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Synthesis of Sulfur-Substituted Phosphatidylethanolamines and Inhibition of Protozoan Cyclopropane Fatty Acid Synthase

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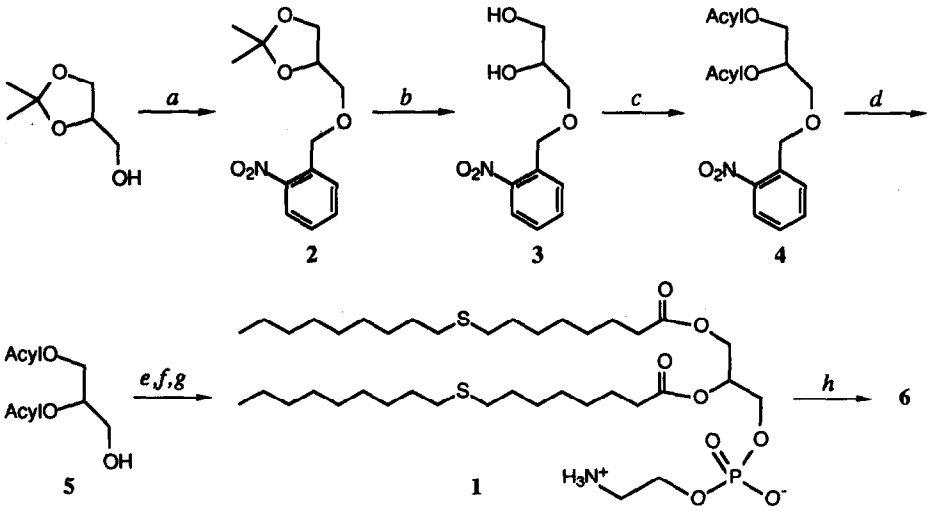
Abstract: A phosphatidylethanolamine (1) containing two 9-thiastearyl groups was prepared by chemical synthesis. This compound and its methylsulfonium derivative were found to be inhibitors of the cyclopropane fatty acid synthase from the parasitic protozoan Crithidia fasciculata.

Dihydrosterculic acid (9,10-methyleneoctadecanoic acid) is a widely distributed cyclopropane fatty acid found in the phospholipids of various bacteria, plants, and parasitic protozoa.[1-3] In recent years we have shown that sulfur-substituted derivatives of stearic acid are potent inhibitors of dihydrosterculic acid biosynthesis in trypanosomatid protozoa, including many pathogenic strains, and that the same compounds inhibit the growth of these organisms at comparable concentrations.[4-6] Dihydrosterculic acid is formed by an S-adenosylmethionine-dependent methylenation of oleyl groups in the 2-position of phosphatidylethanolamines (PEs),[1] and our working hypothesis has been that the inhibitory thiastearic acids are incorporated into the protozoan PEs, and the resulting sulfur-substituted PEs are the actual inhibitors of the cyclopropane fatty acid synthase.[4] We report herein the synthesis of one such sulfur-substituted PE and its inhibition of cyclopropane fatty acid synthase partially purified from the parasitic protozoan *Crithidia fasciculata*.

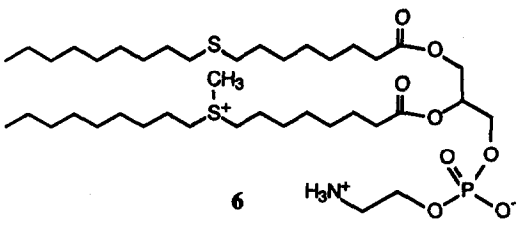
The preparation of bis(thiastearyl) phosphatidylethanolamine **1** is illustrated below. The first stages of the synthesis are patterned after the method of Bruzik et al.[7] except that the photochemically removable 2-nitrobenzyl protecting group was employed (instead of a benzyl group) to avoid the need for catalytic hydrogenation in the presence of thioethers. Thus the fully protected glycerol **2** was formed by alkylation of commercial 2,2-dimethyl-1,3-dioxolane-4-methanol with 2-nitrobenzyl bromide, and then the isopropylidene group was removed with acid to give diol **3**. Acylation of **3** with two equivalents of 9-thiastearyl chloride gave **4**, and photochemical cleavage of the nitrobenzyl group yielded the diacylglycerol **5**. All numbered intermediates were characterized by their ^{1}H and ^{13}C NMR spectra and low and high resolution mass spectra.[8-11] Our initial attempts to convert **5** to the desired bis(thiastearyl) PE **1** gave only complex mixtures or material of inferior quality. Ultimately, however, we found that the simple sequence of Eibl[12] followed by one recrystallization of the product from methylene chloride provided **1** in high purity.[13]



