Inhibition of Cholesterol Biosynthesis by Fluorinated Mevalonate Analogues†

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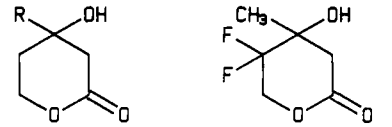
ABSTRACT: The conversion of mevalonate to cholesterol in rat liver homogenates (IC_{50} = 0.01–1.0 mM) is inhibited by 6- (I), 6,6-di- (II), and 6,6,6-trifluoromevalonate (III), as well as 4,4-difluoromevalonate (IV). Addition of compound I, III, or IV to rat liver homogenates results in the accumulation of 5-phospho- and 5-pyrophosphomevalonate. The conversion of isopentenyl pyrophosphate to cholesterol is not inhibited by the fluorinated analogues. It thus appears likely that the decarboxylation of mevalonate 5-pyrophosphate is inhibited. Rat liver homogenates catalyze the phosphorylation of I and III. The inhibition of the decarboxylation of mevalonate 5-pyrophosphate by I and III was demonstrated directly with partially purified decarboxylase. Compound I is a remarkably effective inhibitor of the decarboxylation (K_i = 10 nM). Similar results were reported by Nave et al. [Nave, J. F., d'Orchymont, H., Ducep, J. B., Piriou F., & Jung, M. J. (1985) *Biochem. J. 227*, 247]. It is likely that the phosphorylated or pyrophosphorylated forms of all inhibitors tested are responsible for inhibition. We also describe a chemical method for the synthesis of mevalonate 5-pyrophosphate.

We have previously shown that (*Z*)-3-(trifluoromethyl)-2-butenyl pyrophosphate is a very poor substrate for isopentenyl pyrophosphate isomerase and is also an inhibitor of the isomerization of isopentenyl pyrophosphate (Reardon & Abeles, 1986). 3-(Fluoromethyl)-2-butenyl pyrophosphate and 3-(fluoromethyl)-3-butenyl pyrophosphate are irreversible inactivators of isopentenyl pyrophosphate isomerase (Muehlbacher & Poulter, 1985). Both (*E*)-3-(trifluoromethyl)-2-butenyl pyrophosphate and (*Z*)-3-(trifluoromethyl)-2-butenyl pyrophosphate are very poor substrates for prenyltransferase (Poulter & Satterwhite, 1977). These fluorinated analogues of isopentenyl pyrophosphate and dimethylallyl pyrophosphate could be effective inhibitors of cholesterol biosynthesis. However, the pyrophosphate group would most likely prevent their uptake by intact cells where inhibition of cholesterol synthesis is of primary interest. We, therefore, tried to devise an approach that would allow introduction of these compounds into intact cells. On way of achieving this is by exposing the cell to mevalonate fluorinated at C-6. If these fluorinated mevalonate analogues undergo the same biochemical transformations as mevalonate normally does, then the desired fluorinated inhibitors would be generated by the cell (Figure 1). To test this hypothesis, we synthesized the fluorinated mevalonate analogues I–IV shown in Chart I. While this work was in progress Nave et al. (1985) reported that I inhibits cholesterol biosynthesis in liver homogenates. Their data showed that I was not transformed to 3-(fluoromethyl)-3-butenyl pyrophosphate. Inhibition of cholesterol biosynthesis most probably results from inhibition of mevalonate-5-pyrophosphate decarboxylase by the pyrophosphorylated form of I.

We now report our studies of the inhibition of cholesterol biosynthesis by compounds I–IV. These compounds inhibit cholesterol formation in rat liver homogenates. In all cases, the inhibitor's effect is probably due, at least in part, to the inhibition of mevalonate-5-pyrophosphate decarboxylase. We also show directly, with partially purified decarboxylase, that the phosphorylated and pyrophosphorylated forms of I and III are inhibitory.

Chart I



I R = CH_2F

II R = CHF_2

III R = CF_3

IV

Materials and Methods

Materials. Alkaline phosphatase, ATP,[1] NAD, NADH, and glucose 6-phosphate were purchased from Sigma Chemical Co. [4-^{14}C]Isopentenyl pyrophosphate, [2-^{14}C]mevalonate, [5-^{3}H]mevalonate, and sodium borotritide were pruchased from New England Nuclear. Crystalline orthophosphoric acid was purchased from Fluka Chemical Co. Bis(triethylammonium) phosphate was prepared according to the method of Cornforth and Popjak (1969). Tris(tetrabutylammonium) pyrophosphate was prepared according to the method of Davisson et al. (1985). Triethylammonium bicarbonate, TEAB, was prepared by passing CO_2 gas, from dye ice, through a gas dispersion frit into a cold, stirred 1 M solution of triethylamine until the pH was 7.5. The synthesis of isopentenyl pyrophosphate has been described previously (Reardon & Abeles, 1986). Farnesyl pyrophosphate was synthesized and purified by the procedure used for dimethylallyl pyrophosphate (Reardon & Abeles, 1986) and was further purified on XAD-2

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[1] Abbreviations: FPP, farnesyl pyrophosphate; IPP, isopentenyl pyrophosphate; ATP, adenosine triphosphate; NAD, nicotinamide adenine dinucleotide (oxidized); NADH, nicotinamide adenine dinucleotide (reduced); TEAB, triethylammonium bicarbonate; FPLC, fast protein liquid chromatography; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography; THF, tetrahydrofuran; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; P_i, inorganic phosphate; PEP, phosphoenolpyruvate.

