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Bioorganic & Medicinal Chemistry Letters 14 (2004) 4979–4982
Bioorganic & Medicinal Chemistry Letters

Synthesis of acyloxymethyl ester prodrugs of the transferable protein farnesyl transferase substrate farnesyl methylenediphosphonate

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Received 19 April 2004; revised 7 July 2004; accepted 8 July 2004
Available online 31 July 2004

Abstract—Three isoprenoid diphosphate analogues of farnesyl diphosphate (FPP) where the diphosphate has been replaced by methylene diphosphonate and the negative charges masked by frangible pivaloyloxymethyl (POM) esters were prepared. Farnesyl methylenediphosphonate is a sub-micromolar substrate for protein farnesyl transferase. The tripivaloyloxymethyl esters of isoprenoid methylenediphosphonate have significantly increased lipophilicity and may act as important farnesyl diphosphate prodrugs.

A wide variety of signal transduction proteins, including Ras, require post-translational prenylation for their proper membrane localization and activity.[1–4] Protein farnesyltransferase (FTase) catalyzes the transfer of a farnesyl group from farnesyl diphosphate (FPP, **1**) to the cysteine residue in the carboxy terminus of Ras.[5,6] Prenylation is obligatory for the oncogenic effects of mutant Ras[7–10] and these observations have lead to the development of a number of FTase inhibitors (FTIs) currently in phase I and II clinical trials as anti-cancer agents.[11–15] An alternative anti-neoplastic strategy is to alter the downstream biological function of the prenylated protein by modifying the proteins with unnatural analogues of FPP. Synthetic modification to the farnesyl moiety of FPP has generated both FTIs and alternative substrates transferable by FTase to proteins.[16–20]

The labile phosphoanhydride bond and the multiple negative charges of FPP analogues prevent easy diffusion across cell membranes although naturally occurring isoprenoids such as FPP and geranylgeranyl diphosphate are apparently taken up through an active transport system.[21] The isoprenoids farnesol, geranylgeraniol and the unnatural analogue 3-vinyl-farnesol are converted in mammalian cells to the corresponding diphosphates.[22–24] 3-Vinyl-farnesyl diphosphate is an alternative substrate for FTase and the prodrug 3-vinyl-farnesol is able to block anchorage-independent growth of ras-transformed cells. Presumably, this effect is due to FTase catalyzed modification of proteins with the 3-vinyl-farnesyl group. These observations suggest that alcohol precursors of other FPP analogues may also be useful prodrugs.

However, it is far from clear that arbitrary farnesol analogues will be efficiently converted to the corresponding diphosphates in cells. An alternative strategy for creating membrane permeant molecules is to reduce the overall charge and increase the lipophilicity of the analogue. The bioavailability of membrane impermeant phosphate and phosphonate containing drug molecules has been dramatically increased by masking the charged groups with acyloxymethyl esters.[25–28] The acyloxymethyl esters allow the drugs to diffuse across the cellular membrane into the cytoplasm where general esterases cleave the acyloxymethyl groups revealing the active, charged species (Scheme 1).[29–31] However, this strategy is unlikely to succeed for uncharged diphosphate tetraesters, as the anhydride linkage is rapidly hydrolyzed at physiological

Keywords: Farnesylation; Bisphosphonates; Prodrugs.
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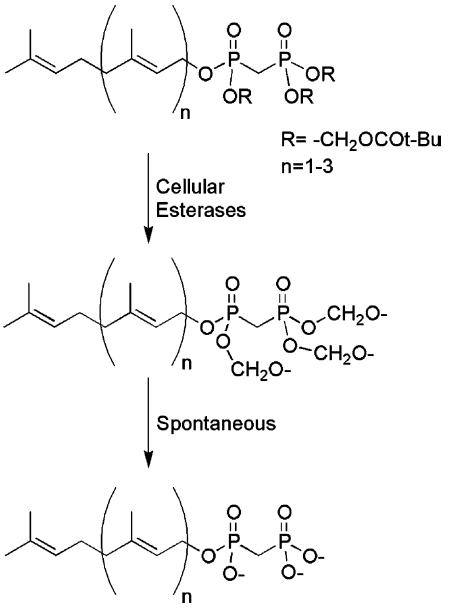
doi:10.1016/j.bmcl.2004.07.017



