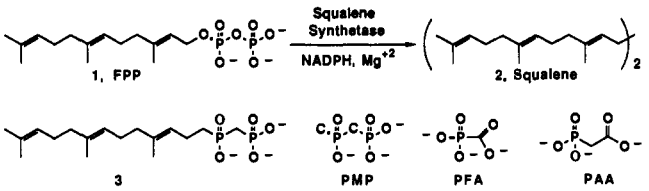
Communications to the Editor

Isoprenyl Phosphinylformates: New Inhibitors of Squalene Synthetase[1]

We report herein a new class of squalene synthetase inhibitors which exploits the phosphinylformate moiety as a surrogate for the allylic diphosphate of farnesyl diphosphate (FPP, 1). Members of this class are the first inhibitors of squalene synthetase shown to block cholesterol biosynthesis in whole cells.

Squalene synthetase is a microsomal protein that catalyzes the reductive dimerization of FPP to squalene (2).[2] This enzyme occupies a unique and strategic site at the final branch point of the cholesterol biosynthetic pathway. We have been pursuing the design, synthesis, and biological evaluation of squalene synthetase inhibitors with the goal of discovering a novel agent for the treatment of hypercholesterolemia.[1,3] Selective inhibition of this enzyme should not directly interfere with essential side pathways to the non-sterol isoprene products (e.g. dolichol, Coenzyme Q_{10}, Heme A, isopentenyl *t*-RNA, isoprenylated proteins).[4]



In the design of inhibitors based on the structure of FPP, pharmacologically stable surrogates for the allylic diphosphate are required. This portion of FPP is crucial for enzyme–substrate binding, presumably via ionic interactions, but is both chemically (high and low pH) and biologically (phosphatases) labile. Additionally, we were concerned that a highly charged molecule resembling FPP might have difficulty penetrating cell membranes. We reported previously that **3** and related compounds were inhibitors of squalene synthetase, demonstrating that the (phosphinylmethyl)phosphonate (PMP) was an effective surrogate for the diphosphate.[3] Although **3** inhibited rat liver microsomal squalene synthetase, it did not affect cholesterol biosynthesis from [^{14}C]acetate in freshly isolated rat hepatocytes[5] (see Table I). We assumed that the lack of activity of **3** in whole hepatocytes was a result of its charge, and were encouraged to search for a new diphosphate surrogate bearing less overall charge.

Table I. Biological Activity of Squalene Synthetase Inhibitors

	I_{50}, μM		
compd	squalene synthetase[3a]	rat hepatocytes[5]	skin fibroblasts[14]
3	31.5	inactive @ 200 μM	inactive @ 100 μM
4a	100	5.7	inactive @ 30 μM
4b	31.8	30	inactive @ 300 μM
4c	−38% @ 300 μM	not determined	not determined
4d	112	24	2.2
5a	8.7	6	inactive @ 15 μM
5b	41.6	not determined	not determined
5d	106	9	12
6	197	not determined	not determined
7a	−40% @ 100 μM	71	inactive @ 100 μM
7b	86	273	not determined

The antiviral agent phosphonoformic acid (PFA) inhibits nucleotide polymerases by binding at the site normally occupied by inorganic pyrophosphate.[6] In this example, the phosphinylformate moiety is serving as a useful diphosphate surrogate for this group of enzymes. We set out to determine whether novel inhibitors of squalene synthetase could be created by replacing one of the charged oxygens of PFA with a C- or O-linked isoprenyl group. The resulting FPP analogues, **4a–c** and **5a,b**, respectively, possess one less ionizable acid function than either FPP or the PMP inhibitors.

Synthetic routes to the phosphinylformate inhibitors are outlined in Scheme I.[7] In order to prepare the C-linked analogues, an expedient route was developed to convert alkyl halides **8a–c**[8] to phosphinylformate triesters **10a–c** without the isolation of intermediates. The alkyl halides were converted to the corresponding lithium (for **8a** and **8c**) or Grignard (for **8b**) reagents, which were reacted with

(1) These results were presented in part at the Xth International Symposium on Drugs Affecting Lipid Metabolism, Houston, TX, November 8–11, 1989 and the 199th National Meeting of the American Chemical Society, Boston, MA, April 22–27, 1990. For the proceedings of the former symposium, see: Biller, S. A.; Forster, C.; Gordon, E. M.; Harrity, T.; Rich, L. C.; Marretta, J.; Scott, W. A.; Ciosek, C. P., Jr. In *Drugs Affecting Lipid Metabolism, X: Proceedings of the Xth International Symposium*; Gotto, A. M., Jr.; Smith, L. C., Eds.; Elsevier: Amsterdam, 1990; pp 213–216.