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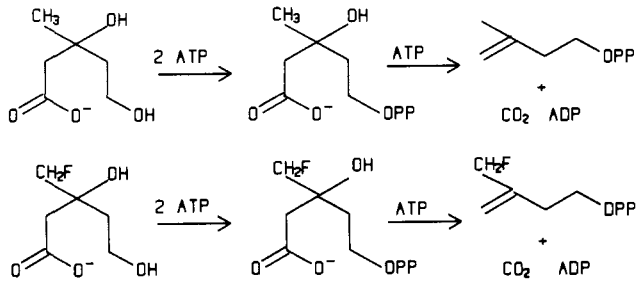

FIGURE 1: Proposed pathway for metabolism of 6-fluoromevalonate.

resin (Cornforth & Popjak, 1969). Mevalonate 5-phosphate was synthesized by the published procedure (Foote & Wold, 1963). 6-Fluoromevalonate (I), 6,6-difluoromevalonate (II), and 6,6,6-trifluoromevalonate (III) were synthesized by the method of Quistad and Cerf (1981). I was purified by flash chromatography on silica gel in ethyl acetate/ether, 2:1; II was purifed by flash chromatography on silica gel in ethyl acetate/hexanes, 2:1; and III was purified by flash chromatography on silica gel in ether/hexanes, 2:1. The purity of all three compounds was checked by ^{1}H NMR, ^{13}C NMR, and TLC. For I in ethyl acetate/ether, 2:1, R_f = 0.28; for II in ethyl acetate/hexanes, 2:1, R_f = 0.25; for III in ethyl acetate/hexanes, 2:1, R_f = 0.33. [5-^{3}H]-6-Fluoromevalonate and [5-^{3}H]-6,6,6-trifluoromevalonate were synthesized by the method of Nave et al. (1985) with one modification. The sodium borotritide reductions were performed in ethanol at room temperature. The lactones were purified as described above. The purity of both compounds was checked by ^{1}H and ^{13}C NMR. The radiochemical purity of both compounds was >99% as determined by TLC in the solvent systems used for the unlabeled compounds. In all cases lactones were converted to the sodium salts according to Popjak (1969).

Syntheses. (*A*) *4,4-Difluoromevalonate* (*IV*). Activated zinc dust (1.3 g, 20 mmol) was placed in a three-neck 250-mL round-bottom flask with 40 mL of dry THF and brought to reflux. Ethyl bromodifluoroacetate (4.1 g, 20 mmol) was added over 1 min. After the zinc was consumed (approximately 10 min), acetylacetaldehyde dimethyl acetal (2.6 g, 20 mmol) was added and the mixture was stirred under reflux for 1 h. After the mixture was cooled to room temperature, 50 mL of ethyl acetate and 50 mL of 0.5 M $NaHSO_4$ were added. The aqueous layer was extracted twice with 75 mL of ethyl acetate. The combined extracts were dried over $MgSO_4$ and concentrated by rotary evaporation in vacuo. The product was purified by flash chromatography on silica gel in ethyl acetate/hexanes, 1:3, to give 2.9 g (60% yield) of ethyl 2,2-difluoro-5,5-dimethoxy-3-hydroxy-3-methylpentanoate as a pale yellow liquid. ^{1}H NMR ($CDCl_3$) δ 1.28 (s, 3 H), 1.29 (t, 3 H), 1.81 (dd, J = 5.6 and 4.6 Hz, 1 H), 2.05 (dd, J = 4.0 and 14.6 Hz, 1 H), 3.31 (s, 3 H), 3.32 (s, 3 H), 4.28 (q, 2 H), 4.69 (t, 1 H). ^{13}C NMR ($CDCl_3$) δ 13.8 (s), 21.9 (s), 37.3 (s), 53.3 (s), 62.8 (s), 73.0 (t, J_{C-F} = 19.3 Hz), 102 (s), 116 (t, J_{C-F} = 258 Hz), 163 (t, J_{C-F} = 19.3 Hz). Ethyl 2,2-difluoro-5,5-dimethoxy-3-hydroxy-3-methylpentanoate (2.2 g, 9 mmol) was dissolved in 10 mL of methanol and stirred at 4 °C. Sodium borohydride (360 mg, 10 mmol) was added in portions over 45 min. Stirring was continued for 30 min as the solution warmed to room temperature. The reaction mixture was acidified to pH 3 with 5 N HCl, and 100 mL of ethyl acetate was added. After the mixture was dried over $MgSO_4$, solvent was removed by rotary evaporation in vacuo. Thirty milliliters of THF and 15 mL of 3 N HCl were added, and the solution was stirred under reflux for 2.5 h. The reaction mixture was cooled to room temperature and extracted twice with 100 mL of ethyl acetate. The combined organic extracts were dried over $MgSO_4$ and concentrated to a syrup by rotary evaporation in vacuo. The cyclic hemiacetal was purified by flash chromatography on silica gel in ethyl acetate/hexanes, 1:1. ^{1}H NMR ($CDCl_3$) δ 1.31 (d, J_{H-F} = 2 Hz, 3 H), 1.99 (br s, 1 H), 2.05 (d, J_{H-H} = 10.3 Hz, 2 H), 3.66 (t, J_{H-H} = 12.3 Hz and J_{H-F} = 12.3 Hz, 1 H), 4.28 (dd, J_{H-H} = 12.3 Hz and J_{H-F} = 31.7 Hz, 1 H), 5.23 (t, 1 H). ^{13}C NMR ($CDCl_3$) δ 20.3 (s), 39.3 (s), 58.2 (dd, J_{C-F} = 26.0 and 36.2 Hz), 71.1 (dd, J_{C-F} = 22.3 and 28.9 Hz), 91.8 (s), 117.7 nt, J_{C-F} = 252 Hz).

The cyclic hemiacetal (670 mg, 4 mmol) was placed in 40 mL of acetone, and Jones reagent (2.67 M CrO_3 in 8 N H_2SO_4) was added dropwise with stirring until the solution was persistently yellow. Excess oxidant was quenched with methanol, and the solution was filtered through a pad of Celite. The residue was extracted with 20 mL of ethyl acetate and filtered. The combined filtrate was dried over $MgSO_4$, filtered, and concentrated to a pale yellow syrup by rotary evaporation in vacuo. The crude product was purified by flash chromatography on silica gel in ethyl acetate/hexanes, 1:3, to give 360 mg of 4,4-difluoromevalonate lactone as an opaque hygroscopic solid. The overall yield from ethyl 2,2-difluoro-5,5-dimethoxy-3-hydroxy-3-methylpentanoate was 25%. ^{1}H NMR ($CDCl_3$) δ 1.39 (d, J_{H-F} = 1.3 Hz, 3 H), 2.79 (m, 2 H), 4.34 (dt, J_{H-H} = 12.3 Hz and J_{H-F} = 12.3 and 1.2 Hz, 1 H), 4.74 (ddd, J_{H-H} = 12.3 Hz and J_{H-F} = 3.6 and 30.2 Hz, 1 H). ^{13}C NMR ($CDCl_3$ 19.8 (s), 41.6 (s), 67.3 (dd, J_{C-F} = 29.0 and 40.5 Hz), 69.9 (dd, J_{C-F} = 23.2 and 28.3 Hz), 117.5 (t, J_{C-F} = 252 Hz), 167. 8 (s).

