Tritioboration and synthesis of tritium-labeled polyunsaturated fatty acids

DEMETRIOS S. SGOUTAS, HOLLY SANDERS, and ESTHER M. YANG

The Burnsides Research Laboratory, University of Illinois, Urbana, Illinois 61801

ABSTRACT Methyl esters of polyunsaturated fatty acids labeled with tritium were prepared by partial stereoselective reduction of the corresponding acetylenic esters with tritiated disiamylborane, followed by protonolysis with tritiated acetic acid. The labeling was strictly specific, and the tritium atoms were located only at the carbon atoms forming the unsaturated bond(s).

Synthesis of some tritiated fatty acid methyl esters with methylene-interrupted *trans-cis* double bonds is also reported.

SUPPLEMENTARY KEY WORDS disiamylborane · *cis*-enynoic acids · *trans*-enynoic acids · polyynoic acids · methyl esters

A FEW YEARS ago the synthesis of oleic acid-9,10-^{3}H from stearolic acid (1) and the partial reduction of 9,12-octadecadiynoic acid in an atmosphere of tritiated hydrogen gas to give linoleic acid-9,10,12,13-^{3}H were reported (2). Since then, several polyunsaturated fatty acids labeled with tritium have also been synthesized through the polyacetylenic approach (3, 4) with subsequent catalytic reduction in tritiated hydrogen gas (5–7). However, some disadvantages associated with the method of catalytic reduction with tritium gas (7, 8) and the preparation and use (3) of Lindlar catalyst have promoted some investigation of alternative methods (8, 9). For example, Nystrom, Nam, and Russo (10) treated methyl stearolate with tritiated disiamylborane and obtained methyl oleate-9,10-^{3}H.

Recently, a need for tritiated polyunsaturated fatty acids prompted us to test disiamylborane (11) for the reduction of methyl esters of enynoic and polyynoic structure. The reaction was found to be suitable for the partial reduction of the triple bond(s) in these molecules, and as a result, tritiated disiamylborane was employed and the synthesis of tritiated polyunsaturated fatty acids was realized. The present work describes these experiments.

Abbreviations: IR, infrared; UV, ultraviolet; GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

EXPERIMENTAL PROCEDURES

Materials

Diethylene glycol dimethyl ether (diglyme) from Aldrich Chemical Co., Inc., Milwaukee, Wis., was dried with CaH_2 overnight and then distilled under reduced pressure over $LiAlH_4$. It was used fresh, immediately after distillation. Metal hydrides were obtained from Metal Hydrides Incorporated, Beverly, Mass., and $NaBH_4$(^{3}H) with a specific activity of 200 mc/mmole was obtained from New England Nuclear Corp., Boston, Mass. Radiopurity was checked by the method of Gahan, Sandford, and Conrad (12). (We wish to thank Mr. M. Veniamin for suggesting this).

Boron fluoride etherate (Eastman Kodak Co., Rochester, N.Y.) was distilled under reduced pressure over CaH_2. 2-Methyl-2-butene from Aldrich Chemical Co. was utilized as received. Tritiated water (approximate specific activity 100 mc/g) from New England Nuclear Corp. was used for the preparation of tritiated acetic acid (CH_3COO^3H) according to the method of Weltner (13). Fatty acid methyl esters used as standards were purchased from The Hormel Institute, Austin, Minn.

Methods

Microanalyses were carried out by Clark Microanalytical Laboratories, Urbana, Ill. IR spectra on 10% carbon disulfide solutions were recorded with a Beckman IR 7 spectrometer, and UV spectra were measured with a Cary model 11M recording spectrometer. GLC was

carried out with a Barber–Colman gas chromatograph, model 5000 series, dual glass column with flame ionization detectors, using 10% EGSS-X (an ethylene glycol succinate polyester combined with a silicone) on Gas-Chrom P, 100–120 mesh, and 1% OV-1 (methyl silicone) on 60–80 mesh Gas-Chrom Q. The U-shaped glass columns were 2.4 m long and were maintained at 175°C for routine analyses. Helium was the carrier gas (60 ml/min). Preparative GLC was carried out with the same instrument on a U-shaped, 2.4 m × 12 mm (I.D.) column, packed with 10% EGSS on 60–80 mesh Gas-Chrom, maintained at 180°C. The outgoing effluent gas was split into two parts (1:9), one part being directed into the detector and the other nine parts into a collecting device that utilized the gradient cooling technique of Schlenk and Sand (14).

