BIOORGANIC CHEMISTRY 17, 281–286 (1989)

A New Method for the Preparation of Carboxy-Labeled Unsaturated Fatty Acids and Its Application to Linoleic Acid

JAMES R. CAMPBELL AND CHARLES H. CLAPP[1]

Department of Chemistry, Bucknell University, Lewisburg, Pennsylvania 17837

Received October 31, 1988

The lithium enolate of methyl linoleate was added at −78°C to diethyl dibromomalonate to yield methyl α-bromolinoleate, which was converted to the corresponding [α-^{13}C]cyanoester by treatment with Na^{13}CN. Mild alkaline hydrolysis of the cyanoester followed by decarboxylation in refluxing pyridine that contained acetic acid and 4-dimethylaminopyridine afforded [1-^{13}C]linoleonitrile, which was hydrolyzed to [1-^{13}C]linoleic acid.

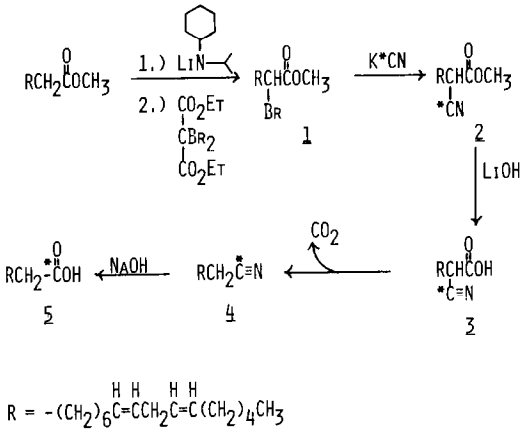
Isotopically labeled unsaturated fatty acids are of great importance in studies of lipid metabolism and biosynthesis. In addition, ^{13}C-labeled fatty acids are potentially useful for NMR studies of membrane dynamics (*1, 2*), and ^{14}C-labeled suicide inactivators are often required for elucidating inactivation mechanisms. All of these areas would benefit from a method of incorporating isotopic carbon into unsaturated fatty acids that are available in unlabeled form, from either natural sources or prior synthesis. In this paper we present a new strategy for accomplishing this objective and describe its application to linoleic acid.

Incorporation of isotopic carbon into saturated fatty acids is straightforward (*3*). Treatment of the silver salt of the unlabeled acid with Br_2 (the Hunsdiecker degradation) replaces the carboxyl group with a bromine atom, which can be displaced with labeled cyanide. Hydrolysis of the resulting nitrile regenerates the acid with isotope in the carboxyl group. Unfortunately, this method is not applicable to unsaturated acids, since Br_2 and intermediates in the Hunsdiecker degradation react with double bonds (*4*). One solution to this problem is to brominate the double bonds and then debrominate with Zn after the Hunsdiecker reaction (*5*). A disadvantage of this approach is that debromination does not always occur with complete anti-stereospecificity (*6*), so that the desired Z fatty acids may require purification from the E isomers (*7*). This problem is particularly serious for polyunsaturated fatty acids. Recently published procedures for preparing labeled samples of naturally occurring monounsaturated (*8*) and polyunsaturated (*9, 10*) fatty acids have followed lengthy routes in which the carbon skeletons were constructed from small molecules.

Our strategy (Scheme 1) follows from the work of Van der Wolf and Pabon (*11*), who demonstrated the feasibility of introducing a bromine atom α to the carbonyl group of a polyunsaturated fatty acid ester without affecting the double bonds. We

[1] To whom all correspondence should be addressed.

0045-2068/89 $3.00



SCHEME 1

reasoned that displacement of bromide by labeled cyanide followed by hydrolysis of the ester would allow decarboxylation of the α-cyano acid, **3,** to yield a labeled nitrile, **4,** which could be hydrolyzed to the labeled acid.

