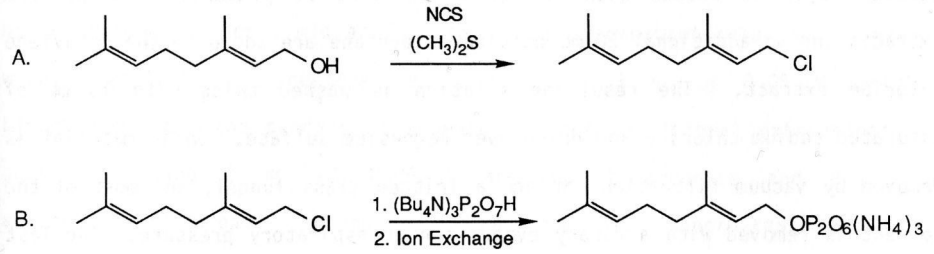
TRISAMMONIUM GERANYL DIPHOSPHATE

(Diphosphoric acid, mono(3,7-dimethyl-2,6-octadienyl) ester (E)-, trisammonium salt)



Submitted by Andrew B. Woodside, Zheng Huang, and C. Dale Poulter.[1]

Checked by Pamela Seaton and James D. White.

1. Procedure

A. Geranyl chloride. To a flame-dried, 100-mL, three-necked, round-bottomed flask equipped with a magnetic stirrer, low temperature thermometer, rubber septum, and nitrogen inlet adapter, is added 1.47 g (11 mmol) of N-chlorosuccinimide (Note 1). The powder is dissolved in 45 mL of dry dichloromethane (Note 2), and the resulting solution is cooled to -30°C with a dry ice/acetonitrile bath. Freshly distilled dimethyl sulfide (0.87 mL, 0.74 g, 12 mmol) is added dropwise by syringe. The mixture is warmed to 0°C with an ice-water bath, maintained at that temperature for 5 min, and cooled to -40°C. To the resulting milky white suspension is added dropwise by syringe 1.54 g (10 mmol) of geraniol (Note 3) dissolved in 5 mL of dry dichloromethane. The suspension is warmed to 0°C with an ice-water bath and stirred for 2 hr. The ice bath is then removed, and the reaction mixture is

allowed to warm to room temperature. Stirring is continued for an additional 15 min. The resulting clear, colorless solution is poured into a 250-mL separatory funnel and washed with 25 mL of saturated sodium chloride. The aqueous layer is washed with two 20-mL portions of pentane. The pentane extracts and an additional 20-mL portion of pentane are added to the methylene chloride extract. The resulting solution is washed twice with 10 mL of saturated sodium chloride and dried over magnesium sulfate. Solid material is removed by vacuum filtration through a fritted glass funnel, and most of the solvent is removed with a rotary evaporator at aspiratory pressure. The last traces of solvent are removed by pumping at high vacuum (0.2 mm) for 1.5 hr. The resulting pale yellow oil (1.61 g, 9.3 mmol, 93%) is used directly in the next step (Note 4).

B. Trisammonium geranyl diphosphate. To a flame-dried, 100-mL, round-bottomed flask equipped with a magnetic stirrer and a nitrogen inlet adapter is added 9.14 g (9.3 mmol) of tris(tetrabutylammonium) hydrogen pyrophosphate trihydrate (Note 5). The flocculant white solid is dissolved in 20 mL of dry acetonitrile (Note 6). To the resulting milky white suspension (Note 7) is added 0.83 g (4.8 mmol) of geranyl chloride. The mixure is allowed to stir at room temperature for 2 hr. Solvent is then removed with a rotary evaporator using a 40°C water bath. The pale yellow residue is dissolved in 3 mL of ion exchange buffer (Note 8), and the resulting clear solution is loaded onto a 4 x 15-cm column of Dowex AG 50W-X8 (100-200 mesh) cation exchange resin (ammonium form) (Note 9). The flask is washed twice with 5 mL of buffer and both washes are loaded onto the column before elution with 360 mL (two column volumes) of ion exchange buffer (Note 10). The eluant is collected in a 500-mL freeze-drying flask, frozen as described in Note 5, and lyophilized for 18-24 hr (Note 11) to yield 2.57 g of a white solid. The material is dissolved