TLC on Silica Gel G and on silica gel impregnated with $AgNO_3$ was performed as reported earlier (2). Radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer, model 314 EX-2. Radioassay of the GLC effluent gas and of TLC scrapings was done as reported elsewhere (2, 15). Mass spectra were obtained with a single focusing Hitachi–Perkin–Elmer RMU-6E instrument.

Syntheses of Acetylenic Fatty Acids

All reactions and the general handling of all compounds containing more than one acetylenic or ethylenic bond were conducted in an argon atmosphere. 9,12-Octadecadiynoic acid was synthesized as previously reported (2, 3). 10,13-Octadecadiynoic acid and its homologues were prepared in a similar fashion. In all cases the formation of the 1,4-diyne system was effected by the reaction of the Grignard reagent of an ω-acetylenic acid [BrMg-C≡C—$(CH_2)_n$COOH] with a substituted propargyl bromide (—C≡C—CH_2Br). 6,9,12-Octadecatriynoic and octadeca-*cis*-12-en-9-ynoic acids were synthesized similarly (3). Octadeca-*trans*-12-en-9-ynoic acid and its homologue were synthesized according to the procedure of de Gaudemaris and Arnaud (16) which is depicted below.

$$CH_3(CH_2)_4CHO + CH_2(COOH)_2 \xrightarrow[\text{pyridine}]{} CH_3(CH_2)_4CH \overset{trans}{=} CHCOOH$$

$$\xrightarrow[LiAlH_4]{} CH_3(CH_2)_4CH \overset{trans}{=} CHCH_2OH \xrightarrow[PBr_3]{} CH_3(CH_2)_4CH \overset{trans}{=} CHCH_2Br$$

$$+$$

$$CH_3(CH_2)_4CH \overset{trans}{=} CHCH_2C{\equiv}C\ (CH_2)_7COOH \leftarrow HC{\equiv}C\ (CH_2)_7COOH$$

The acids were converted to their methyl esters by allowing them to react with methanolic HCl, 5% at room temperature overnight. GLC indicated purities varying from 90 to 98%. Catalytic hydrogenation over PtO_2 in methanol gave the corresponding saturated methyl esters, and through their identification (GLC and standards) the chain length of the parent methyl ester was established. IR spectra gave the expected absorptions, and mass spectra verified the molecular weight. Oxidative cleavage (4) led to the expected (according to the assigned positions of unsaturation) mono- and dicarboxylic acid cleavage fragments.

Reduction with Disiamylborane

The partial reduction of methyl 10,13-octadecadiynoate is given here as representative. In all steps of the reaction, extreme care was taken to exclude moisture. 75 mg of sodium borohydride (2 mmoles) was placed in a dry 50 ml two-necked flask equipped with a magnetic stirring bar. An argon line was connected to one neck, and a rubber septum was fitted on the second. Diglyme (4 ml) was injected through the septum from a syringe, and the sodium borohydride was dissolved by stirring. After the addition of 350 mg of 2-methyl-2-butene (5.0 mmoles), the flask was cooled to 0°C in an ice bath, and 0.3 ml of boron trifluoride etherate (2.7 mmoles) was added drop by drop with constant stirring.

After the addition had been completed, the mixture was allowed to stand at 0–5°C for 2 hr. To the disiamylborane prepared *in situ*, 290 mg of methyl 10,13-octadecadiynoate (1.0 mmole) was added slowly with external cooling (ice bath) and under constant stirring. After another 0.5 hr at 0°C, the solution was allowed to reach room temperature, and the stirring was continued for 2 hr. The mixture was cooled again, and ethylene glycol was added to decompose excess hydride. This was followed by 0.5 ml of glacial acetic acid at 0°C. The protonolysis with the acetic acid was completed overnight at room temperature. The crude product was recovered by pouring the contents into cold water and extracting with hexane. The hexane solution was dried over sodium sulfate and concentrated by evaporation under reduced pressure. The crude product was purified by silicic acid chromatography with 10% anhydrous ether in hexane as the eluting solvent. After the solvent had been evaporated on a rotary evaporator, 266 mg (87.2%) of oily material was recovered, and aliquots subjected to several tests. Elementary analysis gave: C, 77.3; H, 11.2. Calculations for $C_{19}H_{34}O_2$: C, 77.5; H, 11.6%. The IR spectrum indicated 1% of *trans* isomer. UV absorption gave no evidence of conjugated dienes. Catalytic reduction in methanol with PtO_2 catalyst yielded methyl stearate, identified by GLC. Oxidative

