Vol. 81, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1978 Pages 486-492

SYNTHESIS OF 9(12)-OXY-8,11,15-TRIHYDROXYEICOSA-5,13-DIENOIC ACID FROM ARACHIDONIC ACID BY SOYBEAN LIPOXYGENASE-2*

Gary S. Bild, Santhoor G. Bhat, Candadai S. Ramadoss,** and Bernard Axelrod***

Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907

and

Charles C. Sweeley

Department of Biochemistry, Michigan State University

East Lansing, Michigan 48824

Received December 22,1977

SUMMARY: Lipoxygenase-2, an isoenzyme of soybean lipoxygenase catalyzes the formation of a polyhydroxylated furan, 9(12)-oxy-8,11,15-trihydroxyeicosa-5,13-dienoic acid from arachidonic acid.

Pace-Asciak found that preparations from sheep seminal vesicles catalyzed the multiple oxygenation of arachidonic acid to form, in addition to the expected prostaglandins, several new cyclic ethers, one, a prostaglandin compound, prostacyclin, as well as some polyhydroxylated open chain C_{20} compounds, all of which contain a cyclic ether group (1). One of these latter products (the Pace-Asciak compound I) was shown by him to be 9(12)-oxy-8,11,15-trihydroxyeicosa-5,13-dienoic acid. We wish to report that this compound is formed when an isoenzyme of soybean lipoxygenase, L-2,[1] acts on arachidonic acid.

---

*Journal Paper No. 6994, Purdue Agriculture Experiment Station. Supported by NSF Research Grant PCM 09800 (to B.A.) and NIH Research Grant RR0480 from the Biotechnology Resources Branch (to C.C.S.).

**Present address: Forschungsstelle Vennesland der Max-Planck-Gesellschaft, Berlin 33, Harnackstrasse 23, Germany.

***To whom reprint request may be addressed.

[1]Abbreviations used: L-2, soybean lipoxygenase-2; L-1, soybean lipoxygenase-1.

0006-291X/78/0812-0486$01.00/0

Copyright © 1978 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Contrary to the earlier view (2) that lipoxygenase catalyzed but a single dioxygenation of arachidonic acid, we recently showed that soybean lipoxygenase-1 (L-1) could promote the bis-hydroperoxidation of arachidonic acid, consuming approximately two moles of O_2 per mole of arachidonic acid (3). We have now found that L-2, an enzyme known to exhibit a positional specificity for monohydroperoxidation which differs from L-1 (4), also catalyzes double dioxygenation of arachidonate and related compounds. Unlike the bis-hydroperoxidation catalyzed by L-1 which proceeds rapidly with the first oxygen molecule and slowly with the second to give an almost theoretical yield of bis-hydroperoxide of an eicosatetraenoic acid, the reaction catalyzed by L-2 occurs in a mono-phasic manner, while approximately two moles of O_2 are consumed. Moreover a variety of products are obtained of which about 15% appears to be the Pace-Asciak compound I. An analogous compound is formed from 8,11,14-eicosatrienoic acid (Pace-Asciak compound III) (1).

MATERIALS AND METHODS

Enzyme Purification and Assay: Lipoxygenase-2 was purified from soybeans of the Amsoy variety (1974 crop) as previously described (5). Enzyme activities were determined using a Gilson Medical Electronics Oxygraph, model KM, equipped with a Clark oxygen electrode (Yellow Springs) and were performed as described previously (4).

Chemicals: All fatty acids (99% purity) were obtained from NuChek Prep, Inc., Elysian Minnesota. N-Methyl-N-nitroso-p-toluenesulfonamide used to methylate the reaction products was purchased from Aldrich Chemical Company. Pre-coated thin-layer chromatography (TLC) plates of silica gel 60F-254, were purchased from E. Merck. (1-^{14}C)-Arachidonic acid (60.2 mCi/mmol) was purchased from Amersham Searle Corp. Prostaglandin standards were kindly provided by Dr. John Pike of the Upjohn Company, Kalamazoo, Michigan. 8,15-Dihydroxy-5,9,11,13-eicosatetraenoic and 15-hydroxy-5,8,11,13-eicosatetraenoic acid were synthesized by the method of Bild et al. (6). Bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane was obtained from Regis Chemical Company.

