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ISOLATION AND STRUCTURE ELUCIDATION OF PLASMALOPENTAENE-12, THE BIOLOGICAL PRECURSOR OF FECAPENTAENE-12.

David G.I. Kingston,* C.-Y. Duh, T. Piccariello, R. F. Keyes.

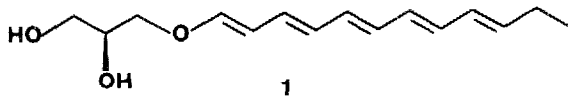
Department of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0212

R. L. Van Tassell, and T. D. Wilkins.*

Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0305

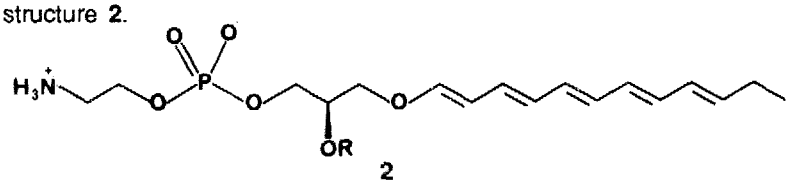
Summary: *The biological precursor of the potent fecal mutagen fecapentaene-12 has been purified and its structure elucidated as the polyunsaturated plasmalogen* ***2****. The occurrence of* ***2*** *in germ-free animals indicates that it is a new mammalian product.*

The potent mutagen fecapentaene-12 was first detected in human feces in 1977,[1] but its structure was not elucidated as **1** until 1982[2,3] due to its instability and the difficulty of its isolation from a complex biological matrix.[4] One fact that materially assisted the isolation was the observation that the yield of fecapentaene-12 could be significantly enhanced by incubating fresh feces prior to



1

isolation;[5] it was found that fecapentaene-12 production was promoted by several species of the common intestinal anaerobes *Bacteroides* and that production was greatly enhanced by bile.[6] These findings indicate that a precursor or precursors of unknown structure must be present in human feces. We now report that these precursors represent a new class of ethanolamine phospholipids with the general structure **2**.



2

R = $CH_3(CH_2)_{16}CO$, $CH_3(CH_2)_7CH{=}CH(CH_2)_7CO$,
$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH)_7CO$, or $CH_3(CH_2)_{14}CO$

Precursors were isolated from freeze-dried feces of a mutagen producer as previously described,[7] using a combination of sequential solvent extractions, acetone precipitation, and HPLC on two different solvent systems. Final chromatography on an amine column with chloroform: methanol:

ammonium hydroxide, 65:35:1, yielded precursors as a symmetrical peak with no impurities detectable by UV absorption at 365 nm. The purified precursors were converted to fecapentaene-12 on incubation with whole cells or cell-free extracts of *Bacteroides thetaiotaomicron* VPI 5482.

The precursors showed an identical UV absorption spectrum to that of fecapentaene-12,[2] thus confirming the presence of the pentaenyl ether moiety. Hydrolysis of the precursors with lipases and phospholipases was initially unsuccessful, but incorporation of the precursors into liposomes or micelles allowed further hydrolysis by a mixture of *Candida* lipase and *B. cereus* phospholipase C, yielding fecapentaene-12.[7] Neither enzyme alone was able to convert the precursors to fecapentaene-12, and this evidence thus identifies the precursors as monoacyl mono(pentaenyl) phospholipids related to the plasmalogens.

The nature of the acyl groups at the sn-2 position of glycerol was determined by methylation analysis. Deacylation of **2** followed by methylation of the resulting fatty acids with diazomethane yielded a mixture of esters which was subjected to capillary GC[8] and GC-MS[9] analysis. GC-MS showed the presence of two major and two minor esters; the major esters had mass spectra consistent with their assignment as methyl hexadecanoate[10] and methyl octadecanoate.[11] Capillary GC comparison with authentic samples confirmed the identity of these two esters.[12] The two minor esters eluted just before methyl octadecanoate and were not observed if the precursor was hydrogenated (Pt/H_2) prior to hydrolysis and methylation. The later eluting of the two was identified as methyl 9-octadecenoate by its mass spectrum[13] and by cochromatography with authentic material,[8,12] and the earlier eluting compound was tentatively identified as methyl 9,12-octadecadienoate by cochromatography with an authentic sample.[8,12,14]

The identity of the group at the sn-3 position of the glycerol unit was determined by a modification of the procedure of Clarke and Dawson.[15] The precursor was hydrolysed with $HgCl_2$ in aqueous methanol, and the resulting monoacyl phospholipid was extracted and deacylated with methylamine. After extraction with butanol/light petroleum/ethyl formate, the aqueous residue was subjected to descending paper chromatography in the solvent system of phenol saturated with H_2O:CH_3COOH:C_2H_5OH, 50:5:6.[16] The resulting glycerylphosphoryl derivative co-chromatographed with authentic 3-glycerophosphoryl ethanolamine, and was well separated from other possible products such as 3-glycerophosphoryl choline or 3-glycerophosphoryl serine.