ozonolysis (5) followed by methylation of the cleavage acids with diazomethane gave methyl pentanoate, dimethyl malonate, and dimethyl decanedioate identified with standards in programmed GLC analysis (nonpolar column, 10 min isothermal run at 50°C, followed by a temperature increase of 10°C/min to 180°C, helium flow rate 60 ml/min).

Reduction with Tritiated Disiamylborane

To prepare the tritiated methyl 10,13-octadecadienoate, we substituted sodium borotritide for sodium borohydride. In a typical experiment, tritiated sodium borohydride was diluted with unlabeled sodium borohydride to a specific activity of 16.4 mc/mmole, and the reaction was carried out as described above. The hexane extract of the crude product contained a total of 29.89 mc, and when an aliquot was chromatographed on a TLC plate, 35.6% of the radioactivity was associated with the methyl ester band. The crude product was passed through a silicic acid column. The recovered methyl ester weighed 231 mg (78.6%) and had a total activity of 7.3 mc (9.3 mc/mmole). It was ultimately purified by preparative GLC to a specific activity of 8.85 mc/mmole (26.9%, radiochemical yield). GLC and radio–gas analysis (Fig. 1) indicated a chemical purity of 97–98% and a radiochemical purity of 96–97%, respectively. Radioassay of the bands from plates coated with silica gel indicated that 96% of the radioactivity was associated with the methyl ester spot. Argentation TLC showed one spot at the position of standard methyl linoleate, and almost all of the radioactivity was associated with that spot. Under the conditions employed, methyl *cis,cis*-8,10-octadecadienoate and methyl *cis,cis*-9,12-octadecadienoate do not separate (17). Radioassay of the cleavage products as they were eluted from the GLC column indicated that they were practically free of tritium, which suggests that the tritium was located at carbons 10, 11, 13, and 14.

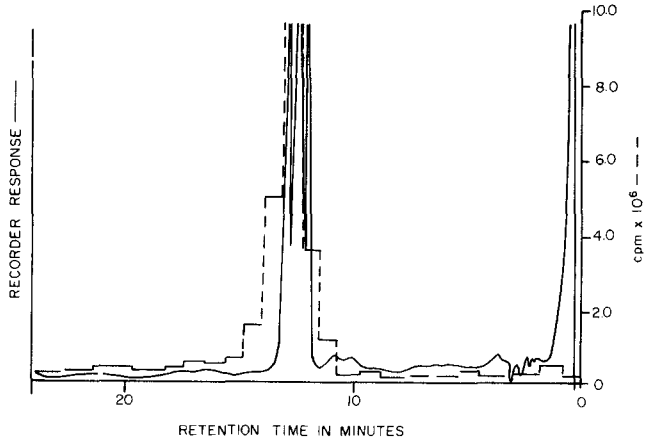

FIG. 1. GLC analysis of methyl 10,13-octadecadienoate-10,11,-13,14-^{3}H and radioassay of the effluent gas. Polar column at 175°C.

Alternatively, bands from TLC (Silica Gel G) corresponding to malonic acid, monoenoic acid, and dienoic acid were radioassayed. For the separation, the solvent benzene–methanol–acetic acid 45:8:4 gave R_f values of 4.2, 8.0, and 9.2 for malonic, decanedioic, and pentanoic acids, respectively. The R_f values were checked with standards. No appreciable amount of radioactivity was associated with any acid. Exhaustive hydrogenation over PtO_2 catalyst gave methyl stearate with a specific activity of 8.8 mc/mmole, which agreed closely with that found for methyl 10,13-octadecadienoate.