Scheme 1. Conditions: (a) n-BuLi/THF; 2-nitrobenzyl bromide; 40% yield; (b) 10% aqueous HOAc/60 °C; 70%; (c) 9-thiastearyl chloride/pyridine; 90%; (d) *hν*/benzene; 55%; (e) $POCl_3/C_2HCl_3$; (f) ethanolamine/Et_3N/THF; (g) 10:4:1 i-PrOH:water:HOAc; 40% overall e-g; (h) $CH_3I/CHCl_3$/HCl.

Cyclopropane fatty acid (CFA) synthase was partially purified from *C. fasciculata* by using Method A of Taylor and Cronan (who described the purification of this enzyme from *E. coli*).[14] The resulting lipid-free preparation must be reconstituted with phospholipid vesicles to obtain active enzyme. Our most consistent results were obtained with vesicles containing phosphatidylcholine (PC), dioleyl phosphatidylethanolamine (OPE), and distearyl phosphatidylethanolamine (SPE) in a 4:2:3 ratio. The OPE is the olefinic substrate for the enzyme, and various concentrations of the inhibitor were introduced by substituting all or part of the SPE with **1** during the reconstitution procedure. The enzyme was assayed by the method of Taylor and Cronan,[14] which monitors the incorporation of tritium from [*methyl*-^{3}H]-*S*-adenosyl-L-methionine (SAM) into the phospholipid, except that the assay was conducted at 26 °C (the growth temperature of *C. fasciculata*) rather than 37 °C. In the standard assay mixture the SAM concentration was 2.0 mM, the total phospholipid concentration was 2.0 mM, and the inhibitor concentration (a part of the total phospholipid) was varied from 0 to 0.67 mM. The assay was linear for at least 90 minutes. Under these conditions, compound **1** gave 30-50% inhibition of the CFA synthase at 0.67 mM, and the I_{50} for this compound was estimated to be 0.79 ± 0.20 mM.[15] Interestingly, when CFA synthase was preincubated for 40 minutes with inhibitor-containing phospholipid in the presence of unlabeled SAM before initiating the radioassay, the degree of inhibition was roughly twice as great. Preincubation with the standard phospholipid had no effect on enzyme activity.

We have previously proposed[4] that inhibition of dihydrosterculic acid biosynthesis by various thiastearates is due to the formation of thiastearyl PEs such as **1**, followed by methylation of **1** by CFA synthase to give the sulfonium compound **6**. This species resembles the presumed cationic intermediate in the normal methylenation reaction (**7**)[16] and should bind tightly to the enzyme active site. Ator et al.[17] have discussed related mechanisms



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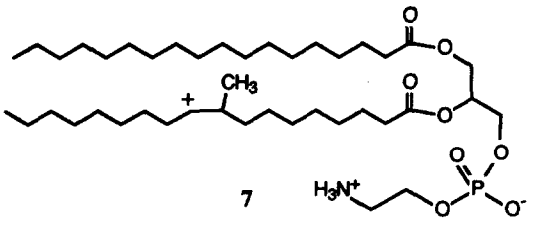