(2) Poulter, C. D.; Rilling, H. C. In *Biosynthesis of Isoprenoid Compounds*; Porter, J. W., Spurgeon, S. L., Eds.; Wiley: New York, 1981; Vol. 1, Chapter 8.

(3) (a) Biller, S. A.; Forster, C.; Gordon, E. M.; Harrity, T.; Scott, W. A.; Ciosek, C. P., Jr. *J. Med. Chem.* **1988**, *31*, 1869. (b) Biller, S. A.; Forster, C. *Tetrahedron* **1990**, *46*, 6645. (c) Related studies: McClard, R. W.; Fujita, T. S.; Stremler, K. E.; Poulter, C. D. *J. Am. Chem. Soc.* **1987**, *109*, 5544–5545.

(4) Recent studies suggest that toxicity in rabbits and some of the clinical side effects exhibited by the HMG-CoA reductase inhibitor Lovastatin may be due to the inhibition of certain non-sterol products of the isoprene pathway (e.g. Coenzyme Q_{10}): Kornbrust, D. J.; MacDonald, J. S.; Peter, C. P.; Duchai, D. M.; Stubbs, R. J.; Germershausen, J. I.; Alberts, A. W. *J. Pharmacol. Exp. Ther.* **1989**, *248*, 498. Willis, R. A.; Folkers, K.; Tucker, J. L.; Ye, C.-Q.; Xia, L.-J.; Tamagawa, H. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 8928. Folkers, K.; Langsjoen, P.; Willis, R.; Richardson, P.; Xia, L.-J.; Ye, C.-Q.; Tamagawa, H. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 8931. Tobert, J. A. U.S. Patent 4 929 437, 1990. Brown, M. S. U.S. Patent 4 933 165, 1990.

(5) Cells were isolated by a modification of: Berry, M. N. *Methods Enzymol.* **1974**, *32B*, 625. Cholesterol biosynthesis was measured by a modification of: Capuzzi, D. M.; Margolis, S. *Lipids* **1971**, *6*, 601.

(6) Öberg, B. *Pharmacol. Ther.* **1989**, *40*, 213. Hutchinson, D. W. *Antiviral Res.* **1985**, *5*, 193.

(7) All new compounds exhibited ^{1}H NMR, ^{13}C NMR, and infrared spectra, as well as mass spectra and/or combustion analysis, which were consistent with the assigned structure. The final target salts were purified by chromatography on CHP20P gel, as described in ref 3b.

(8) **8a** was prepared by the published method: Sandifer, R. M.; Thompson, M. D.; Gaughan, R. G.; Poulter, C. D. *J. Am. Chem. Soc.* **1982**, *104*, 7376. **8b** was prepared as described in ref 3b. **8c** was derived from farnesyl chloride by the following route: (a) $ClMgOCH_2CH_2CH_2MgCl$, HMPA, THF; (b) MsCl, Et_3N; (c) NaI, acetone.

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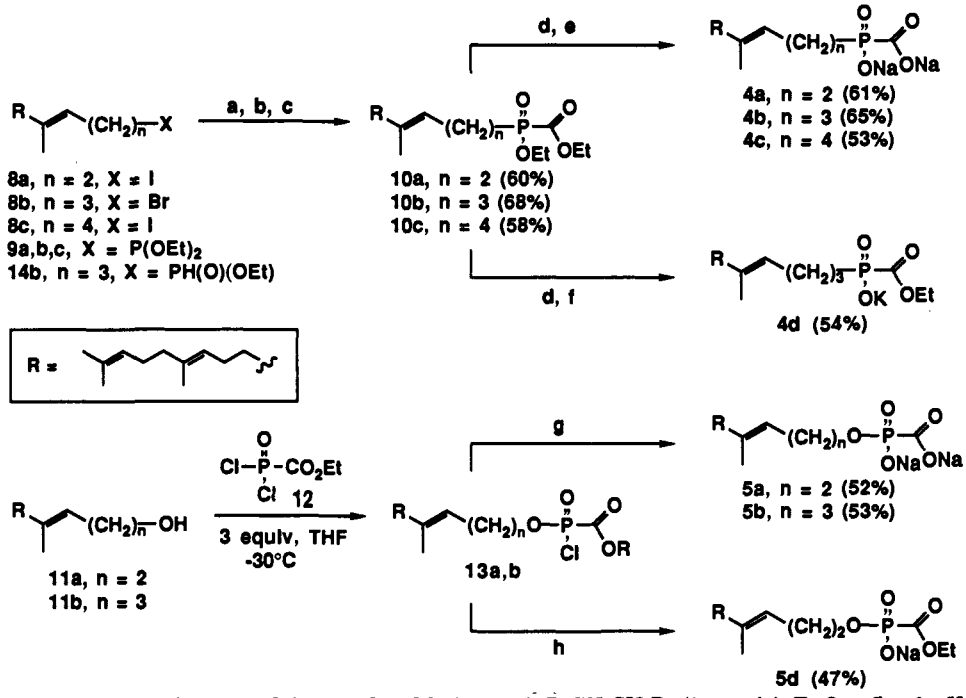
Scheme I[a,7]