RESULTS AND DISCUSSION

Reactions with disiamylborane are summarized in Table 1. Yields of 82–94% were observed, and they were similar to those reported by Osbond, Philpott, and Wickens (3), namely 85–90%, for the catalytic partial reduction of polyacetylenic fatty acids.

Tritiated disiamylborane gave the results recorded in Table 2. Yields were somewhat lower than those recorded with unlabeled $NaBH_4$, and the yield seems to decrease with increasing specific activity of the tritiated sodium borohydride employed. Radioactive damage and decomposition of the hydride may have accounted for the lower values. The radiochemical yield based on the radioactivity of $NaBH_4(^3H)$ varied from 25.7 to 29.0%. Assuming a 100% formation of diisoamylborane, theory would predict a 33.3% yield.

It is of interest to note that when methyl 9,12-octadecadiynoate was partially reduced in an atmosphere of tritiated hydrogen gas over Lindlar catalyst, a 70% yield was obtained (2). In that case, the specific activity of the final product was 357 mc/mmole. When tritiation was effected by using unlabeled disiamylborane and tritiated acetic acid, higher yields were obtained (Table 2). The activity of the tritiated acetic acid was very low (1.2 mc/mmole), however, and because tritiated water of a

TABLE 1 DISIAMYLBORANE REDUCTION OF ACETYLENIC ACID METHYL ESTERS

Methyl Ester*	Yield†
	%
$CH_3(CH_2)_4C{\equiv}CCH_2C{\equiv}C(CH_2)_7COOCH_3$	86.1
$CH_3(CH_2)_3C{\equiv}CCH_2C{\equiv}C(CH_2)_8COOCH_3$	87.2
$CH_3(CH_2)_4C{\equiv}CCH_2C{\equiv}C(CH_2)_8COOCH_3$	91.6
$CH_3(CH_2)_5C{\equiv}CCH_2C{\equiv}C(CH_2)_8COOCH_3$	82.3
cis $CH_3(CH_2)_4CH{=}CHCH_2C{\equiv}C(CH_2)_7COOCH_3$	94.2
trans $CH_3(CH_2)_4CH{=}CHCH_2C{\equiv}C(CH_2)_7COOCH_3$	93.1
trans $CH_3(CH_2)_5CH{=}CHCH_2C{\equiv}C(CH_2)_8COOCH_3$	93.2
$CH_3(CH_2)_4C{\equiv}CCH_2C{\equiv}CCH_2C{\equiv}C(CH_2)_4COOCH_3$	85.6

* 1 mmole of each.
† Total yield calculated on the starting methyl ester.

TABLE 2 SYNTHESIS OF TRITIATED POLYUNSATURATED FATTY ACID METHYL ESTERS

Starting Acetylenic Compound*	Tritiating Agent†	Yield†		Specific Activity
		Chemical	Radiochemical	
		%		*mc/mmole*
$CH_3(CH_2)_4C{\equiv}CCH_2C{\equiv}C(CH_2)_7COOCH_3$	$NaBH_4(^3H)$	82.3	27.8	9.25
$CH_3(CH_2)_3C{\equiv}CCH_2C{\equiv}C(CH_2)_8COOCH_3$	"	78.6	26.9	8.80
"	$CH_3COOH(^3H)$	82.8	—	1.92
"	$NaBH_4(^3H)$	72.6	25.7	26.20
trans $CH_3(CH_2)_4CH{=}CHCH_2C{\equiv}C(CH_2)_7COOCH_3$	"	85.2	29.0	11.60
trans $CH_3(CH_2)_5CH{=}CHCH_2C{\equiv}C(CH_2)_7COOCH_3$	"	81.7	28.5	11.40
$CH_3(CH_2)_4C{\equiv}CCH_2C{\equiv}CCH_2C{\equiv}C(CH_2)_4COOCH_3$	"	70.7	26.0	30.12
"	$CH_3COOH(^3H)$	75.4	—	2.52

* Approximately 1 mmole of each.
† Specific activity: $NaBH_4(^3H)$, 20–50 mc/mmole; $CH_3COOH(^3H)$, 1.2 mc/mmole.
Chemical yield based on starting methyl ester. Radiochemical based on $NaBH_4(^3H)$.

higher specific activity was not available we did not investigate the subject any further.