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of inhibition of steroidal SAM-dependent methyltransferases by thioethers, and other sulfonium compounds have been shown to be potent inhibitors of SAM-dependent methyltransferases.[17,18] Thus we prepared compound **6**[19] by treatment of **1** with methyl iodide in the presence of HCl (to prevent possible methylation of the amine and phosphate). Compound **6** is a more effective inhibitor of CFA synthase than **1**, and the I_{50} was determined to be 0.47 ± 0.09 mM.[15] However, compound **6** is undoubtedly a mixture of isomers, probably including the two possible sulfonium regioisomers (and four diastereomers) that may result from methylation of **1** at sulfur, and it is unlikely that all of these species are inhibitors of CFA synthase. Thus the active component(s) may be a much more potent inhibitor than **1**.

The precise mechanisms of inhibition of CFA synthase by compounds **1** and **6** remain to be elucidated. The finding that preincubation of **1** with the enzyme gives greater inhibition suggests the possibility of slow-binding inhibition or mechanism-based inactivation, but a more precise method for the assay of CFA synthase, and possibly also enzyme preparations of higher purity, will be required to address this issue.

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8. For **2**: ^{1}H NMR ($CDCl_3$, 270 MHz) δ 1.37 (s, 3H), 1.43 (s, 3H), 3.63 (m, 2H), 3.78 (dd, 1H, J = 8, 6 Hz), 4.08 (dd, 1H, J = 8, 6 Hz), 4.35 (quintet, 1H, J = 6 Hz), 4.94 (s, 2H), 7.43 (dd, 1H, J = 8, 8 Hz), 7.63 (dd, 1H, J = 8, 8 Hz), 7.78 (d, 1H, J = 8 Hz), 8.03 (d, 1H, J = 8 Hz); ^{13}C NMR ($CDCl_3$) δ 25.3, 26.7, 66.6, 70.0, 72.1, 74.6, 109.5, 124.6, 127.9, 128.7, 133.6, 134.8, 147.3; MS, *m/z* 267 (M^+, 2), 252 (54, M-CH_3), 136 (86), 101 (100). Exact mass 252.0883 (M-CH_3 ion), calcd for $C_{12}H_{14}NO_5$ 252.0872.
9. For **3**: ^{1}H NMR ($CDCl_3$, 270 MHz) δ 3.68 (m, 4H), 3.97 (tt, 1H, J = 6, 4 Hz), 4.97 (s, 2H), 7.46 (ddd, 1H, J = 8, 8, 1 Hz), 7.65 (ddd, 1H, J = 8, 8, 1 Hz), 7.72 (d, 1H, J = 8 Hz), 8.04 (dd, 1H, J = 8, 1 Hz);

^{13}C NMR ($CDCl_3$) δ 63.8, 70.0, 70.8, 72.4, 124.6, 128.2, 128.7, 133.5, 134.2, 147.4; MS, *m/z* 227 (M^+, 1.5), 136 (100), 120 (93), 92 (43), 78 (72). Exact mass 227.0754, calcd for $C_{10}H_{13}NO_5$ 227.0794.