One concern throughout this work was for the purity of the precursor preparation, since it was isolated by HPLC with UV detection and small amounts of saturated phospholipids might conceivably have co-chromatographed with the precursor. We thus carried out a control experiment in which the material eluting from the final HPLC separation immediately before and immediately after the UV-absorbing precursor was collected and subjected to the same analysis as described above. The fraction eluting immediately prior to the precursor was clean, but the fraction that eluted immediately after the precursor contained modest amounts, averaging 10%, of the same acids as the precursor. This indicates some contamination of the sample, presumably with normal plasmalogens. Due to the

instability of the precursor, it has not yet proved possible to obtain a sample free of such contamination, but the modest amounts of contaminating material do not invalidate our basic findings.

These results thus establish the structure of the precursor as the new ethanolamine phospholipid **2**, for which we propose the name plasmalopentaene-12. The acyl group at the 2 position is estimated to be made up of 18:0, 18:1, 18:2, and 16:0 fatty acids in the approximate ratio 28:19:31:22, as determined by GC analysis after correction for contaminating material. The stereochemistry at the 2-position is confirmed as (R) by the conversion of **2** into fecapentaene-12 (**1**) of known absolute stereochemistry,[2] and the positions of attachment of the acyl and phosphoethanolamine groups are confirmed both by the identification of 3-glycerophosphoryl ethanolamine and by the conversion of **2** into **1** by the lipase/phospholipase C mixture described earlier.[7]

The finding that the precursors of fecapentaene-12 are ether phospholipids related to the plasmalogens raises significant questions concerning their origin and function. As far as their origin is concerned, recent studies show that plasmalopentaenes are present in germ-free pigs,[17] indicating that they are produced by the host.[18] The plasmalopentaenes are thus *new mammalian metabolites*. Their biological function is as yet unknown, other than serving as precursors to fecapentaene-12. However, ether lipids have been shown to have a variety of important biological activities in the cancer area,[19] and it seems probable that the plasmalopentaenes will also transpire to have a significant activity in this area. One attractive possibility is that the plasmalopentaenes serve as antioxidants to protect the colon from injury by oxygen radicals.

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4. The mutagen as isolated is actually a mixture of E and Z isomers. The form shown is one of the major isomers present in the mixture: Kingston, D. G. I.; Piccariello, T.; Duh, C.-Y.; Govindan, S. V.; Wilkins, T. D.; Van Tassell; R. L. Van der Gen, A.; de Wit, P. P.; Van der Steeg, M. *J. Nat. Prod.* **1988**, *51*, 176.

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8. G. C. conditions: 30mx0.25mm RSL-200 column (Alltech), 0.9mL/min He flow, split ratio 50:1, FID detector, program 150-300°C at 15°C/min.

9. GC-MS conditions: 25m x 0.32 mm HP5 column, program 150-295° at 10°C/min, HP5740 gas chromatograph. VG 7070E-HF mass spectrometer, 70ev, 200°C source temperature, scan *m/z* 50-450 at 2 sec/decade.

10. Mass spectrum (*m/z*, relative intensity): 270(10,M^+), 241(2), 239(4), 227(4), 143(8), 129(5), 101(5), 98(3), 97(6), 87(88), 83(11), 75(32), 74(100), 69(20), 59(13), 57(28), 55(40).

11. Mass spectrum (*m/z*, relative intensity): 298(32,M^+), 269(3), 267(11), 255(12), 143(21), 129(12), 101(11), 97(18), 86(18), 87(95), 83(24), 75(72), 74(100), 71(22), 69(48), 59(25), 57(68), 55(78).

12. Lipid standards were purchased from Sigma Chemical Co., St. Louis, Missouri.

13. Mass spectrum (*m/z*, relative intensity): 296(4,M^+), 265(13), 264(22), 222(11), 180(7), 125(7), 124(8), 123(11), 111(15), 110(15), 109(12), 101(6), 98(25), 97(40), 96(32), 95(22), 87(43), 84(33), 83(55), 82(17), 81(24), 74(68), 70(20), 69(79), 68(20), 67(30), 59(16), 57(24), 56(23), 55(100).

14. The mass spectrum of this minor compound indicated it to be a mixture of methyl 9-octadecenoate and methyl 9,12-octadecadienoate, presumably due to incomplete separation on the GC column.

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18. The possibility that the precursors are present in the feed was excluded by direct analysis and by the fact that ether lipids are only minor constituents of animal feed: Mangold, H. K. in *Ether Lipids. Biochemical and Biomedical Aspects.* (Mangold, H. K.; Pattauf, F., eds.) pp 231-238, Academic Press, New York.

19. See, for example, the symposium on *Ether Lipids in Oncology,* Gottingen, FRG, 1986, published in *Lipids* **1987**, 22, 787-974.

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