The bulk of radioactive contaminants were removed from the crude product with the aid of silicic acid column chromatography. However, a considerable amount of radioactivity (5–10%) was still present as a contaminant, which became evident when the material was subjected to preparative GLC. A decrease in specific activity was consistently observed. Tritiated methyl 10,13-octadecadienoate with a specific activity of 9.3 mc/mmole after silicic acid column chromatography fell to 8.85 mc/mmole after preparative GLC (see Methods). Apparently, the contaminant was eluted together with the methyl ester from a silicic acid column but not from the GLC column. These observations indicate that for the final purification of the product, preparative GLC is mandatory.

Oxidative cleavage products did not show any appreciable amount of tritium, which suggests that in the parent tritiated methyl 10,13-octadecadienoate tritium atoms were located on the carbon atoms forming the unsaturated double bonds. Catalytic tritiation of methyl 9,12-octadecadiynoate (2) gave a 5% distribution of tritium, scattered over the molecule as a result of exchange. This percentage might have been higher, since malonic acid was not isolated from the oxidation cleavage products, and it was not radioassayed in that study. Considering that malonic acid is generated from the allylic position of linoleic acid, the activated hydrogens might have been extensively exchanged with tritium gas, and a large amount of tritium could have been incorporated in that position.

The method of tritioboration does not require a high vacuum line and Toepler pump and therefore offers a more convenient way for labeling polyunsaturated fatty acid methyl esters than previously reported. As the present examples demonstrated, activities sufficient for biological experiments can readily be attained.

We wish to thank Donald Lighter for the synthesis of some acetylenic intermediates and Dr. F. A. Kummerow for his encouragement during the course of this study.

This work was supported by Grant HE 10779-01 from the National Institutes of Health, United States Public Health Service, and Grant RN 68-31 from the Chicago and Illinois Heart Association.

Manuscript received 2 April 1969; accepted 8 July 1969.

REFERENCES

1. Tenny, K. S., S. C. Gupta, R. F. Nystrom, and F. A. Kummerow. 1963. *J. Amer. Oil Chem. Soc.* **40:** 172.
2. Sgoutas, D. S., and F. A. Kummerow. 1964. *Biochemistry.* **3:** 406.
3. Osbond, J. M., P. G. Philpott, and J. C. Wickens. 1961. *J. Chem. Soc. (London).* 2779.
4. Osbond, J. M. 1966. *In* Progress in the Chemistry of Fats and Other Lipids. Ralph T. Holman, editor. Pergamon Press, Inc., New York. **9** (Pt. 1): 119.
5. Stoffel, W. 1965. *J. Amer. Oil Chem. Soc.* **42:** 583.
6. Van Dorp, D. A., R. K. Beerthuis, D. H. Nugteren, and H. Vonkeman. 1964. *Biochim. Biophys. Acta.* **90:** 204.
7. Kunau, W. H. 1968. *Hoppe-Seyler's Z. Physiol. Chem.* **349:** 333.
8. Evans, E. A. 1966. Tritium and Its Compound. D. Van Nostrand Co., Inc., Princeton, New Jersey. 143–146.
9. Tenny, K. S. 1961. *Diss. Abstr.* **22:** 1574.
10. Nystrom, R. R., N. H. Nam, and A. J. Russo. 1964. Proceedings of the Congress on Methods of Preparing and Storing Marked Molecules, Brussels, November 1963. European Atomic Energy Community, Euratom. EUR 1625e, 47.
11. Brown, H. C., and G. Zweifel. 1961. *J. Amer. Chem. Soc.* **83:** 3834.
12. Gahan, L. C., P. A. Sandford, and H. E. Conrad. 1966. *Biochemistry.* **6:** 2755.
13. Weltner, W., Jr. 1955. *J. Amer. Chem. Soc.* **77:** 3941.
14. Schlenk, H., and D. M. Sand. 1963. *Anal. Chem.* **34:** 1676.
15. Sgoutas, D. S., M. J. Kim, and F. A. Kummerow. 1965. *J. Lipid Res.* **6:** 383.
16. de Gaudemaris, M., and P. Arnaud. 1962. *Bull. Soc. Chim. Fr.* **28:** 315.
17. Christie, W. W. 1968. *J. Chromatogr.* **34:** 405.