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Synthesis of Geranyl *S*-Thiolodiphosphate. A New Alternative Substrate/Inhibitor for Prenyltransferases

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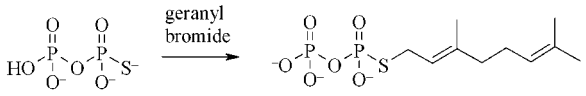
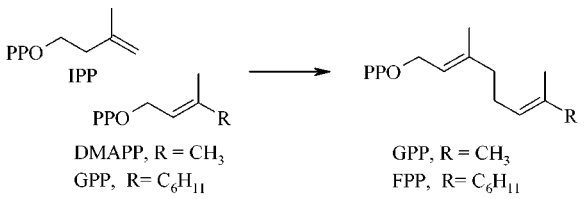
ABSTRACT



5 **6**

The tris(tetra-*n*-butylammonium) salt of thiopyrophosphate 5 was prepared from trimethyl phosphate in four steps. Treatment of geranyl bromide with 5 gave an 80% yield of geranyl *S*-thiolodiphosphate (6). Thiolodiphosphate 6 is substantially less reactive than geranyl diphosphate (7) in the prenyl transfer reaction catalyzed by farnesyl diphosphate synthase and is a good inhibitor of the enzyme.

Prenyltransferases catalyze the electrophilic alkylation of electron-rich acceptor substrates by the hydrocarbon moiety in allylic isoprenoid diphosphates.[1] Some of the more common acceptors are carbon−carbon double bonds (synthesis of isoprenoid chains),[1c] aromatic rings (synthesis of respiratory quinones),[2] amino groups (modification of tRNAs),[3] and sulfhydryl moieties (modification of proteins).[4] The products of prenyl transfer reactions are ultimately converted into over 30 000 naturally occurring isoprenoid compounds. Farnesyl diphosphate synthase (FPPase) is the best characterized of the prenyltransferases.[5] The enzyme catalyzes two sequential chain elongation reactions. Isopentenyl diphosphate (IPP) is first condensed with dimethylallyl diphosphate (DMAPP) to form geranyl diphosphate (GPP, **7**), followed by condensation of GPP with a second IPP to form farnesyl diphosphate (FPP). FPP is required for the biosynthesis of numerous essential metabolites, including ubiquinones, sterols, and dolichols, and FPPase activity is apparently present in all organisms.



DMAPP, R = CH_3
GPP, R= C_6H_{11}

GPP, R = CH_3
FPP, R= C_6H_{11}

The chain elongation reaction is a stepwise electrophilic alkylation.[1c] The carbon−oxygen bond in the allylic diphosphate is cleaved and the resulting allylic carbocation alkylates the double bond in IPP to form a tertiary carbocation. In the final step a proton, originally at C2 of IPP, is removed to give FPP.[6] Several different analogues of the allylic substrates, in which the hydrocarbon moiety is attached to a

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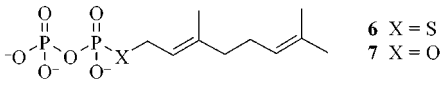
diphosphate mimic, have been synthesized. These include phosphonophosphates,[7] phosphonophosphinates,[8] and bisphosphonates,[9] where methylene groups replace the oxygen between phosphorus and carbon and the bridging oxygen between the two phosphorus atoms. Both classes of compounds are inhibitors, and the bisphosphonates are alternate substrates as well. The lower binding affinities these molecules typically have relative to the normal substrates have been attributed to differences in the pK_as of the phosphate and phosphonate groups.[10] Difluoromethylene analogues have more closely matched pK_as,[10] but the fluorine atoms introduce steric interactions not found in the normal substrates.

Thiophosphate derivatives are widely used as inhibitors and alternate substrates for nucleotides.[11] For these applications, the analogues typically replace substrates for phosphoryl transfer reactions where bonds are being made and broken at the phosphorus atom. Model solvolysis studies with allylic isoprenoid thiolo- and thionophosphates, where the bond to the allylic moiety is cleaved, suggest substantially different patterns of reactivity.[12] For simple dimethylallyl derivatives, the reactivity of the thiono isomer is similar to that of the corresponding phosphate, while the thiolo isomer is approximately 10^6-fold less reactive. These results translate to enzyme-catalyzed reactions, where the P(1) thiono analogue of GPP is a good alternate substrate for FPPase with steady-state kinetic parameters similar to those of the GPP itself.[12] On the basis of the model studies, we reasoned that the thiolo isomer would be substantially less reactive in the prenyl transfer reaction but that its binding affinity would be similar to that of the natural substrate. We now describe a synthesis of tris(tetrabutylammonium)thiopyrophosphate (**5**) with sulfur in a nonbridging position, the subsequent preparation of geranyl *S*-thiolodiphosphate (**6**), and preliminary studies of **6** as a substrate for FPPase.