in 5 mL of 0.05 M ammonium bicarbonate, and the clear solution is transferred to a 50-mL centrifuge tube. Twenty milliliters of 1:1 (v/v) acetonitrile: isopropyl alcohol is added, and the contents are mixed thoroughly on a vortex mixer, during which time a white precipitate forms. The suspension is cleared by centrifugation for 5 min at 2000 rpm. The supernatant solution is removed with a pipette, the residue is suspended in 5 mL of 0.05 M ammonium bicarbonate, and the process is repeated. Three additional extractions are performed using 2 mL of 0.05 M ammonium bicarbonate and 8 mL of acetonitrile:isopropyl alcohol. The combined supernatant solutions (approximately 80 mL) are concentrated to approximately 5 mL with a rotary evaporator at 40°C (Note 12).

One half of the concentrated extract, dissolved in an equal volume of chromatography buffer (Note 13), is loaded onto a 5.5 x 18-cm cellulose flash column[2] (Note 14). The flask is rinsed with three 5-mL portions of chromatography buffer and each is loaded onto the column. The column is then eluted with 900 mL of chromatography buffer. After a 50-mL forerun, twenty-eight 30-mL fractions are collected, and every second fraction is analyzed by thin layer chromatography (Note 15). Fractions containing trisammonium geranyl diphosphate (typically 12-23) are pooled and concentrated to approximately 120 mL with a rotary evaporator at 40°C. The concentrate is transferred to a 250-mL freeze-drying flask and lyophilized for 18-24 hr as previously described in Note 5. The resulting flocculant white solid is collected and stored at -78°C. The cellulose chromatography is repeated to yield a total of 1.51-1.55 g (85-87%) of trisammonium geranyl diphosphate from geraniol (Note 16).

2. Notes

1. N-Chlorosuccinimide (from the Aldrich Chemical Company, Inc.) is recrystallized from benzene (*CAUTION: CARCINOGENIC*).

2. Methylene chloride is distilled from phosphorus pentoxide immediately before use.

3. Geraniol (from the Aldrich Chemical Company, Inc.) is distilled before use, bp 90-92°C at 3 mm.

4. The IR, ^{1}H NMR, and ^{13}C NMR spectra of this material are identical with those for distilled geranyl chloride (bp 49-51°C at 0.2 mm). Distillation on a small scale significantly reduces the yield, and there is no improvement in the yield of the phosphorylation reaction using distilled material. A synthesis of geranyl chloride was reported earlier in this series.[3] We find, however, that the procedure of Corey, Kim, and Takeda[4] is more convenient.

5. Disodium dihydrogen pyrophosphate (3.13 g, 14 mmol) (from Sigma Chemical Co.) is dissolved in 25 mL of deionized water containing 1 mL of concentrated ammonium hydroxide. The resulting clear solution is loaded onto a 2 x 30-cm column of Dowex AG 50W-X8 cation exchange resin (100-200 mesh, H^+) and eluted with deionized water. The first 150 mL of eluant is collected in a 250-mL freeze-drying flask. A magnetic stirring bar is added, and the solution is titrated to pH 7.3 by slow addition of tetrabutylammonium hydroxide (Aldrich Chemical Company, Inc.). The stirring bar is removed, and the flask is placed in a dry ice/propanol bath. The flask is spun slowly in a manner to uniformily freeze its contents to the walls. Water is removed by lyophilization for 24 hr. The resulting flocculant white solid (10.6 g, 83%) contains 3 to 4 waters of hydration and is used without further purification. The

material is extremely hygroscopic and can be stored in a desiccator over phosphorus pentoxide.