[a] *t*-BuLi (2.2–2.4 equiv), pentane-ether, −78 °C for X = I, or Mg (2 equiv), $BrCH_2CH_2Br$ (0.1 equiv), Et_2O, reflux for X = Br; (b) $ClP(OEt)_2$ (2–2.5 equiv for X = Li, 1 equiv for X = MgBr); (c) $ClCO_2Et$ (10 equiv); (d) TMSI (2–2.5 equiv), 2,4,6-collidine (1 equiv), CH_2Cl_2, 0 °C; (e) 1 M NaOH (5–6 equiv), 65–80 °C; (f) Et_3N, H_2O quench; CH_2Cl_2/1 M HCl partition; 1 M KOH (1 equiv), MeOH; (g) 2 M NaOH (16 equiv), 0 °C to RT; (h) Et_2O/water partition; 1 M NaOH (1 equiv), EtOH, H_2O.

diethyl chlorophosphite to provide the intermediate phosphonous diesters **9a–c**.[9] Treatment of **9a–c** in situ with excess ethyl chloroformate gave **10a–c** in 58–68% overall yield.[10] Diesters **10a–c** were converted to the corresponding di-Na^+ salts **4a–c** by cleavage of the phosphorus ester with TMSI,[11] followed by hydrolysis of the carboxylate ester with excess NaOH. Selective cleavage of the phosphorus ester of **10b** and then careful neutralization of the monoacid with 1 equiv of KOH provided monoester **4d**. The O-linked derivatives were prepared by the addition of isoprenyl alcohols **11a,b**[12] to a solution of 3 equiv of dichloride **12**[13] in THF at −30 °C, thereby generating the phosphonyl monochlorides **13a,b**. The intermediate acid chlorides were either hydrolyzed with excess NaOH to provide disodium salts **5a,b** or subjected to aqueous workup followed by neutralization of the free acid with 1 equiv of NaOH to form monoester **5d**.

The final targets were evaluated for their ability to inhibit rat microsomal squalene synthetase,[1,3a] as well as their ability to block cholesterol biosynthesis from [^{14}C]acetate in freshly isolated rat hepatocytes[5] and in human skin fibroblasts.[14,15] Phosphonoformic acid (PFA) itself was

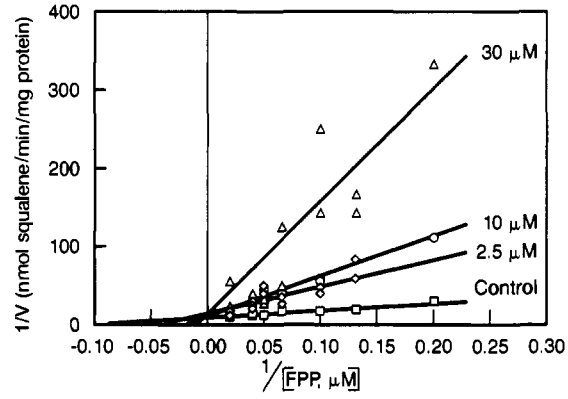


Figure 1. Lineweaver–Burk analysis of the inhibition of rat microsomal squalene synthetase by **5a**. Concentration of **5a**: 0 (□), 2.5 (◇), 10 (○), and 30 μM (△).

inactive as a squalene synthetase inhibitor at up to 1 mM. The C-linked phosphinylformate **4b**, which has the same overall length as both **3** and FPP, was found to be equipotent to **3** in the enzyme assay despite its lower negative charge (Table I). In concordance with our initial assumptions about charge and cell penetration, **4b** inhibited cholesterol biosynthesis from [^{14}C]acetate in rat hepato-

(9) Sander, M. *Chem. Ber.* **1960**, *93*, 1220. Kabachnik, M. I.; Tsvetkov, E. N.; Chang, C.-Y. *Doklady Akad. Nauk S.S.S.R.* **1959**, *125*, 1260.