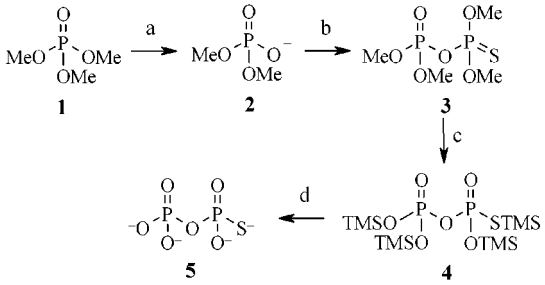


6 X = S
7 X = O

Allylic isoprenoid diphosphates are most easily prepared by a direct displacement of the corresponding chloro or bromo derivative with inorganic pyrophosphate.[13] We based our synthesis of **6** on the displacement procedure, assuming that the nucleophilicity of the sulfur atom in the thiopyrophosphate nucleophile would be sufficiently dominate to give the thiolo derivative as the sole regioisomer.[14]

The synthesis of thiopyrophosphate (**5**) is outlined in Scheme 1. Trimethyl phosphate was heated at 90−100 °C

Scheme 1



[a]nBu_4NOH [b]$(MeO)_2P(S)Cl$ [c]TMSI [d]nBu_4NOH/H_2O

with 1 equiv of tetrabutylammonium hydroxide (TBAH) for 24 h to give dimethyl phosphate in 95% yield. We used TBAH for the hydrolysis because the resulting salt is highly soluble in organic solvents[13] and can be used directly in the next steps without having to exchange the counterion. Treatment of **2** with dimethyl thiophosphochloridate gave tetramethyl thiolodiphosphate **3** in a 30% yield. The tetramethyl ester was unstable to the reaction conditions. The best yields were obtained when the reaction was conducted at −35 °C for 30 min, and the mixture was immediately transferred to a silica column to separate **3** from unreacted **2**. The proton-decoupled ^{31}P NMR spectrum of **3** was an AB quartet with doublets at 55.6 and −13.8 ppm (J = 20 Hz) for P(S) and P(O), respectively.

The methyl groups in tetraester **3** were removed at −35 °C in 97% yield using a 5-fold excess of trimethyliodosilane (TMSI). Initial attempts using trimethylbromosilane (TMSBr) or TMSI generated in situ by treating TMSBr with NaI gave poorer yields. The mechanism for dealkylation, displacement of iodide from TMSI by the phosphoryl oxygen or thiophosphoryl sulfur followed by a second displacement at the methyl groups by iodide,[15] predicts formation of thiolo-TMS diphosphate **4**. The TMS derivative was too unstable to be chromatographed, but a proton-decoupled ^{31}P NMR spectrum of the reaction mixture had a four-line pattern consistent with an AB quartet with peaks at −32.64 and 29.94 ppm (J = 16.5 Hz) for P(O) and P(S) resonances, respectively. Diphosphate **4** was desilylated by slow addition of 40% (v/v)

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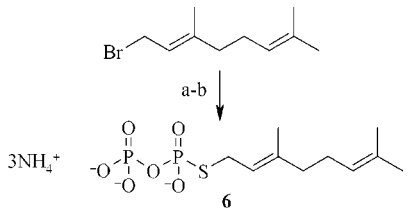
tetrabutylammonium hydroxide to give the isomer of thiopyrophosphate with sulfur in a nonbridging position. Thiopyrophosphate **5** is sensitive to strongly basic conditions. The pH of the solution was monitored as the TMS groups were removed, and the addition of tetrabutylammonium hydroxide was stopped at pH 7−7.5, where **5** is a trianion. The tris(tetra-*n*-butylammonium) salt was extracted into benzene, and solvent was removed at reduced pressure to give a viscous pale yellow oil. A proton-decoupled ^{31}P NMR showed an AB quartet with resonances at 36.88 and −7.68 ppm (J = 30 Hz) for P(S) and P(O), respectively. The resulting salt was used directly for the thiolophosphorylation of geranyl bromide without additional purification.