6. Reagent grade acetonitrile is distilled from phosphorus pentoxide immediately before use.

7. Clear solutions can be obtained by filtration. Residual water can be removed from freeze-dried salt by repeated evaporation of dry acetonitrile (rotary evaporator). This material is not noticeably more effective than the salt obtained after lyophilization.

8. Ammonium bicarbonate (2.0 g) is dissolved in 1 L of 2% (v/v) isopropyl alcohol/water. The resulting solution is 25 mM in ammonium bicarbonate.

9. The ammonium form of the resin is generated by placing 188 mL of Dowex AG 50W-X8 (100-200 mesh, H^+ form) in an 1-L feitted glass funnel and washing the material with four 200-mL portions of concentrated ammonium hydroxide. The resin is washed with 200-mL portions of deionized water until the pH of the filtrate drops to pH 7, then with two 200-mL portions of ion exchange buffer. The washed resin is suspended in 200 mL of buffer and slurry-packed into the column.

10. Dowex AG 50W-X8 (100-200 mesh) from BioRad has a capacity of 1.7 meq per mL of resin bed. This represents approximately a 10-fold excess of exchangeable ions in the resin over material loaded onto the column. However, the tetrabutylammonium cation has a lower affinity for the resin than the ammonium cation. To optimize the exchange, it is important to maintain a low concentration of ammonium ion in the exchange buffer, to elute the material slowly (less than 9 mL/min on the 4 x 15-cm column), and to elute with only two column volumes of exchange buffer; otherwise previously exchanged tetrabutylammonium cation will begin to elute from the column. Incomplete exchange

dramatically reduces the efficiency of the subsequent purification on cellulose. The efficiency of the exchange can be determined by ^{1}H NMR.

11. Trisammonium geranyl diphosphate will decompose if left under vacuum for extended periods. It is important to remove the sample from the freeze-drier within a few hours after water has been removed.

12. This material is stored at -20°C until chromatography on cellulose.

13. Chromatography buffer is prepared by dissolving 4.0 g of ammonium bicarbonate in 250 mL of deionized water and adding 500 mL of isopropyl alcohol and 250 mL of acetonitrile. The resulting solution is approximately 50 mM in ammonium bicarbonate.

14. Whatman CF11 fibrous cellulose powder is prepared for chromatography by the following procedure. Cellulose powder (1 L, dry volume) is mixed with 700 mL of deionied water in a 2-L beaker by vigorous stirring with a glass rod. The suspension is allowed to stand for 30 min, and the water is removed by decantation. The same procedure is followed as the cellulose is washed in succession with two 700-mL portions of 0.1 N hydrochloric acid, two 700-mL portions of deionized water, two 700-mL portions of 0.1 N sodium hydroxide, two 700-mL portions of deionized water, and two 700-mL portions of 1:1 (v/v) isopropyl alcohol:water. The material is stored at 4°C in 1:1 isopropyl alcohol:water until used. The column is slurry-packed in 1:1 isopropyl alcohol:water and washed with 1.3 L (approximately three colunn volumes) of acetonitrile. The column is then washed with 1.3 L of 1:1 isopropyl alcohol:water and equilibrated with 1.3 L of chromatography buffer (Note 13).

15. E. Merck cellulose thin-layer chromatography plates (available from American Scientific Products) are developed with chromatography buffer (Note 13) and visualized with sulfosalicylic acid/ferric chloride spray.[5] The system consists of a solution of 1.0 g of sulfosalicylic acid (from Aldrich

Chemical Co., Inc.) dissolved in 100 mL of 3:2 (v/v) ethanol:water and a solution of 0.20 g of ferric chloride in 100 mL of 4:1 (v/v) ethanol:water. Plates are first sprayed with the sulfosalicylic acid solution (thoroughly wetted but not dripping) and allowed to air dry. The ferric chloride solution is lightly sprayed onto the plates. Phosphate-containing compounds appear as white spots on a pink background. A second light spraying with ferric chloride may be necessary to make the spots pronounced. It is *important* to prepare both sprays freshly. Their shelf life is only about 6 hr. Under these conditions the trisammonium geranyl diphosphate has an R_f of 0.35. Residual tetrabutylammonium salt moves with the solvent front, and ammonium inorganic pyrophosphate remains at the origin.