(*B*) *Mevalonate 5-Pyrophosphate.* The synthesis is based upon the procedure described by Davisson (1985). Mevalonate lactone (1.3 g, 10 mmol) was dissolved in 8 mL of dry methylene chloride and stirred under nitrogen at −30 °C. Trimethylsilyl iodide (2.2 g, 11 mmol) was added dropwise via syringe over 1 min, and stirring was continued for 45 min as the reaction mixture was allowed to reach room temperature. Methanol (2 mL) was added via syringe, and the reaction was stirred for 10 min at room temperature. The product mixture was added dropwise to a stirred solution of diazomethane (approximately 20 mmol) in 50 mL of ether at 4 °C. After the addition was complete, stirring was continued for 20 min at 4 °C. Excess diazomethane was removed under a stream of nitrogen, and the sample was concentrated to a syrup by rotary evaporation in vacuo. Purification by flash chromatography on silica gel in hexanes/ether, 2:1, gave 2.0 g (76% yield) of methyl 3-hydroxy-5-iodo-3-methylpentanoate as a colorless liquid. ^{1}H NMR ($CDCl_3$) δ 1.19 (s, 3 H), 2.12 (m, 2 H), 2.40 (d, 1 H), 2.48 (d, 1 H), 3.18 (m, 2 H), 3.67 (s, 3 H). ^{13}C NMR ($CDCl_3$) δ 1.66 (s), 26.1 (s), 44.3 (s), 46.6 (s), 51.8 (s), 71.8 (s), 172.8 (s).

Tris(tetrabutylammonium) pyrophosphate (1.06 g, 1.17 mmol) was placed in a 25-mL round-bottom flask and covered with a septum. Acetonitrile (1.5 mL) was added and the solution was stirred under an atmosphere of nitrogen. Methyl 3-hydroxy-5-iodo-3-methylpentanoate (130 mg, 0.5 mmol) was dissolved in 200 μL of acetonitrile and added to the reaction flask. The mixture was stirred for 3 h at room temperature. The product mixture was diluted with 10 mL of water and passed through a 1.5 × 7 cm column of Dowex 50 in the ammonium ion form. The column was washed with 10 mL of water, and the combined eluent was applied to a 1.5 × 25 cm column of QAE-Sephadex A-25 in the bicarbonate form. The column was eluted with a 500-mL linear TEAB gradient from 2.2 to 0.6 M. The column fractions containing methyl

mevalonate 5-pyrophosphate were pooled and concentrated to dryness by rotary evaporation in vacuo. The residue was diluted with 3 mL of water and passed through a 1.5 × 5 cm column of Dowex 50 in the Li^+ form. The eluent was lyophilized to dryness. ^1H NMR (D_2O) δ 1.27 (s, 3 H), 1.92 (t, 2 H), 2.56 (d, 1 H), 2.62 (d, 1 H), 3.65 (s, 3 H), 4.03 (q, 2 H). ^{13}C NMR (D_2O) δ 28.6 (s), 43.8 (d, J_{C-P} = 7.2 Hz), 48.7 (s), 54.8 (s), 64.7 (d, J_{C-P} = 5.5 Hz), 73.7 (s), 176.7 (s). ^{31}P NMR (D_2O) broad-band decoupled δ 4.27 (d, J_{P-P} = 19.8 Hz), 8.33 (dt, J_{P-P} = 19.8 Hz and J_{H-P} = 6.2 Hz).

Trilithium methyl mevalonate 5-pyrophosphate (100 mg, 0.29 mmol) was dissolved in 3 mL of 0.5 M LiOH and stirred for 18 h at 4 °C. The pH was adjusted to 8 with 1.0 N HCl, and the sample was lyophilized to dryness. The white powder was suspended in 10 mL of ethanol/ether, 1:2, and collected by centrifugation. This washing procedure was repeated 3 times with ethanol/ether, 1:2, and 1 time with ether. After drying in vacuo, 85 mg of tetralithium mevalonate 5-pyrophosphate was obtained. The overall yield from mevalonate lactone was 25%. ^1H NMR (D_2O) δ 1.22 (s, 3 H), 1.87 (t, 2 H), 2.30 (d, 1 H), 2.36 (d, 1 H), 4.01 (q, 2 H). ^{13}C NMR (D_2O) δ 28.6 (s), 43.7 (d, J_{C-P} = 5.8 Hz), 50.9 (s), 65.0 (d, J_{C-P} = 5.7 Hz), 73.6 (s), 183.2 (s). ^{31}P NMR (D_2O) broad-band decoupled δ 4.15 (d, J_{P-P} = 20.0 Hz), 8.18 (d, J_{P-P} = 20.0 Hz); coupled δ 4.15 (d, J_{P-P} = 20.0 Hz), 8.18 (dt, J_{P-P} = 20.0 Hz and J_{H-P} = 6.3 Hz).

The same procedure was used to synthesize [5-^{3}H]-mevalonate 5-pyrophosphate from 1.0 mmol (1.0 MCi) of [5-^{3}H]mevalonate. 6,6-Difluoromevalonate 5-phosphate was also synthesized from 6,6-difluoromevalonate by the above procedure. ^1H NMR (D_2O) δ 1.98 (t, 3 H), 2.47 (s, 2 H), 4.06 (dt, J_{H-H} = 6.3 Hz and J_{H-P} = 6.3 Hz, 2 H), 5.71 (t, J_{H-F} = 55.4 Hz, 1 H). ^{13}C NMR (D_2O) δ 37.3 (d, J_{C-P} = 5.3 Hz), 42.2 (s), 63.7 (d, J_{C-P} = 5.0 Hz), 75.2 (t, J_{C-F} = 19.8 Hz), 119.5 (t, J_{C-F} = 246 Hz), 181.6 (s). ^{31}P NMR (D_2O) broad-band decoupled δ 4.18 (d, J_{P-P} = 19.3 Hz), 8.39 (d, J_{P-P} = 19.3 Hz); coupled δ 4.18 (d, J_{P-P} = 19.8 Hz), 8.39 (dt, J_{P-P} = 19.3 Hz and J_{H-P} = 6.3 Hz).