Incubation and Product Isolation: Incubations were conducted at 24° for 5 minutes in a Dubnoff metabolic shaker in a mixture containing 93 e.u. of L-2 (linoleic acid units (5)), 0.1 mM (1-^{14}C) arachidonic acid (900,000) cpm and 0.1 M sodium phosphate buffer pH 6.8 in a total volume of 50 ml. The reaction products were reduced by the procedure of Nugteren and Hazelhof (7) by adding 25 ml of a 20% aqueous solution of $Na_2S_2O_4$ and shaking for 15 minutes at 25°. The pH was adjusted to 3.0 with 1.0 M citric acid and the mixture was extracted twice with 2 volumes of diethyl ether. The extract was dried over anhydrous sodium sulfate and evaporated to dryness under vacuum. The material was taken up in diethyl ether containing 10% methanol and was chromatographed on Merck silica gel (60F-254) thin-layer chromatography plates 250 μ thickness) for

1 hour at 25° in a developing solvent containing diethyl ether:methanol:acetic acid, 90:5:0.1. Plates were scanned in a Packard Radiochromatogram Scanner. Products were then visualized on the plates by spraying with anisaldehyde-ethanol-H_2SO_4. For the gas chromatographic-mass spectral analysis of the reaction products a 2 cm wide band of material chromatographing with standard $PGF_{2\alpha}$ was scraped from the silica gel plate and extracted with diethyl ether containing 10% methanol. The solution was dried over anhydrous sodium sulfate, filtered and taken to dryness under vacuum. The residue was methylated in the dark with ethereal diazomethane containing 10% methanol at 25°. Excess reagent and solvent were removed by evaporation and the residue was taken up in 25 µl of pyridine and silylated for 20 minutes at 80° with 25 µl of bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane. Gas chromatographic-mass spectral analysis of the product was done with a Finnigan 4000 Gas Chromatograph/El-Cl Mass Spectrometer equipped with a 6100 MS Data System. The gas chromatograph column was packed with 3% SE-30 and was programmed to run isothermally at 200° for 1 min and then increased to 300° at 6°/min. The flow rate was 20 ml/min and injection temperature was 225°. Electron energy voltage in the mass spectrometer was 70.0 e.v.

RESULTS AND DISCUSSION

It can be seen (Fig. 1) that the L-2 catalyzed uptake of oxygen by arachidonic acid proceeds to a ratio of about two moles of oxygen per mole of substrate. In contrast to the experience with L-1 where a sharp break in the oxygen uptake curve occurs when one mole of oxygen has been incorporated (3), the curve with L-2 is not biphasic. In the case of the L-1 oxygenation of arachidonic acid the biphasic kinetics have been correlated with the rapid appearance of the monohydroperoxide followed by the slower conversion of this product to the bis-hydroperoxide (3). With L-2 it is quite clear that multiple dioxygenation is occurring.

Thin-layer radiochromatograms of the sodium dithionite reduced reaction products obtained from the L-2 incubation with arachidonic acid yielded three main regions of radioactivity. The area of lowest mobility, R_f = 0.24, corresponding to peak A described elsewhere (8) was scraped from the plates and eluted with diethyl ether containing 10% methanol. Following methylation and silylation, the material was subjected to gas-liquid chromatography. Two distinct peaks separated with elution times of 9.84 min (Peak I) and 10.87 min (Peak II) (8). The mass fragmentation pattern (Fig. 2) of the material in Peak II was nearly identical to that seen by Pace-Asciak for the cyclic ether 9(12)-oxy-8,11,15-trihydroxyeicosa-5,13-dienoic acid (1). The

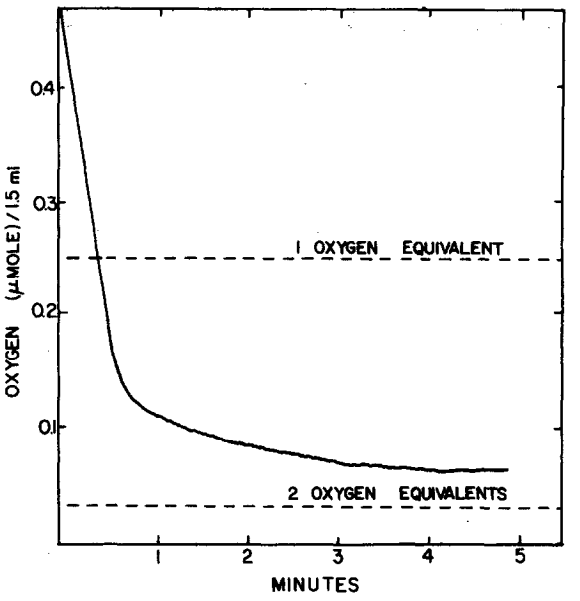

Fig. 1. Oxygraph recording of O_2 consumption in a reaction mixture containing 0.17 e.u. of lipoxygenase-2 and a final concentration of arachidonic acid of 1.42×10^{-4} M in 0.20 M sodium phosphate buffer, pH 6.8 at 15°C. Total volume of reaction mixture was 1.50 ml.

