyl-acetate; m.p. 108—110°C. Yield: 1.64 g (4.34 mmoles) 52% of theory. N: found 3.72%; calc. 3.71%.

*[7-^{3}H]erythro-*DL*-sphingosine:* 2 g of $LiAlH_4$ was refluxed in 100 m*l* of dry ether for 10 minutes. The suspension was cooled to 10°C and 1.34 g (3.55 mmoles) of *ethyl [7-^{3}H]2-amino-3-hydroxy-4t-octadecenoate* hydrochloride dissolved in 70 m*l* of dry tetrahydrofuran added to it dropwise over 30 minutes. The reaction proceeded at room temperature for 30 minutes and boiled under reflux for a further 90 minutes. Finally excess $LiAlH_4$ was decomposed at 0°C with water and then with 10% Na_2CO_3. The product was extracted with ether, the ether extracts washed with water, dried over Na_2SO_4 and concentrated to dryness; yield: 0.937 g (3.13 mmoles) 88% of theory, m.p. 67°C.

The *triacetyl derivative* prepared in the presence of acetic anhydride and pyridine in 80% yield, crystallized from hexane, m.p. 89,5°C.

The sphingosine base proved to be pure by TLC. Oxidative ozonolysis of the triacetyl derivative yielded exclusively [^{3}H]myristic acid, which was identified by radio-GLC.

Ozonolysis was preformed in acetic acid/methylacetate (1:1) at —15°C followed by the oxidation with perhydrol at 40°C for 3 days. Excess H_2O_2 was decomposed with PtO_2 and the solvent evaporated. The residue was esterified with diazomethane.

When the two [5-^{3}H]dihydrosphingosines were oxidized with $KMnO_4$—KIO_4 according to SWEELEY et al.[19] [^{3}H]palmitic acid was the sole radioactive degradation product.

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