Preparation of Rat Liver Homogenate. Male Sprague-Dawley rats (150–200 g) were used as the source of liver. The rats were killed by decapitation, and a 10000*g* (S_{10}) supernatant was prepared in 0.1 M Tris-HCl, pH 7.4, and 5 mM $MgCl_2$, as described by Popjak (1969). For some experiments a 100000*g* (S_{100}) supernatant was used (Popjak, 1969).

NMR Spectra. All NMR spectra were recorded on a Varian XL-300 spectrometer. ^1H NMR spectra were recorded in $CDCl_3$ or D_2O, and tetramethylsilane was used as an external reference. ^{19}F NMR spectra were recorded in D_2O, and $CFCl_3$ was used as an external reference. ^{13}C NMR spectra were recorded in $CDCl_3$ or D_2O, and tetramethylsilane was used as an external reference. ^{31}P NMR spectra were recorded in D_2O, and 85% H_3PO_4 was used as an external reference.

Purification of Mevalonatepyrophosphate Decarboxylase. The enzyme was purified by a modification of a published procedure (Alvear et al., 1982). This procedure was used through the DEAE step. Thereafter, the enzyme was concentrated by $(NH_4)_2SO_4$ precipitation, dialyzed against 3 L of buffer A, and purified further by FPLC. The following buffers were used: (A) 10 mM potassium phosphate, pH 7, 10 mM mercaptoethanol, and 0.1 mM EDTA; (B) 500 mM potassium phosphate, pH 7, and other additions as above.

Protein (50 mg in 6 mL) was applied to a Mono Q 10/10 column (Pharmacia). The column was washed with 16 mL of buffer A, followed by a gradient to 20% B over 160 mL. Elution is then continued with 20% B. The enzyme elutes between 155 and 170 mL. The eluted enzyme was precipitated by addition of $(NH_4)_2SO_4$ to 85% saturation. The precipitate was then dialyzed overnight against buffer A (20% glycerol was added before loading on column). The dialyzed protein (2 mL or 10.6 mg of protein) was then put on a hydroxylapatite column, 1.3 × 4.5 cm (Ultrogel HA, LKB), which had been equilibrated against 13 mM potassium phosphate, pH 7.0, 10 mM mercaptoethanol, 0.1 mM EDTA, and 20% glycerol. The column was eluted with the same buffer. The enzyme is not absorbed under these conditions. The eluted enzyme (12 mL) was dialyzed against buffer A containing 20% glycerol. The dialyzed enzyme was concentrated by ultrafiltration to 12 mL. The specific activity of the enzyme so obtained was 10.3 units/mg. Enzyme activity was determined by measuring the formation of ADP in a coupled assay. Experimental conditions are described in footnote *a* of Table II.

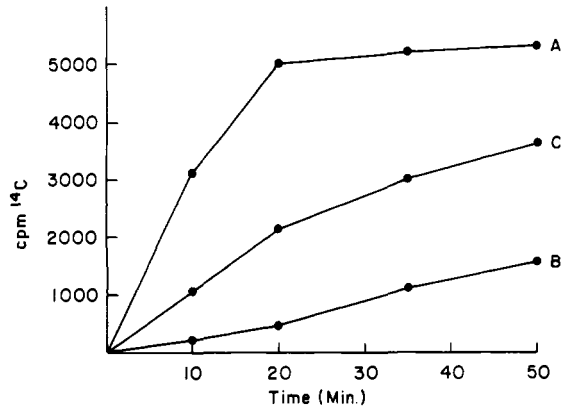

FIGURE 2: Inhibition of nonsaponifiable lipid biosynthesis by fluorinated mevalonate analogues. Incubation mixtures (2.2 mL) contained 1.9 mL of S_{10} homogenate, 20 μM [2-^{14}C]sodium mevalonate (5 × 10^4 cpm), and the following additions: (A) none, (B) 10 μM sodium 6-fluoromevalonate (I), or (C) 300 μM sodium 6-trifluoromevalonate (III). Cofactors to (A) and (B) as in Table I; the samples were incubated at 37 °C. At the time intervals indicated, 500-μL aliquots were removed and analyzed for nonsaponifiable lipid content according to Popjak (1969).

Table I: Inhibition of Mevalonate Incorporation into Nonsaponifiable Lipids[a]

compound	I_{50} (μM)[b]
6-fluoromevalonate (I)	6.5
6,6-difluoromevalonate (II)	10.0
6,6,6-trifluoromevalonate (III)	300
4,4-difluoromevalonate (IV)	1000

[a] Incubation mixtures contained 0.85 mL of S_{10} homogenate, 5 mM ATP, 3 mM glucose 6-phosphate, 1 mM NAD, 1 mM NADP, 5% 1,2-propanediol (v/v), 20 μM [2-^{14}C]sodium mevalonate (5 × 10^4 cpm), and inhibitor (I–IV). After 10 min at 37 °C the samples were analyzed for nonsaponifiable lipid content according to Popjak (1969). [b] The I_{50} value is defined as the inhibitor concentration that results in 50% inhibition of nonsaponifiable lipid biosynthesis from 20 μM mevalonate after 10 min at 37 °C. The values reported are for the *R,S* mixture.