molecular ion at m/e 600 is consistent with a molecular composition $C_{21}H_{33}O_3(OSi(CH_3)_3)_3$. Fragments, with their probable origins indicated, appear at m/e 585 (M-15; loss of $\cdot CH_3$), 510 (M-90; loss of Me_3SiOH), 495 (M-(90+15); loss of Me_3SiOH and $\cdot CH_3$), 479 (M-(90+31); loss of Me_3SiOH and $\cdot OCH_3$), 459 (M-141; loss of $\cdot CH_2-CH=CH-(CH_2)_3-CO_2CH_3$), 439 (M-(90+71); loss of Me_3SiOH and C_5H_{11}), 420 (M-(2x90)), 369 (M-(141+90)), 357 (M-243; loss of $Me_3SiOCHCH_2CH=CH(CH_2)_3CO_2CH_3$), 337 (M-(173+90); loss of $Me_3SiOCH-(CH_2)_4CH_3$), 279 (M-141+(2x90)), 267 (M-(243+90)), 243 ($Me_3SiOCHCH_2CH=CH(CH_2)_3CO_2CH_3$), 211 (243-32), 199 ($CH=CH-CH(OSi(CH_3)_3)(CH_2)_4CH_3$), 173 ($Me_3SiOCH(CH_2)_4CH_3$).

Similar treatment of 8,11,14-eicosatrienoic acid and 6,9,12-linolenic acid (γ-linolenic acid) yielded fragmentation patterns consistent with the corresponding analogs. The mass spectrum produced from the reaction product derived from 6,9,12-linolenic acid lacked a molecular ion (m/e 574), but did contain fragments at m/e 484 (M-90), 413 (M-(90+71)), 394 (M-(2x90)), 369