RESULTS AND DISCUSSION

Van der Wolf and Pabon added diethyl dibromomalonate to a cold solution of the lithium enolate of *tert*-butyl 8(Z), 11(Z), 14(Z)-eicosatrienoate to obtain the 2-bromo derivative of this ester. Our attempts to apply this procedure to methyl linoleate yielded two TLC-detectable materials in a ratio of approximately 2 : 1. The major component was found to be the desired 2-bromo derivative, **1.** The second material was not completely characterized, but its ir and NMR spectra suggested a mixture of products derived from Claisen condensations between **1** and lithium enolate. This pathway could probably be suppressed by using the more sterically hindered *tert*-butyl ester, but this approach appeared undesirable, since Van der Wolf and Pabon reported low yields for both the introduction and the removal of the *tert*-butyl group (*11*). Instead, the side reaction was reduced to about 10% by reversing the order of addition, that is, by adding the cold lithium enolate solution to a cold solution of diethyl dibromomalonate in THF. This was accomplished by forcing the enolate solution under nitrogen pressure through a double-tipped syringe needle. When the reaction was carried out in this manner, the desired product was obtained in 70% yield after purification by flash chromatography. This material was homogeneous by GC/MS, and its ^{1}H NMR resonances for the vinyl and allylic protons were identical to those in methyl lineolate. These results indicate that no isomerization of the 1,4-diene had occurred. In contrast, if the lithium enolate was allowed to warm to room temperature prior to transfer, the α-bromo ester that was obtained contained at least four isomers by GC/MS, and the ^{1}H NMR spectrum indicated that about one-third of the 1,4-diene molecules had isomerized to conjugated dienes.

The α-bromo ester, **1**, was treated at 18–20°C with a mixture of $K^{13}CN$ and $K^{12}CN$ (41 : 100) to afford the ^{13}C-enriched α-cyanoester, **2**, in 73% yield, and the ester was hydrolyzed to **3** in 88% yield by stirring with 1 M LiOH/THF (3 : 1) for 5 h at room temperature. Decarboxylation was accomplished in 76% yield by refluxing the α-cyano acid, **3**, for 6 h in pyridine that contained acetic acid and 4-dimethylaminopyridine (DMAP). If **3** was refluxed in pyridine alone, decarboxylation was very slow. Addition of acetic acid increased the rate, and inclusion of DMAP resulted in a further increase of about sevenfold.

Alkaline hydrolysis of **4** to ^{13}C-enriched linoleic acid was accomplished in 84% yield. The product was homogeneous by GC, and its mass spectrum exhibited a parent peak at 280 and an M + 1 peak with an intensity (0.62 relative to the parent) that agreed well with the value of 0.60 calculated from the molar ratio of [^{12}C]- to [^{13}C]cyanide used in the second step, with correction for natural abundance ^{13}C. The ir spectrum was identical to that of unlabeled linoleic acid except for a shoulder (1670 cm^{-1}) on the carbonyl peak due to the ^{13}C-containing material. No signal was present from 960 to 1000 cm^{-1} where *E* olefins absorb strongly (*12*). The ^1H NMR spectrum was identical to that of unlabeled linoleic acid except for the position of the signal due to the acidic proton.

In summary, our method provided [1-^{13}C]linoleic acid with no detectable alteration of the Z,Z-1,4-diene, a unit that is found in many naturally occurring fatty acids. Since the expected level of enrichment was obtained, the method is suitable for preparation of material with very high enrichment. On the basis of the isolated yields for each intermediate, the overall yield was 29%, and the yield after introduction of isotope was 41%. It is likely that the overall yield could be improved by not isolating every intermediate; we have already found that isolation of **3** is unnecessary.

The method appears to be applicable to unsaturated fatty acids bearing a hydroxyl group. Treatment of methyl 12-hydroxy-(Z)-9-octadecenoate (methyl ricinoleate) with 2.2 eq of lithium isopropylcyclohexylamide followed by addition of the resulting dianion to diethyl dibromomalonate yielded the 2-bromo derivative in 45% yield. Studies with unlabeled materials indicate that the remaining reactions in Scheme 1 proceed as described for linoleic acid. We are currently adapting this route to the small-scale synthesis of [1-^{14}C]ricinoleic acid, which will be described in a future paper.

EXPERIMENTAL

The ir spectra were obtained on a Perkin–Elmer 1310 spectrometer or a Nicolet 5DXC FT instrument. NMR spectra were recorded on a Jeol FX 90Q FT spectrometer with tetramethylsilane as internal standard. GC/MS analyses were carried out on a Finnigan 4021 system with a 12.5 m × 0.2 mm column of crosslinked dimethylsilicone. Flash chromatography was performed according to Still *et al.* (*13*) on grade-60 silica gel from Aldrich. TLC was carried out on Baker silica gel 1 B-F strips, and spots were visualized with iodine. Anhydrous $MgSO_4$ was used to dry organic extracts.

Methyl linoleate was purchased from Sigma Chemical Co., and diethyl dibromomalonate was prepared as described by Van der Wolff and Papon (*11*). *n*-Butyllithium and DMAP were obtained from Aldrich, and $K^{13}CN$ was obtained from Cambridge Isotope Labs. THF was distilled from potassium.