RESULTS

The S_{10} supernatant of a rat liver homogenate contains all of the enzymes required for biosynthesis of cholesterol from mevalonate (Popjak, 1969). Addition of compounds I–IV to the incubation results in a decreased rate of steroid biosynthesis. The time course for incorporation of mevalonate into nonsaponifiable lipids as well as the effect on this incorporation by I and III is shown in Figure 2. The I_{50} values for compounds I–IV, determined during the linear portion of the assay, are shown in Table I. Although compounds I–IV inhibit the

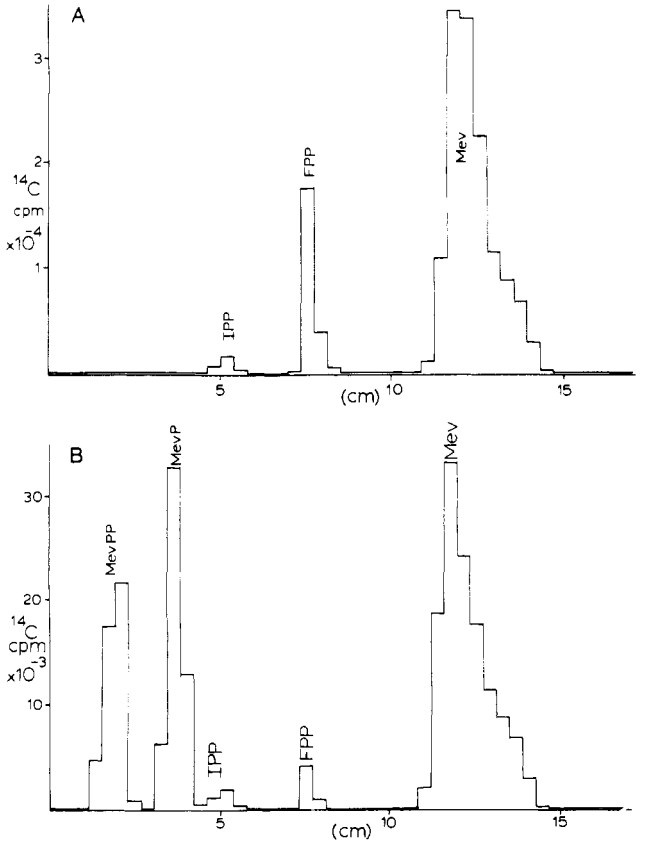

FIGURE 3: Water-soluble metabolites of mevalonate in the presence of fluorinated mevalonate analogues. Incubation mixtures (0.15 mL) contained 100 μL of S_{10} homogenate, 60 μM [2-^{14}C]sodium mevalonate (0.5 μCi), and the following additions: (A) none or (B) 120 μM sodium 6-fluoromevalonate (I). Cofactors to (A) and (B) as in Table I; samples were incubated at 37 °C for 15 min. Incubations were stopped by immersion in a boiling water bath for 5 min. After centrifugation to remove protein, the samples were supplemented with approximately 1 μmol of each of the following: mevalonate, mevalonate 5-phosphate, mevalonate 5-pyrophosphate, IPP, and FPP. The samples were concentrated to 25 μL under a stream of nitrogen and applied in two spots to a 6 × 20 cm Kieselgel 60F-254 preparative TLC plate. The samples were chromatographed in 1-propanol/NH_4OH/H_2O, 60:30:10, dried with a stream of warm air, and rechromatographed in the same solvent. One lane was sprayed with ammonium molybdate–perchloric acid (Hanes & Isherwood, 1949) to visualize the position of the phosphorylated carriers. The plate was then cut into 0.4-mm strips, and the unsprayed lane was scraped into scintillation vials. The samples were suspended in 0.5 mL of water prior to scintillation counting in 4 mL of Aquasol.

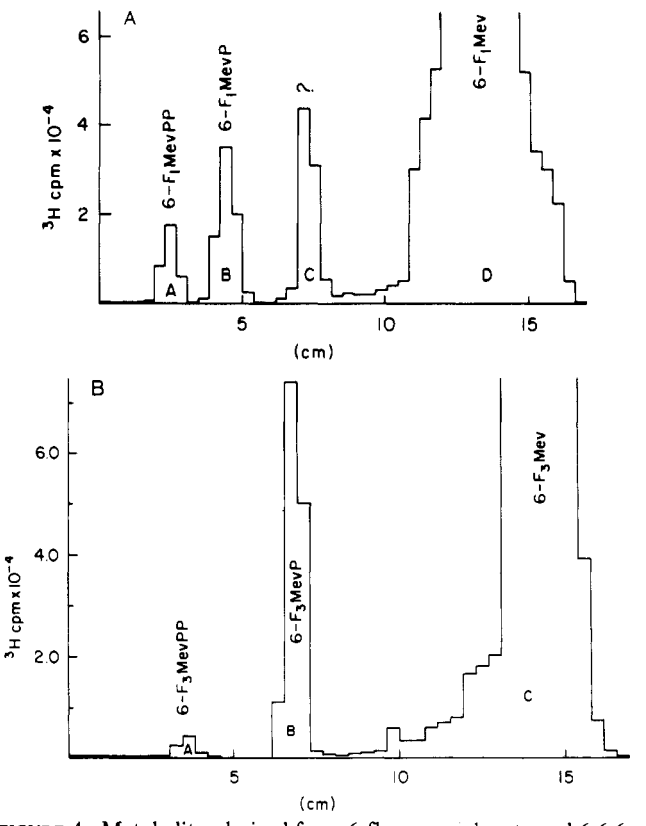

FIGURE 4: Metabolites derived from 6-fluoromevalonate and 6,6,6-trifluoromevalonate. Incubation mixtures (0.25 mL) contained 200 μL of the S_{10} homogenate and the following: (A) 13 mM [5-^{3}H]-sodium 6-fluoromevalonate (2.5 × 10^7 cpm) or (B) 20 mM [5-^{3}H]sodium 6,6,6-trifluoromevalonate (2.5 × 10^7 cpm). Cofactors to (A) and (B) as in Table I; samples were incubated for 2 h at 37 °C. Fifty microliters of sample A and 100 μL of sample B were mixed with the carriers listed in the legend to Figure 3 and chromatographed as described in Figure 3.

incorporation of [2-^{14}C]mevalonate into nonsaponifiable lipids, they do not affect the rate of incorporation of [4-^{14}C]IPP (data not shown). This result suggests that the site of action of compounds I–IV is prior to isopentenylpyrophosphate isomerase.

To further pinpoint the site of action of these compounds, the distribution of radioactivity among the water-soluble metabolites of mevalonate was examined (Figure 3). In the control incubation the only water-soluble metabolites detected were IPP and FPP (Figure 3A). Addition of compounds I, III, and IV results in a dramatic reduction in the levels of FPP, and two new peaks corresponding to mevalonate 5-phosphate and mevalonate 5-pyrophosphate are observed. The data for I are shown in Figure 3B. Data for the other compounds (III and IV) are not shown. The fluorinated mevalonate analogues appear to inhibit the conversion of mevalonate 5-pyrophosphate to IPP.