(10) Issleib, K.; Stiebitz, B. *Synth. React. Inorg. Met.-Org. Chem.* **1986**, *16*, 1253.

(11) Zygmunt, J.; Kafarski, P.; Mastalerz, P. *Synthesis* **1978**, 609. Blackburn, G. M.; Ingleson, D. *J. Chem. Soc., Perkin Trans. 1* **1980**, 1150.

(12) **11a** was synthesized from farnesol according to: Leopold, E. J. *Org. Synth.* **1985**, *64*, 164. **11b** was prepared as described in ref 3b.

(13) Vaghefi, M. M.; McKernan, P. A.; Robins, R. K. *J. Med. Chem.* **1986**, *29*, 1389.

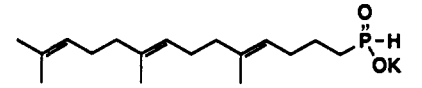
(14) A modification of: Faust, J. R.; Goldstein, J. L.; Brown, M. S. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 5018.

(15) It has been suggested that the selective inhibition of cholesterol biosynthesis in hepatic tissue, as opposed to extrahepatic tissue, may lead to clinical advantages: Karanewsky, D. S.; Badia, M. C.; Ciosek, C. P., Jr.; Robl, J. A.; Sofia, M. J.; Simpkins, L. M.; DeLange, B.; Harrity, T. W.; Biller, S. A.; Gordon, E. M. *J. Med. Chem.* **1990**, *33*, 2952. Parker, R. A.; Clark, R. W.; Sit, S. Y.; Lanier, T. L.; Grosso, R. A.; Wright, J. J. K. *J. Lipid Res.* **1990**, *31*, 1271. Koga, T.; Shimada, Y.; Kuroda, M.; Tsujita, Y.; Hasegawa, K.; Yamazaki, M. *Biochim. Biophys. Acta* **1990**, *1045*, 115. Mosley, S. T.; Kalinowski, S. S.; Schafer, B. L.; Tanaka, R. D. *J. Lipid Res.* **1989**, *30*, 1411. Germershausen, J. I.; Hunt, V. M.; Bostedor, R. G.; Bailey, P. J.; Karkas, J. D.; Alberts, A. W. *Biochem. Biophys. Res. Commun.* **1989**, *158*, 667.

cytes, whereas PMP 3 was inactive. Both decreasing and increasing the length of the $(CH_2)_n$ linker between the isoprenyl subunit and the diphosphate surrogate (**4a**, n = 2; **4c**, n = 4) led to a loss of potency in the enzyme assay. Introduction of an oxygen atom in the linker in the form of a phosphorus ester (**5a** and **5b**) resulted in more active enzyme inhibitors than the corresponding C-linked isosteres (**4b** and **4c**, respectively). The optimal overall chain length of both the C- and O-linked inhibitor series (**4b** and **5a**) corresponds to that which is isosteric to FPP. Phosphinylformate **5a** (I_{50} = 8.7 μM), the most potent enzyme inhibitor in this study, also exhibited commensurate activity in the rat hepatocyte assay (I_{50} = 6 μM).

The inhibition of microsomal squalene synthetase by **5a** (0–30 μM) was studied at increasing FPP (5–50 μM) and saturating NADPH (0.9 mM) concentrations. Double-reciprocal analysis of the kinetic data (Figure 1) indicates that **5a** is a competitive inhibitor with respect to FPP (K_i = 2.6 μM). In comparison, the K_i of **3** is 10 μM and the apparent K_m of FPP is 12.7 μM.[1,3a]

Carboxylate esters **4d** and **5d** are weak inhibitors of squalene synthetase compared to the corresponding salts **4b** and **5a**, but the salts and esters exhibited similar activity in the hepatocyte assay. We speculate that **4d** and **5d** serve as effective precursors to **4b** and **5a** in whole cells but not in the enzyme assay. In addition, **4d** and **5d** inhibited the conversion of [^{14}C]acetate to cholesterol in human skin fibroblasts under conditions where the salts are inactive, suggesting that the lipophilic esters can more readily enter this cell type.[15]