16. This material migrates as a single spot on the cellulose thin-layer system and has no extraneous peaks in the ^1H, ^{13}C, and ^{31}P NMR spectra. The IR and NMR spectral properties of trisammonium geranyl diphosphate are as follows: IR (KBr) cm^{-1}: 3100-3500 (br), 2800,2990 (br), 1650, 1450, 1400, 1200, 1120, 1080, 1015, and 900; ^1H NMR (300 MHz, D_2O/ND_4OD) δ: 1.62 (s, 3 H, methy), 1.68 (s, 3 H, methyl) 1.72 (s, 3 H, methyl), 2.11 (m, 4 H, CH_2 at C_4 and C_5), 4.47 (t, 2 H, $J_{^1H,^1H}$ = 6.5, $J_{^1H,^{31}P}$ = 6.5, CH_2 at C_1), 5.22 (broad, 1 H, $J_{^1H,^1H}$ = 6.5, H at C_6), and 5.47 (t, 1 H, $J_{^1H,^1H}$ = 6.5, H at C_2); ^{13}C NMR (75 MHz, D_2O/ND_4OD, ^1H decoupled): 18.28 (CH_3), 19.69 (CH_3), 27.55 (CH_3), 28.60 (CH_2), 41.55 (CH_2), 65.42 (CH_2, d, $J_{^{13}C,^{31}P}$ = 4.0), 122.85 (CH, d, $J_{^{13}C,^{31}P}$ = 7.5), 127.10 (CH), 136.68 (C), and 145.76 (C); ^{31}P NMR (32 MHz, D_2O/ND_4OD, ^1H decoupled): -11.23 (d, 1 P, $J_{^{31}P,^{31}P}$ = 20) and -9.10 (d, 1 P, P_2).

3. Discussion

Previous methods for the preparation of salts of geranyl diphosphate and other allylic isoprenoid diphosphates are based on condensation between the alcohol and inorganic phosphate by trichloroacetonitrile as originally reported by Cramer[6] and modified by Popjak.[7] The reaction generates a complex mixture of organic and inorganic polyphosphates which must be separated by chromatography. The desired diphosphate ester has been prepared on small scales in yields of up to 30%,[8] but in our experience, the yields of pure material obtained by this procedure are usually less than 10%.

The direct displacement reaction can be used to prepare many of the common diphosphate esters in the isoprene biosynthetic pathway,[9] including isopentenyl diphosphate.[10,11] The yields are typically 60-90% from the alcohol, and the absence of phosphate polymers found in the Cramer procedure simplifies the purification step. We have also used the displacement procedure to prepare radio-labeled material for biosynthetic studies.[12]

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Appendix

Chemical Abstracts Nomenclature (Collective Index Number); (Registry Number)

Geranyl chloride: 2,6-Octadiene, 1-chloro-3,7-dimethyl-, (E)- (8,9); (5389-87-7)

N-Chlorosuccinimide: Succinimide, N-chloro- (8); 2,5-Pyrrolidinedione, 1-chloro- (9); (128-09-6)

Geraniol: 2,6-Octadien-1-ol, 3,7-dimethyl-, (E)- (9); (106-24-1)

Tris(tetrabutylammonium) hydrogen pyrophosphate: 1-Butanaminium, N,N,N-tributyl-, diphosphate (3:1) (10); (76947-02-9)

Disodium dihydrogen pyrophosphate: Pyrophosphoric acid, disodium salt (8); Diphosphoric acid, disodium salt (9); (7758-16-9)

Tetrabutylammonium hydroxide: Ammonium tetrabutyl-, hydroxide (8); 1-Butanaminium, N,N,N-tributyl-, hydroxide (9); (2052-49-5)