This inhibition could be due to the fluoromevalonate analogues or, more likely, to metabolic derivatives of these analogues, possibly pyrophosphorylated compounds. To determine whether the analogues were metabolically modified, the water-soluble products formed upon incubation of [5-^{3}H]-I and [5-^{3}H]-III with the S_{10} supernatant were examined. Three water-soluble metabolites of [5-^{3}H]-I were observed (Figure 4A). Peak A cochromatographed with carrier mevalonate 5-pyrophosphate, and peak B cochromatographed with carrier mevalonate 5-phosphate. Peak C did not cochromatograph with any of the carriers added. Peak D is the unreacted I. When the incubation mixture was treated with alkaline phosphatase prior to chromatography, only peaks C and D are observed.

A similar experiment was done with [5-^{3}H]-III. Two water-soluble metabolites were observed (Figure 4B). Peak A cochromatographed with carrier mevalonate 5-phosphate while peak B did not cochromatograph with any of the carriers added. Peak C is the unreacted [5-^{3}H]-III. Both peaks A and B were sensitive to prior treatment with alkaline phosphatase.

Determination of the structures of the water-soluble metabolites of I and III was achieved by isolation of the compounds from a large-scale incubation with the S_{100} supernatant, followed by NMR spectroscopy. Figure 5A shows the QAE Sephadex A-25 elution profile for the incubation of the S_{100} supernatant with compound I. Peak I is unreacted I. Three other radioactive peaks were detected. Peak II was shown by NMR to be unlabeled glycerol 3-phosphate contaminated with a trace of [5-^{3}H]-I. Peak III gave the following: ^{1}H NMR (D_2O) δ 1.77 (t, 2 H), 2.35 (s, 2 H), 3.74 (dt, J_{H-H} = 6.3 Hz and J_{H-P} = 6.3 Hz, 2 H), 4.32 (d, J_{H-F} = 48 Hz, 2 H). ^{13}C NMR (D_2O) 39.1 (dd, J_{C-F} = 3.8 Hz and J_{C-P} = 7.6 Hz), 44.8

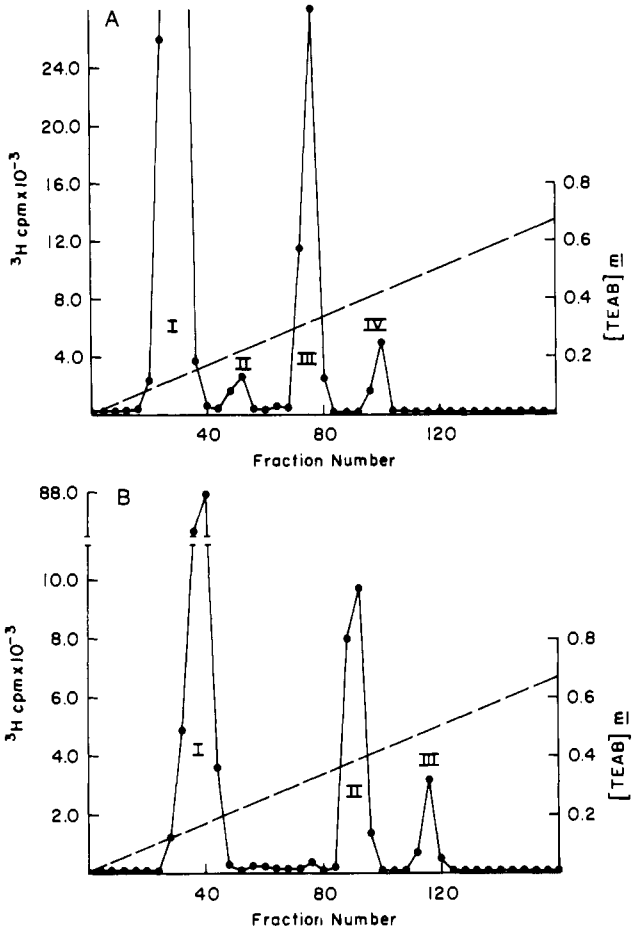

FIGURE 5: QAE-Sephadex A-25 separation of 6-fluoromevalonate and 6,6,6-trifluoromevalonate metabolites. Incubation mixtures (100 mL) contained 80 mL of S_{100} supernatant, 20 mM ATP, 10 mM NaF, 10 mM cysteine, and the following: (A) 29 mM [5-^{3}H]sodium 6-fluoromevalonate (1.0×10^7 cpm) or (B) 29 mM [5-^{3}H]sodium 6,6,6-trifluoromevalonate (1.0×10^7 cpm). Samples were incubated at 37 °C for 6 h. Incubation was stopped by immersion in a boiling water bath for 10 min. After the mixture was cooled in an ice bath, the protein was removed by centrifugation and the supernatant was poured into 400 mL of ice-cold ethanol. After centrifugation to remove precipitated nucleotides, the samples were concentrated to 50 mL by rotary evaporation in vacuo. Samples were diluted to 200 mL with water, adjusted to pH 8 with 5 N NaOH, and applied to a 2.5 × 25 cm column of QAE-Sephadex A-25 in the bicarbonate form. The column was washed with 50 mL of water and then eluted with a 3-L linear TEAB gradient from 0.0 to 0.67 M. Fractions of 20 mL were collected at a flow rate of 2 mL/min. One hundred microliter aliquots were used for liquid scintillation counting of the column fractions.