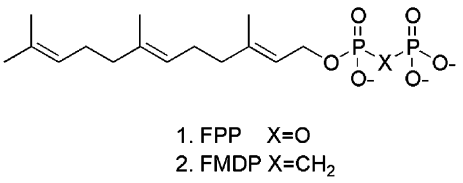
Scheme 1. Proposed mechanism of in vivo activation.[25]

pH. Methylenediphosphonate (MDP) is a structural analogue of diphosphate, which does not contain a labile anhydride linkage. Replacing the labile diphosphate with MDP and masking the negative charges with acyloxymethyl esters may result in membrane permeant FPP analogues. We found no description of monoallylic triacyloxymethyl methylenediphosphonate tetraesters in the literature. We report the preparation of three such isoprenoids where the diphosphate has been replaced by MDP and the negative charges are masked by frangible pivaloyloxymethyl (POM) esters.

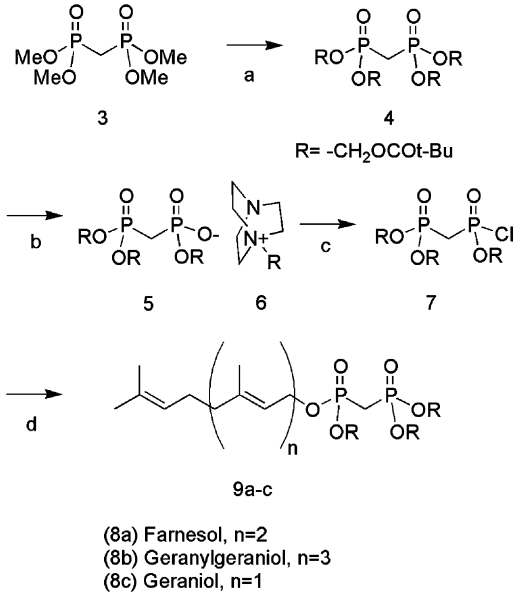
The strategy outlined above requires that the substitution of MDP for the diphosphate in FPP and other analogues results in molecules that are substrates for FTase. Farnesyl methylene diphosphonate (FMDP) **2** has previously been prepared and was shown by Eummer et al. to be a competitive inhibitor (IC_{50} = 160 nM) of FTase.[32] However, the assay used in that study was unable to distinguish whether FMDP was an alternative substrate for FTase or an FTI. To resolve this question, FMDP, **2** was synthesized as previously described, and was shown to be a substrate for FTase in an in vitro fluorescent assay varying isoprenoid concentration.[33] The catalytic efficiency for transfer of the farnesyl group from FMDP **2** to dansyl-GCVLS peptide substrate by FTase was found to be an order of magnitude lower than that for FPP **1** (Table 1). The previous study also reported that treatment of NIH3T3 cells with **2** had no effect on their growth. Eummer at al. suggested that this might be due to **2** acting as an alternative substrate for FTase, leading to normal farnesylation of Ras. The results of the in vitro fluorescent assay are consistent with this observation.

The synthesis of triPOM esters of isoprenoid diphosphonates is summarized in Scheme 2. Tetrapivaloyloxymethyl methylenediposphonate (**4**) was prepared from tetramethyl methylenediphosphonate (**3**) and pivaloyloxymethyl chloride (POM-Cl) in 47% yield as previously described.[34] In analogy to previously reported methods, treatment of **4** with DABCO provided the tripivaloyloxymethyl methylenediphosphonate salt **5** in 80–95% yield. The crude DABCO salt **5**, was converted to the corresponding chloride **7** with oxalyl chloride and catalytic DMF in toluene in 90–95% yield. The purity of the air and moisture sensitive chloride **7** was established by examining the ^{1}H and ^{31}P NMR spectra of the crude filtrate. The ^{1}H NMR spectrum of **7** showed no trace of the quaternary DABCO cation **6**.[36] TriPOM-methylenediphosphonate isoprenoids **9a–c** were prepared by combining the corresponding allylic alcohols **8a–c** with chloride **7** in either dilute toluene or methylene chloride solution containing a threefold excess of DBU and catalytic DMAP.[37] Isoprenoids **9a–c** were isolated in 40–50% yield after purification by silica gel flash chromatography using 1% TEA added to a hexane:ethyl acetate mobile phase. Significantly lower yields of the isoprenoids **9a–c** were obtained if TEA was omitted from the eluant. Isoprenoids **9a–c** were quite labile and no product was recovered from attempts to purify them using alumina and fluorosil. Purification by RP-HPLC