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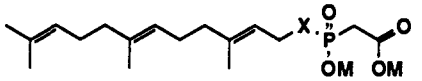

7a, X = CH_2, M = K
7b, X = O, M = Li

Phosphinylformates are unstable at low pH,[16] undergoing decarboxylation to the corresponding phosphonous acids (e.g. **4b** to **6**). We prepared **6** independently via the acid-catalyzed hydrolysis of **9b** to **14b** (78% from 8, n = 3, X = I) followed by treatment with KOH.[9] This material was found to be identical with that which is obtained upon reaction of **4b** with aqueous HCl. Phosphonous acid **6** is 6.2-fold less potent than **4b** in the squalene synthetase assay, suggesting that the activity of **4b** is not due to its conversion to **6**.

The phosphinylacetate isosteres **7a** and **7b**,[17] based on the related antiviral agent PAA,[6] are less potent squalene synthetase inhibitors than the corresponding phosphinylformates of the same chain length (**4b** and **5a**). For comparison, PAA is inactive in the squalene synthetase assay at up to 1 mM.

In summary, we have demonstrated that the phosphinylformate moiety is a novel, dianionic diphosphate surrogate for the construction of inhibitors of squalene synthetase. In addition, isoprenyl phosphinylformates and their esters are the first inhibitors of this enzyme to block the synthesis of cholesterol in whole cells. We anticipate that the phosphinylformate function, and the chemistry described herein, will find utility in the synthesis of analogues of other diphosphate-containing molecules of biological significance.[13,19]

Acknowledgment. We would like to thank the Bristol-Myers Squibb Analytical Chemistry Department for obtaining elemental analyses, mass spectra, IR spectra, and certain NMR spectra. In addition, we would like to acknowledge helpful discussions with Drs. Donald S. Karanewsky, William H. Koster, and William A. Scott.

Supplementary Material Available: Representative procedures for the synthesis of **4a** and **5a** are available (2 pages). Ordering information is given on any current masthead page.

(16) Warren, S.; Williams, M. R. *J. Chem. Soc. B* **1971**, 618.
(17) **7a** was prepared from phosphonyl chloride **5a** of ref 3b: (a) $LiCH_2CO_2$-*t*-Bu, THF, -78 °C; (b) KOH. **7b** was prepared by from farnesyl chloride: (a) $(n\text{-}Bu_4N^+)_2[O_3PCH_2CO_2Et]^{2-}$, CH_3CN;[18] (b) AG 50W-X8 (Li form); (c) LiOH.
(18) Davisson, V. J.; Woodside, A. B.; Neal, T. R.; Stremler, K. E.; Muehlbacher, M.; Poulter, C. D. *J. Org. Chem.* **1986**, *51*, 4768.
(19) Griengl, H.; Hayden, W.; Penn, G.; De Clercq, E.; Rosenwirth, B. *J. Med. Chem.* **1988**, *31*, 1831. Lambert, R. W.; Martin, J. A.; Thomas, G. J.; Duncan, I. B.; Hall, M. J.; Heimer, E. P. *J. Med. Chem.* **1989**, *32*, 367.

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Targeting Renal Dipeptidase (Dehydropeptidase I) for Inactivation by Mechanism-Based Inactivators

Renal dipeptidase (dehydropeptidase I, EC 3.4.13.11) is a zinc-containing hydrolytic enzyme that shows preference for dipeptide substrates with dehydro amino acids (e.g., 2-amidoacrylic acid derivatives) at the carboxy position; however it can accommodate substrates with both D or L amino acids at that position as well.[1] While the physiological function of this enzyme is not known presently, it has been implicated in metabolism of glutathione and its derivatives.[2] In addition, it is responsible for the hydrolytic scission of the lactam bond in carbapenems, potent broad-spectrum antibiotics that are resistant to the action of microbial β-lactamases.[3] Enzymic turnover of carbapenems in vivo poses a serious obstacle to clinical efficacy of these bactericidal agents; therefore specific inhibitors for this enzyme are widely sought.[4]

We have reported recently on the ability of carboxypeptidase A, a prototypic zinc protease, to carry out a deprotonation reaction that was exploited in inactivation of the enzyme.[5,6] We report here our studies of the first two mechanism-based inactivators designed specifically for porcine renal dipeptidase, which take advantage of the

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