(d, J_{C-F} = 3.8 Hz), 62.3 (d, J_{C-P} = 4.5 Hz), 74.9 (d, J_{C-F} = 17.1 Hz), 90.4 nd, J_{C-F} = 170 Hz), 182.3 (s). ^{31}P NMR (D_2O) broad-band decoupled δ −4.62 (s); coupled δ −4.62 (t, J_{H-P} = 5.2 Hz). ^{19}F NMR (D_2O) δ −228.6 (t, J_{H-F} = 48 Hz). These data correspond unambiguously to 6-fluoromevalonate 5-phosphate. This purified compound cochromatographed with peak B in Figure 4A. Peak IV gave the following: ^{1}H NMR (D_2O) δ 1.90 (t, 2 H), 2.41 (s, 2 H), 3.99 (dt, J_{H-H} = 6.3 Hz and J_{H-P} = 6.3 Hz), 4.39 (d, J_{H-F} = 47.3 Hz). ^{13}C NMR (D_2O) δ 38.9 (dd, J_{C-F} = 3.8 Hz and J_{C-P} = 7.6 Hz), 44.8 (d, J_{C-P} = 4.0 Hz), 64.1 (d, J_{C-P} = 5.7 Hz), 74.8 (d, J_{C-F} = 17.1 Hz), 90.3 (d, J_{C-F} = 171 Hz), 182.2 (s). ^{31}P NMR (D_2O) broad-band decoupled δ 4.25 (d, J_{P-P} = 19.9 Hz), 8.38 (d, J_{P-P} = 19.9 Hz); coupled δ 4.25 (d, J_{P-P} = 19.9 Hz); 8.38 (dt, J_{P-P} = 19.9 Hz and J_{H-P} = 6.2 Hz). ^{19}F NMR (D_2O) δ −228.5 (t, J_{H-F} = 47 Hz). These data correspond unambiguously to 6-fluoromevalonate 5-pyrophosphate. This compound cochromatographed with peak A in Figure 4A. Under these experimental conditions no compound corresponding to peak C (Figure 3A) was detected.

Table II: Inhibitors of Mevalonate-5-pyrophosphate Decarboxylase[a]

inhibitor	K_i (μM)
6-fluoromevalonate 5-pyrophosphate	0.01
6,6,6-trifluoromevalonate 5-pyrophosphate	0.5
6-fluoromevalonate 5-phosphate	0.9
mevalonate 5-phosphate	105
mevalonate 5-pyrophosphate	9.9 (K_m)

[a] Reaction contained 5 mM ATP, 0.5 mM PEP, 0.3 mM NADH, 10.5 units of pyruvate kinase, 20 units lactic dehydrogenase, 0.1 M Tris-HCl, pH 7.0, 5 mM $MgCl_2$, mevalonate-5-pyrophosphate decarboxylase, and 3.5–70 μM mevalonate pyrophosphate. Mevalonate analogues were phosphorylated enzymically by use of rat liver homogenates. Compounds were isolated and purified chromatographically as described in Figure 5.

Figure 5B shows the QAE-Sephadex A-25 elution profile for the incubation of III with the S_{100} supernatant. Peak I is unreacted III. Peak II gave the following: ^{1}H NMR (D_2O) δ 1.92 (dt, J_{H-H} = 6.7 Hz and J_{H-F} = 1.8 Hz, 2 H), 2.35 (d, J_{H-H} = 15.8 Hz, 1 H), 2.51 (d, J_{H-H} = 15.8 Hz, 1 H), 3.72 (dt, J_{H-H} = 6.7 Hz and J_{H-P} = 9.0 Hz, 2 H). ^{13}C NMR (D_2O) δ 34.0 (d, J_{C-P} = 6.9 Hz), 37.9 (s), 58.4 (d, J_{C-P} = 4.1 Hz), 72.7 (q, J_{C-F} = 27.5 Hz), 125.5 (q, J_{C-F} = 287 Hz), 178.1 (s). ^{31}P NMR (D_2O) broad-band decoupled δ −4.57 (s); coupled δ −4.57 (t, J_{H-P} = 6.7 Hz). ^{19}F NMR (D_2O) δ −82.6 (s). These data correspond unambiguously to 6,6,6-trifluoromevalonate 5-phosphate. This compound cochromatographed with peak B in Figure 4B. Peak III gave the following: ^{1}H NMR (D_2O) δ 2.07 (dt, J_{H-H} = 6.7 Hz and J_{H-F} = 1.8 Hz, 2 H), 2.46 (d, J_{H-H} = 15.8 Hz, 1 H), 2.64 (d, J_{H-H} = 15.8 Hz, 1 H), 4.08 (dt, J_{H-H} = 6.7 Hz and J_{H-P} = 9.0 Hz, 2 H). ^{13}C NMR (D_2O) δ 33.9 (d, J_{C-P} = 7.4 Hz), 37.8 (s), 60.4 (d, J_{C-P} = 4.1 Hz), 72.6 (q, J_{C-F} = 27.5 Hz), 125. 6 (q, J_{C-F} = 287 Hz), 178.1 (s). ^{31}P NMR (D_2O) broad-band decoupled δ 6.39 (d, J_{P-P} = 20.0 Hz), 9.06 (d, J_{P-P} = 20.0 Hz); coupled δ 6.39 (d, J_{P-P} = 20.0 Hz), 9.06 (dt, J_{P-P} = 20.0 Hz and J_{H-P} = 6.2 Hz). ^{19}F NMR (D_2O) δ −82.6 (s). These data correspond unambiguously to 6,6,6-trifluoromevalonate 5-pyrophosphate. This compound cochromatographed with peak A in Figure 4B. These results establish that the two mevalonate analogues tested (I and III) are phosphorylated and pyrophosphorylated in the rat liver preparation.

The results obtained so far suggested that phosphorylated or pyrophosphorylated derivatives of the fluoromevalonate analogues I–IV inhibit conversion of mevalonate to FPP. A likely point of inhibition is mevalonate decarboxylase. It has already been shown that pyrophosphorylated I inhibits the decarboxylase (Nave et al., 1985). We, therefore, examined the effect of phosphorylated and pyrophosphorylated I and III on the decarboxylase. The results are summarized in Table II. The pyrophosphorylated forms of the inhibitors are effective inhibitors of the decarboxylase. The phosphorylated species are less effective, and essentially no inhibition is seen with unphosphorylated compounds.

Discussion

The synthesis of mevalonate 5-pyrophosphate reported here is to the best of our knowledge the first reported chemical synthesis of this compound. The overall yield from commercially available mevalonate lactone is 25%. The commercial availability of mevalonate lactone radiolabeled in several positions makes this synthesis a useful method for preparation of radiolabeled mevalonate 5-pyrophosphate. The synthesis of 6,6-difluoromevalonate 5-pyrophosphate further illustrates the utility of this method for the synthesis of me-

valonate 5-pyrophosphate analogues.