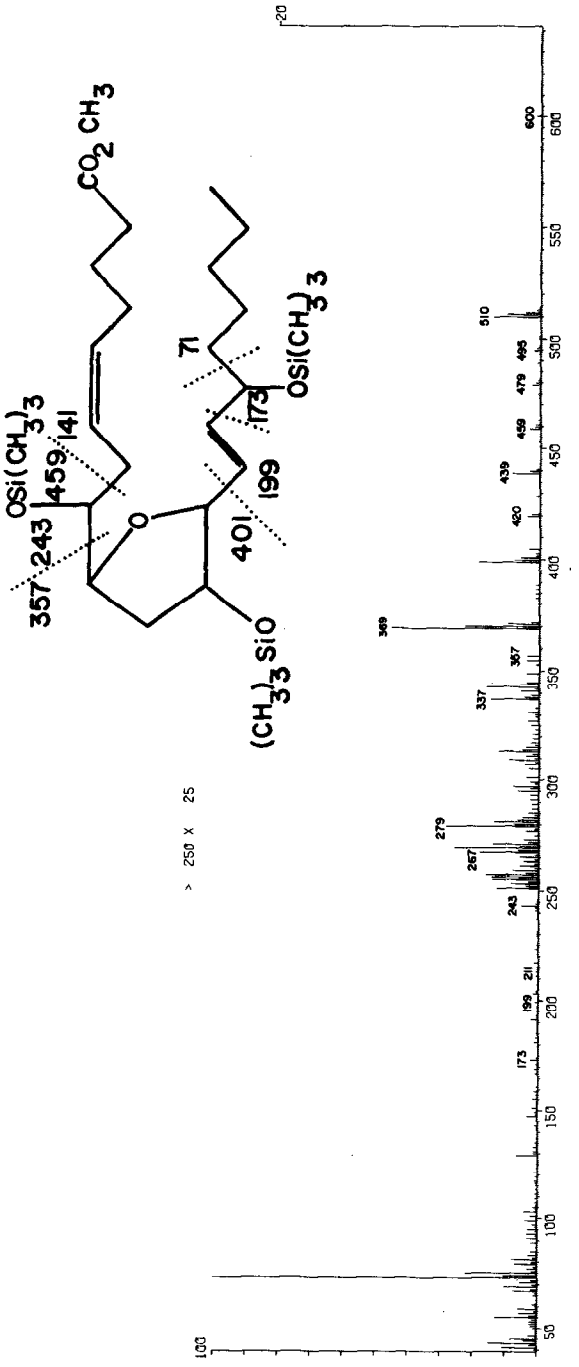

Fig. 2. Mass spectrum of the Peak II material eluted from the 3% SE-30 gas chromatography column, Figure 2.

(M-(115+90); loss of $\cdot(CH_2)_4COOCH_3$ and Me_3SiOH), 357 (M-(217; loss of $Me_3SiOCH-(CH_2)_4COOCH_3$), 311 (M-(90+173)), 267 (M-(217+90)), 217, 199, 173 and 129 ($(CH_3)_3SiOCHCH_2-CH$). When 9,12,15-linolenic acid (α-linolenic acid) was incubated with lipoxygenase-2 in a manner identical to that for the above three substrates no compound analogous to that of identified polyhydroxy cyclic ethers was detected.

The corresponding product obtained from 8,11,14-eicosatrienoic acid was examined with an LKB 9000 mass spectrometer employing a PDP 11 data system. The product gave a molecular ion two mass units greater than the arachidonic acid product. The fragmentation patterns of the two products were essentially similar except that the fragments from the former, which contained both carbons 5 and 6, were two mass units heavier.

The band of material scraped from the silica gel plate containing the polyhydroxy cyclic ether obtained from arachidonic acid accounted for approximately 35% of the total radioactivity. This material separated into two peaks upon injection into the gas chromatograph, the second of which, containing the polyhydroxy cyclic ether, accounted for 40% of the total material. The polyhydroxy cyclic ether appears, therefore, to be produced in an approximately 14% yield under the incubation conditions employed.

The 9(12)-oxy-8,11,15-trihydroxyeicosa-5,13-dienoic acid which we have obtained from arachidonic acid was isolated and identified following treatment of the reaction products with the reducing agent sodium dithionite, and may have therefore been formed from the corresponding hydroperoxide or endoperoxide. It should be recognized that the mass spectral fragmentation pattern does not distinguish stereoisomers.

Pace-Asciak has more recently designated the compound, which is both a trihydroxy and furan derivative, as ThF_2 and called attention to its structural resemblance to thromboxane B_2 (9). The 9(12)-oxy-8,11,15-trihydroxyeicosa-5,13-dienoic acid was originally obtained by Pace-Asciak in a 1% yield, using the sheep seminal vesicle preparation and appears not be have been tested

for physiological activity. The present work indicates the route to an improved synthesis of the compound and reveals a new and unusual specificity of a lipoxygenase.

ACKNOWLEDGEMENTS

We are indebted to Betty Hess for skillful technical assistance in the preparation of the enzymes, to Karen Gooding for providing some of the mass spectra and to Dr. J. E. Pike of the Upjohn Company for samples of authentic prostaglandins.

REFERENCES

1. Pace-Asciak, C. (1971) Biochemistry, 10, 3664-3669.
2. Hamberg, M. and Samuelsson, B. (1967) J. Biol. Chem., 242, 5329-5335.
3. Bild, G. S., Ramadoss, C. S. and Axelrod, B. (1977) Arch. Biochem. Biophys., 184, 36-41.
4. Christopher, J. P., Pistorius, E. K., Regnier, F. E. and Axelrod, B. (1972) Biochim. Biophys. Acta, 289, 82-87.
5. Christopher, J., Pistorius, E., and Axelrod, B. (1970) Biochim. Biophys. Acta, 198, 12-19.
6. Bild, G. S., Ramadoss, C. S., Lim, S. and Axelrod, B. (1977) Biochem. Biophys. Res. Comm., 74, 949-954.
7. Nugteren, D. H. and Hazelhof, E. (1973) Biochim. Biophys. Acta, 326, 448-461.
8. Bild, B., Bhat, S. G., Ramadoss, C. S., and Axelrod, B. (1978) J. Biol. Chem. (in press).
9. Pace-Asciak, C. R. (1977) Prostaglandins, 13, 811-817.