Table 1. Kinetics of FMDP



Analogue	K_m (nM)	K_{cat} (s^{-1})	K_{cat}/K_m ($M^{-1}s^{-1}$)
FPP (1)	40	0.09	2×10^6
FMDP (2)	180	0.06	3×10^5



Scheme 2. Synthesis of POM protected MDP's. Reagents and yields: (a) POMCl, NaI, acetonitrile, reflux (47%). (b) DABCO, acetonitrile, reflux (90%). (c) $(COCl)_2$, DMF (cat), rt (96%). (d) ROH **8(a–d)**, DBU, DMAP (cat) toluene/methylene chloride (40–50%).

was unsuccessful due to progressive decomposition of the tetraesters **9a**–**c** in water or buffer (see below).

Tetraesters **9a**–**c** are water sensitive and can only be stored for a few days at −20 °C without significant decomposition. There is evidence that indicates acyloxymethyl group deprotection occurs chemically at the phosphorous atom in $H_2{}^{18}O$.[30] If the chemical hydrolysis occurs at the allylic alcohol linkage rather than the acyloxymethyl group linkage then these compounds may not be able to traverse a cell membrane as the MDP analogue, but rather the parent alcohol. Tetraester stability was monitored by RP-HPLC for the disappearance of methylenediphosphonates **9a**–**b** and the concomitant appearance of the parent alcohols **8a**–**b** (Fig. 1a–b). Due to low aqueous solubility of the tetraesters, decomposition was measured in mixed aqueous/organic solution. 50% of tetraester **9b** had decomposed after 200 min in water/acetonitrile (99:1) and 500 min in pH = 7.4 buffered cell culture media:acetonitrile (99:1). Under these conditions only 10% of the tetraester **9b** was converted to the parent alcohol **8b** after 500 min. ^{31}P NMR spectra of the time dependent breakdown of methylenediphosphonate **9a** in 9:1 water/acetonitrile is consistent with the formation of the monoallylic diPOM salt. Niemi et al. prepared and tested the aqueous stability of tetra, tri and P,P′-diPOM esters of dichloromethylenediphosphonate.[35] They found that the triPOM ester had a half-life of 78 min in serum and that the insoluble tetraPOM was converted to the P,P′-diPOM and triPOM esters in pH 7.4 serum. Together these data suggest that the charged monoallylic diPOM salts are the primary decomposition products of tetraesters **9a**–**c**. Niemi et al. have also shown that pivaloxymethyl esters of prodrug bisphosphonates are fully deprotected in the presence of 10% rabbit liver homogenates.

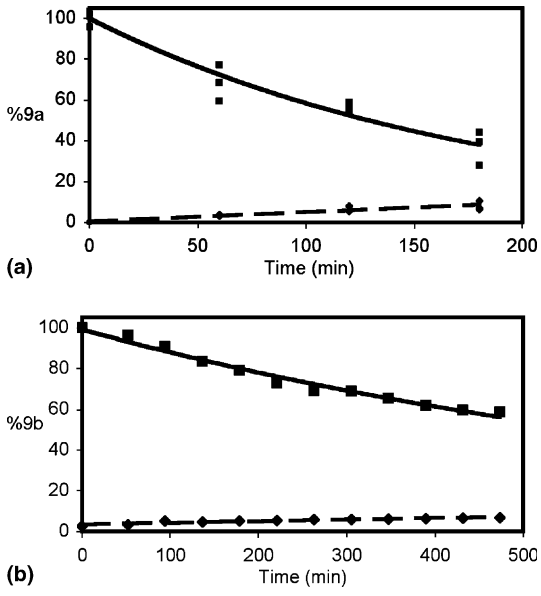


Figure 1. All RP-HPLC data calculated based on relative peak integrals of initial tetraester verses tetraester or alcohol produced at each time point and fit to exponential curve. (a) **9a** decomposition in water (b) **9b** decomposition in cell media.