Inhibition of the cholesterol biosynthetic pathway by I has been previously demonstrated (Nave et al., 1985). In their study it was shown that 6-fluoromevalonate was phosphorylated by mevalonate kinase and mevalonate-5-phosphate kinase and that the pyrophosphorylated compound inhibits mevalonate-5-pyrophosphate decarboxylase. We have obtained similar results with compound I. In addition, we examined other fluorinated mevalonate analogues (II–IV). It was also shown with partially purified decarboxylase that the pyrophosphorylated form and, to a lesser extent, the phosphorylated form of I and III inhibit the decarboxylase. Pyrophosphorylated I is a good inhibitor (K_i = 10 nM). It is very likely that all analogues tested (I–IV) inhibit cholesterol biosynthesis primarily by blocking the decarboxylase and that the pyrophosphorylated form is actually responsible for this inhibition. The ability of I and III to be phosphorylated by the liver homogenate supernatant was shown directly by using the 5-^{3}H-labeled inhibitors. IV causes a reduction in FPP levels but leads to a smaller buildup of predominantly mevalonate 5-phosphate. This finding suggests that fluorine atoms β to the C_5 alcohol, which is pyrophosphorylated, lead to a reduced rate of phosphorylation and the compound may actually inhibit the phosphorylation of mevalonate.

Particularly noteworthy is the observation that pyrophosphorylated I has a remarkably low K_i (10 nM). The basis for this low K_i is not clear. Presumably the decarboxylation involves phosphorylation of the 3-hydroxy group of mevalonate pyrophosphate, followed by decarboxylation with elimination of P_i. Possibly the presence of fluorine on the carbon α to the carbon bearing the OH group that becomes phosphorylated reduces the rate of phosphorylation. Under these conditions, an enzyme–mevalonate pyrophosphate–ATP complex may accumulate, and K_{diss} for that complex may be relatively low. Some evidence in support of the retarding effect of adjacent fluorine in phosphorylation is provided by these results with IV. Our data suggest that IV was phosphorylated more slowly than I or III. If fluorine adjacent to the carbinol indeed retards the rate of phosphorylation of the carbinol, two effects could be responsible: (1) The larger size of the fluorine compared to that of the hydrogen could prevent the proper alignment of the –OH group. If that were the case, this reaction shows unusually high steric requirements. (2) The reaction does not involve general-base catalysis; i.e., the OH group does not lose a proton in the transition state. Under these conditions positive charge develops in the transition state and the electron-withdrawing effect of the fluorine would destabilize the transition state. However, until the reaction is further characterized, it is premature to speculate further concerning the basis of the low K_i for I.

When [5-^{3}H]-III was incubated with the liver homogenate supernatant, two derivatives of III were observed. In Figure 4B, peak A cochromatographed with carrier mevalonate 5-phosphate; however, this compound was shown by NMR spectroscopy to be 6,6,6-trifluoromevalonate 5-pyrophosphate. Similarly, peak B which chromatographed with a higher R_f than the carrier mevalonate 5-phosphate was shown by NMR spectroscopy to be 6,6,6-trifluoromevalonate 5-phosphate. The effect of the trifluoromethyl group on the R_f of these compounds is surprising and illustrates the need for careful identification of the radiolabeled compounds by a method other than cochromatography with nonfluorinated carriers.

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Registry No. I, 2822-77-7; II, 83191-82-6; III, 83191-83-7; IV, 108868-96-8; IPP, 358-71-4; FPP, 372-97-4; ethyl bromodifluoroacetate, 667-27-6; acetaldehyde dimethyl acetal, 5436-21-5; ethyl 2,2-difluoro-5,5-dimethoxy-3-hydroxy-3-methylpentanoate, 108895-14-3; cyclic hemiacetal, 108868-97-9; mevalonate 5-pyrophosphate, 1492-08-6; mevalonate lactone, 503-48-0; methyl 3-hydroxy-5-iodo-3-methylpentanoate, 108868-98-0; trilithium methyl mevalonate 5-pyrophosphate, 108868-99-1; tetralithium mevalonate 5-pyrophosphate, 108869-00-7; [5-^{3}H]mevalonate 5-pyrophosphate, 108869-01-8; [5-^{3}H]mevalonate, 108869-02-9; 6,6-difluoromevalonate 5-phosphate, 108869-03-0; mevalonate-5-pyrophosphate decarboxylase, 9024-66-2; 6-fluoromevalonate 5-pyrophosphate, 96643-92-4; 6,6,6-trifluoromevalonate 5-pyrophosphate, 108869-04-1; 6-fluoromevalonate 5-phosphate, 108869-05-2; cholesterol, 57-88-5; mevalonate, 150-97-0; mevalonate 5-phosphate, 1189-94-2; 6,6,6-trifluoromevalonate 5-phosphate, 108869-06-3.

REFERENCES

Alvear, M., Jabalquinto, A. M., Eyzaguirre, J., & Cardemil, E. (1982) *Biochemistry 21*, 4646.

Cornforth, R. H., & Popjak, G. (1969) *Methods Enzymol. 15*, 359.

Davisson, V. J., Woodside, A. B., & Poulter, C. D. (1985) *Methods Enzymol. 110*, 130.

Foote, C. D., & Wold, F. (1963) *Biochemistry 2*, 1254.

Hanes, C. F., & Isherwood, F. H. (1949) *Nature (London) 164*, 1107.

Muehlbacher, M., & Poulter, C. D. (1985) *J. Am. Chem. Soc. 107*, 8307.

Nave, J. F., d'Orchymont, H., Ducep, J. B., Piriou, F., & Jung, M. J. (1985) *Biochem. J. 227*, 247.

Popjak, G. (1969) *Methods Enzymol. 15*, 393.

Poulter, C. D., & Satterwhite, D. M. (1977) *Biochemistry 16*, 5470.

Quistad, G. B., & Cerf, D. C. (1981) *Pr. Nauk. Inst. Chem. Org. Fiz. Politech. Wroclaw 22*, 163.

Reardon, J. E., & Abeles, R. H. (1986) *Biochemistry 25*, 5609.