Methyl 2-bromo-9(Z), 12(Z)-octadecadienoate (**1**). *n*-Butyllithium (4.9 ml of a 2.5 M solution in hexanes) was added by syringe to a cold (−78°C), magnetically stirred solution of isopropylcyclohexylamine (1.58 g, 11.2 mmol) in THF (30 ml) under N_2 in a three-necked flask fitted with two serum caps and an addition funnel. A solution of methyl linoleate (3.0 g, 10.2 mmol) in THF (10 ml) was then added dropwise from the addition funnel over 30 min. The reaction mixture was maintained at −78°C throughout the addition and subsequently stirred at −78°C for 55 min. The cold lithium enolate solution was then transferred under nitrogen pressure through an 18-gauge double-tipped needle (Aldrich Chemical Co.) into a second flask that contained a magnetically stirred solution of diethyl dibromomalonate (8.7 g, 27 mmol) in THF under N_2 at −78°C. The transfer was carried out dropwise over 70 min. The reaction was stirred for an additional 2 h at −78°C and then quenched at −78°C with 2% HCl (14 mL). After being warmed to room temperature, the mixture was added to 150 ml of saturated NaCl and extracted with hexanes (4 × 75 ml). The combined organic layers were dried and concentrated to an oil, which was applied to a 21.5 × 5.3-cm column of silica gel (Davisil 62). Elution under nitrogen pressure with hexanes (3.3 liter) followed by concentration of the eluate yielded 3.8 g of an oil, which showed a major spot (R_f = 0.52) and a minor spot (R_f = 0.62) by TLC in hexanes/ethyl acetate (19 : 1). Excess reagent and diethyl bromomalonate remained on the column. A portion of the oil (482 mg) was subjected to flash chromatography on a 21 × 2.3-cm column with hexanes/ethyl acetate (19 : 1) to yield 339 mg (70%) of the major product, which was homogeneous by TLC and GC/MS and gave the expected spectroscopic data for **1:** ^1H NMR ($CDCl_3$, 90 MHz) δ 5.34 (m, 4H, vinyl), 4.20 (t, J = 7.5 Hz, 1 H, H-2), 3.77 (s, 3H, OCH_3), 2.76 (t, J = 5.3 Hz, 2H, H-11), 2.05 (m, 6H, H-3, H-8, H-14); ir (film) 1750 cm^{-1}; MS, *m/z*, 372 (M, ^{79}Br), 374 (M, ^{81}Br).

Methyl 2-([^{13}C]cyano)-9(Z),12(Z)-octadecadienoate (**2**). A solution of **1** (311 mg, 0.83 mmol) in DMSO (3 ml) was added to a mixture of $K^{13}CN$ (23 mg, 0.35 mmol) and $K^{12}CN$ (56 mg, 0.86 mmol) in DMSO (12 ml) at 18–20°C. The mixture was stirred at 18–20°C for 40 min, added to 100 ml of saturated NaCl, and extracted with ether (3 × 100 ml). The combined organic layers were washed with water (100 ml), dried, and concentrated to 265 mg of an oil. TLC of this material in hexanes/ethyl acetate (19 : 1) showed a major product (R_f = 0.25), a minor product (R_f = 0.34), and a trace of starting material. Purification of the major product by flash chromatography on a 21.6 × 2.3-cm column with hexanes/ethyl acetate, 97 : 3, afforded 194 mg (73%) of **2:** NMR ($CDCl_3$, 90 Mz) δ 5.35 (m, 4H, vinyl), 3.81 (s, 3H, OCH_3), 3.49 (t, J = 7.6 Hz, 1H, H-2), 2.76 (t, J = 6 Hz, 2H, H-11), δ 2.08 (m, 6H, H-3, H-8, H-14); ir (film) 2250 ($^{12}C{\equiv}N$), 2200 ($^{13}C{\equiv}N$), 1750 cm^{-1} (C=O); MS, *m/z*, 319 (M), 320 (M + 1).