10. For **4**: ^1H NMR ($CDCl_3$, 270 MHz) δ 0.85 (t, 6H, J = 6 Hz), 1.24 (m, 36H), 1.54 (m, 12H), 2.29 (t, 2H, J = 8 Hz), 2.31 (t, 2H, J = 8 Hz), 2.46 (overlapping t's, 8H), 3.71 (m, 2H), 4.19 (dd, 1H, J = 12, 6 Hz), 4.35 (dd, 1H, J = 12, 4 Hz), 4.89 (d, 1H, J = 15 Hz), 4.91(d, 1H, J = 15 Hz), 5.27 (quintet, 1H, J = 6 Hz), 7.42 (ddd, 1H, J = 8, 8, 1 Hz), 7.63 (ddd, 1H, J = 8, 8, 1 Hz), 7.73 (dd, 1H, J = 8, 1 Hz), 8.04 (dd, 1H, J = 8, 1 Hz), ^{13}C NMR ($CDCl_3$) δ 14.0, 22.6, 24.7, 24.8, 28.7, 28.81, 28.87, 28.92, 29.2, 29.4, 29.6, 29.7, 31.8, 32.0, 32.2, 33.9, 34.2, 62.4, 69.5, 69.9, 124.6, 128.0, 128.4, 133.6, 134.5, 147.0, 172.9, 173.2; MS, *m/z* 795 (M^+, 1), 643 (M-$OCH_2C_6H_4NO_2$, 10) 533 (33), 359 (34), 285 (100), 159 (45), 136 (51). Exact mass 795.5152, calcd for $C_{44}H_{77}NO_7S_2$ 795.5141.
11. For **5**: ^1H NMR ($CDCl_3$, 500 MHz) δ 0.87 (t, 6H, J = 7 Hz), 1.26 (m, 36H), 1.60 (m, 12H), 2.32 (t, 2H, J = 8 Hz), 2.34 (t, 2H, J = 8 Hz), 2.49 (t, 8H, J = 7 Hz), 3.71 (m, 2H), 4.22 (dd, 1H, J = 12, 6 Hz), 4.31 (dd, 1H, J = 12, 4 Hz), 5.08 (quintet, 1H, J = 5 Hz), ^{13}C NMR ($CDCl_3$) δ 14.1, 22.6, 24.7, 24.8, 28.7, 28.8, 29.2, 29.5, 29.6, 29.7, 31.8, 32.1, 32.2, 34.0, 34.2, 61.5, 62.0, 72.1, 173.2, 173.6; MS, *m/z* 660 (M^+, 11), 533 (67), 483 (16), 359 (23), 302 (38), 285 (100), 199 (24), 159 (55). Exact mass 660.4800, calcd for $C_{37}H_{72}O_5S_2$ 660.4821.
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13. For **1**: ^1H NMR ($CDCl_3$ and CD_3OD, 500 MHz) δ 0.85 (t, 6H, J = 7 Hz), 1.24 (m, 36H), 1.56 (m, 12H), 2.27 (t, 2H, J = 8 Hz), 2.29 (t, 2H, J = 8 Hz), 2.47 (t, 8H, J = 7 Hz), 3.12 (br s, 2H), 3.93 (t, 2H, J = 6 Hz), 4.06 (br s, 2H), 4.12 (dd, 1H, J = 12, 7 Hz), 4.36 (dd, 1H, J = 12, 4 Hz), 5.18 (m, 1H); ^{13}C NMR ($CDCl_3$ and CD_3OD) δ 13.7, 22.4, 24.5, 28.5, 28.65, 28.71, 29.0, 29.2, 29.35, 29.43, 31.6, 31.8, 31.9, 33.7, 33.9, 40.3, 61.5, 62.3, 63.6, 70.0, 173.2, 173.5. Analysis: C, 59.3; H, 9.6. Calcd for $C_{39}H_{78}NO_8PS_2$: C, 59.7; H, 10.0.
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15. Molar concentrations have limited meaning when one refers to suspensions of phospholipid vesicles. It may be better to specify the per cent of total phospholipid; in the present assays, 0.79 mM corresponds to 39.5% of the total phospholipid, and 0.47 mM to 23.5%.
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19. For **6**: ^1H NMR ($CDCl_3$, 270 MHz) δ 0.86 (t, 6H, J = 6 Hz), 1.2-1.6 (m, 44H), 1.82 (m, 6H), 2.31 (m, 3H), 2.48 (t, 1H, J = 7 Hz), [overlapping 3.20 (m) and 3.24 (s, S-CH_3), 7H], 3.74 (m, 7H), 4.13 (m, 2H), 4.36 (m, 1H), 5.19 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 14.0, 22.5, 22.7, 24.2, 24.4, 24.5, 28.0, 28.3, 28.6, 28.7, 29.0, 29.1, 29.2, 29.4, 29.6, 29.7, 31.7, 31.8, 32.1, 33.9, 41.1, 41.3, 62.3, 64.0, 70.2, 172.9, 173.2.

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