In conclusion, we report synthetic methodology to prepare monoallylic tripivaloyloxymethyl tetraesters of methylenediphosphonic acid and have shown that farnesyl methylenediphosphonate is a substrate for protein farnesyl transferase. The lipophilicity of isoprenoid methylenediphosphonates is significantly increased by masking the phosphonic acid groups with acyloxymethyl esters. Results of the ongoing biological evaluation of these isoprenoid diphosphate analogues will be reported elsewhere.

Acknowledgements

This work was supported in part by the Kentucky Lung Cancer Research Program (to H.P.S. and D.A.A.), the National Institutes of Health (GM66152-01 to H.P.S.), and the NMR instruments used in this work were obtained with support from NSF CRIF Grant No. CHE-9974810. We thank Dr. Carol Fierke and Jennifer Pickett for the gift of rat protein farnesyltransferase.

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36. Tri(pivaloyloxymethyl) methylenediphosphonyl chloride (**7**) TriPOM salt **5** (546 mg, 0.73 mmol) was dissolved in 20 mL of dry acetonitrile and the solvent evaporated under reduced pressure (3×) followed by the addition of 10 mL dry toluene, 40 μL anhydrous DMF and 0.3 mL oxalyl chloride (3.3 mmol). A white precipitate formed immediately with evolution of heat and the reaction was stirred at rt for 45 min. Solids were removed by filtration through a pad of Na_2SO_4, which was washed with 5 mL of dry toluene. The organic extracts were combined and immediately evaporated under reduced pressure to give a pale yellow oil used without further purification (377 mg, 96%). ^{1}H NMR 5.64–5.85 (m, 6H), 3.03 (t, 2H, J = 20 Hz), 1.23 (s, 27H); ^{31}P NMR 28.74 (d, 1P, J = 10.95 Hz), 15.74 (d, 1P, J = 10.95 Hz).
37. General synthesis of TriPOM esters (**9a–c**): Farnesyl tri(pivaloyloxymethyl) methylenediphosphonate (**9a**): Chloride **7** (697 mg, 1.3 mmol) diluted in 5 mL of dry toluene or anhydrous methylene chloride was added dropwise to a cooled (−20 °C), stirred solution of farnesol **8a** (94 mg, 0.42 mmol) DBU (198 mg, 1.3 mmol) and DMAP (5 mg, 0.04 mmol) in 10 mL of dry toluene. Stirring was continued for 2 h followed by the addition of 50 mL of ether. The organic layer was washed two times each with 10 mL of 0.1 N HCl, water and freshly prepared 5% $NaHCO_3$, then dried over Na_2SO_4 and evaporated under reduced pressure. Farnesyl ester **9a** was obtained in 40% yield (121 mg) after silica gel flash chromatography with 7:3 hexane/EtOAc + 1% triethylamine. ^{1}H NMR 5.61–5.71 (m, 6H), 5.35 (t, 1H), 5.04 (t, 2H), 4.62 (m, 2H), 2.57 (t, 2H, J = 21.6 Hz), 1.90–2.08 (m, 8H), 1.67 (s, 3H), 1.63 (s, 3H), 1.55 (s, 6H), 1.16 (s, 27H); ^{13}C NMR 177.09, 177.01, 143.80, 135.80, 131.55, 124.46, 123.65, 118.57, 82.46, 82.40, 82.14, 63.59, 39.87, 39.74, 38.91, 27.02, 26.89 (t, J = 138 Hz), 26.40, 25.80, 17.90, 16.02, 16.50; ^{31}P NMR 20.02 (d, 1P, J = 7.29 Hz), 19.30 (d, 1P, J = 6.16 Hz).