Geranyl *S*-thiolodiphosphate was prepared by the protocol of Davisson et al.[13] with minor modifications (see Scheme 2). Geranyl bromide was added dropwise to a well-stirred

Scheme 2



[a]Tris-(tetra-n-butylammonium)thiopyrophosphate, CH_3CN.
[b]Dowex AG 50W-X8 (NH_4^+ form).

solution of 2.3 equiv of **5** in CH_3CN at −30 °C over a period of approximately 30 min. The salt was then passed through a short ion exchange column (NH_4^+ form), and the material was chromatographed on cellulose to afford geranyl *S*-thiolodiphosphate (**6**) in 80% yield. The proton-decoupled ^{31}P NMR spectrum of **6** had a characteristic AB pattern with doublets (J = 29 Hz) at 7.97 and −7.43 ppm for P(S) and P(O), respectively. In an ^{1}H-coupled ^{31}P spectrum, each peak of the doublet at 7.97 ppm was further split into triplets (J = 8.6 Hz) by the C(1) protons in the geranyl moiety. An ^{1}H NMR spectrum of **6** confirmed the thiolo structure. The C(1) methylene protons in GPP appear as a triplet (coupling to the proton at C(2) and the P(1) phosphorus) at 4.46 ppm. In **6** the C(1) protons also appear as a triplet at 3.5 ppm, as expected for the C−S−P linkage.

Although sulfur is substantially more nucleophilic than oxygen, we used a 2.3-fold excess of **5** in the displacement reaction with geranyl bromide to guard against competing displacement by one of the four anionic oxygens in the tris-salt. Under these conditions geranyl *S*-thiolodiphosphate (**6**) was the only product.

We conducted preliminary biochemical experiments with **6** as a substrate for the chain elongation reaction catalyzed by recombinant avian FPPase.[16] As anticipated, the thiolodiphosphate is a good inhibitor of the enzyme with IC_{50} = 28 μM. In comparison, K_M^{GPP} = 4.5 μM under similar conditions. Although IC_{50} and K_M are not strictly comparable kinetic constants, the somewhat larger values for IC_{50} for **6** may reflect the larger C−S and P−S bonds in the thio analogue as well as differences in the C−S−P and C−O−P bond angles. Preliminary studies also indicate that **6** is a substrate for FPPase, although the thiolo analogue is *substantially* less reactive than GPP. Recombinant FPPase was incubated with [^{14}C]IPP (20 μM, 10 μCi/μmol) and **6** or GPP (50 μM), and the rates of the reactions were determined by the standard acid lability assay.[17] Under conditions in which the condensation of [^{14}C]IPP and GPP gave 2471 dpm in a 10 min incubation, no detectable formation of product was seen for [^{14}C]IPP (20 μM, 10 μCi/μmol) and thio analogue **6** (50 μM). When the concentration on FPPase was increased 100-fold and the time of the incubation was lengthened 10-fold, 130 dpm were seen above background. When extrapolated back to the normal assay conditions, the reaction of IPP with **6** is > 10^5 times slower than that for GPP.

FPPase (34 μg) was incubated with [^{14}C]IPP (20 μM, 55 μCi/μmol) and **6** (50 μM) in 200 μL of the assay buffer at 37 °C overnight (20 h).[18] A parallel incubation in which **6** was replaced by **7** was performed for 10 min. A 10 μL portion of each sample was co-spotted with cold FPP on a silica gel 60 F_{254} TLC plate, which was developed with 25:15:4:2 (v/v/v) $CHCl_3$/methanol/water/acetic acid. When the products were detected by phosphorimaging, a single new spot (R_f = 0.37), corresponding to FPP, was seen. In the incubation with **7**, all of the [^{14}C]IPP was consumed, while only 4% of the IPP radioactivity appeared in FPP for the incubation with **6**. Thus, thiolodiphosphate **6** is a slow reacting alternative substrate for GPP that binds with similar affinity to avian FPPase.

In summary, we describe a new useful synthesis of tris(tetra-*n*-butylammonium)thiopyrophosphate (**5**), the subsequent synthesis of geranyl *S*-thiolodiphosphate (**6**), and preliminary results demonstrating that the analogue for GPP is a slowly reacting alternate substrate. This procedure should provide a practical route to thiolodiphosphate esters that can be synthesized by the displacement protocol, including a wide variety of isoprenoid and nucleotide derivatives useful in biological studies.[11]

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Supporting Information Available: Complete experimental procedures for the synthesis of compounds **2**−**6** and conditions for acid-labile activity assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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(17) The acid-lability assay is described in the Supporting Information.

(18) The reaction mixture was prepared in the same manner as the assay mixture for the acid-lability assay.