2-([^{13}C]Cyano)-9(Z),12(Z)-octadecadienoic acid (**3**). A solution of **2** (172 mg, 0.54 mmol) in THF (5 ml) was treated with 1 M LiOH (15 ml) and stirred at room temperature for 5 h. The reaction mixture was then added to 100 ml of 1 M HCl

and extracted with ether (3 × 50 ml). The combined organic layers were washed with 100 ml of water, dried, and concentrated to yield 200 mg of an oil. (This material can be used directly in the next step.) The major component was purified by flash chromatography on a 20.3 × 1.8-cm column with hexanes/ethyl acetate/acetic acid (87 : 10 : 3) to give 144 mg (88%) of **3.** TLC (hexanes/ethyl acetate/acetic acid, 81 : 15 : 4) R_f = 0.48; NMR ($CDCl_3$, 90 MHz) δ 5.35 (m, 4H, vinyl), 3.62 (t, 1H, H-2), 2.77 (t, 2H, H-11), 2.08 (m, 6H); ir (film) 2500–3500 (br), 2250 ($^{12}C{\equiv}N$), 2200 ($^{13}C{\equiv}N$), 1715 cm^{-1} (C=O).

9(Z),12(Z)-Octadecadieno[1-^{13}C]nitrile (**4**). A solution of **3** (113 mg, 0.37 mmol), in pyridine (5 ml) that contained acetic acid (0.6 g) and DMAP (2.0 g) was refluxed for 6 h. After being cooled, the mixture was added to 1 M HCl (150 ml) and extracted with ether (3 × 50 ml). The combined organic layers were washed with 100 ml of water, dried, and concentrated to yield 115 mg of an oil. Flash chromatography on a 20.3 × 1.8-cm column with hexanes/ethyl acetate (98 : 2) yielded 74 mg (76%) of **4.** TLC (hexanes/ethyl acetate/acetic acid, 87 : 10 : 3) R_f = 0.58; NMR ($CDCl_3$, 90 MHz) δ 5.37 (m, 4H, vinyl), 2.79 (t, 2H, H-11), 2.34 (t, 2H, H-2), 2.05 (m, 4H, H-8, H-13); ir (film) 2250 ($^{12}C{\equiv}N$), 2190 ($^{13}C{\equiv}N$) cm^{-1}; MS, *m/z*, 261 (M), 262 (M + 1).

[1-^{13}C]-9(Z),12(Z)-Octadecadienoic acid (**5**). A solution of **4** (65 mg, 0.25 mmol) and NaOH (1.0 g) in ethanol (7 ml) and H_2O (10 ml) was refluxed for 8.5 h. After being cooled, the solution was added to 75 ml of 1 M HCl and extracted with ether (3 × 50 ml). The combined organic layers were washed with 100 ml of water, dried, and concentrated to 75 mg of an oil. Purification by flash chromatography on a 20.3 × 1.8-cm column with hexanes/acetic acid (97 : 3) yielded 59 mg (84%) of **5.**

ACKNOWLEDGMENTS

We are grateful to Mr. Jimmy Spriggle for carrying out the GC/MS analyses reported here and to Mr. Robert Zimmermann for obtaining the FT NMR spectra. The work was supported by NIH Grant GM 37289.

REFERENCES

1. Brown, M. F., Riberio, A. A., and Williams, G. D. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 4325–4329.
2. Brown, M. F., and Williams, G. D. (1985) *J. Biochem. Biophys. Methods* **11,** 71–81.
3. Murray, A., and Williams, D. L. (1958) Organic Syntheses with Isotopes, p. 62, Interscience, New York.
4. Wilson, C. V. (1957) *Org. React.* **9,** 332–387.
5. Howton, D. R., Davis, R. H., and Nevenzel, J. C. (1954) *J. Amer. Chem. Soc.* **76,** 4970–4974.
6. March, J. (1985) Advanced Organic Chemistry, pp. 924–925, Wiley, New York.
7. Stoffel, W. (1965) *J. Amer. Oil Chem. Soc.* **42,** 583–589.
8. Valicenti, A. J., Pusch, F. J., and Holman, R. T. (1985) *Lipids* **20,** 234–242.
9. Do, U. H., Sundaram, M. G., Ramachandran, S., and Bryant, R. W. (1979) *Lipids* **14,** 819–821.

10. SPRECHER, H., AND SANKARAPPA, S. K. (1982) *in* Methods in Enzymology (Lands, W. E. M., and Smith, W. L., Eds.), Vol. 86, pp. 357–366, Academic Press, San Diego, CA.
11. VAN DER WOLF, L., AND PABON, H. J. J. (1977) *Recl. Trav. Chim. Pays-Bas* **96,** 72–74.
12. SILVERSTEIN, R. M., BASSLER, G. C., AND MORRILL, T. C. (1981) Spectrometric Identification of Organic Compounds, p. 110, Wiley, New York.
13. STILL, W. C., KAHN, M., AND MITRA, A. (1978) *J. Org. Chem.* **43,** 2923–2925.