SYNTHESIS OF A TRITIUM LABELED PHOTOLABILE ANALOGUE OF FARNESYL DIPHOSPHATE: (E,E)-[1-^{3}H]-(2-DIAZO-3-TRIFLUOROPROPIONYLOXY)GERANYL DIPHOSPHATE (DATFP-GDP)

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SUMMARY

Tritiated (E,E)-(2-diazo-3-trifluoropropionyloxy)geranyl diphosphate (DATFP-GDP) has been used as a photolabile analogue of (E,E)-farnesyl diphosphate (E,E-FDP) for an aid in isolating enzymes utilizing E,E-FDP as a substrate. We now report an alternative method of synthesizing this probe in which the tritium label is introduced in the step just before the introduction of the diphosphate group. Thus, DATFP-geraniol is oxidized to DATFP-geranial with activated manganese dioxide. The tritium label is introduced by reduction of the aldehyde with $NaBT_4$. The DATFP-group successfully withstands both of these steps. The overall yield for these two steps is 69%. Diphosphorylation of the resulting alcohol afforded DATFP-[1-^{3}H]-GDP in 8% yield with a specific activity of 48.6 μCi/μmol and radiochemical purity of 94%.

Key words: Farnesyl Diphosphate Analogue, DATFP-GDP, Tritium, (E,E)-(2-Diazo-3-trifluoropropionyloxy)geranyl Diphosphate.

INTRODUCTION

Allen and coworkers (1,2) and Benedict and coworkers (3) have successfully utilized (E,E)-(2-diazo-3-trifluoropropionyloxy)geranyl diphosphate (DATFP-GDP) as a photolabile analogue of (E,E)-farnesyl diphosphate (E,E-FDP). E,E-FDP is the biosynthetic precursor of gossypol (**1**) (4,5), and δ-cadinene (**2**) has been proposed as a biosynthetic intermediate leading to gossypol (6,7). Our interest in the isolation of δ-cadinene cyclase led us to consider DATFP-GDP as a potential photoaffinity probe to assist in the isolation of this enzyme. We now report a

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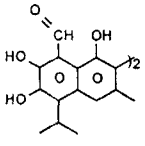
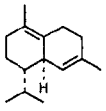
modification of Baba and Allen's synthesis (1) which allows the introduction of tritium at a latter step in the synthesis. ^{1}H-, ^{13}C-, and ^{31}P-NMR data are provided to fully characterize the synthetic intermediates.



(1)
Gossypol



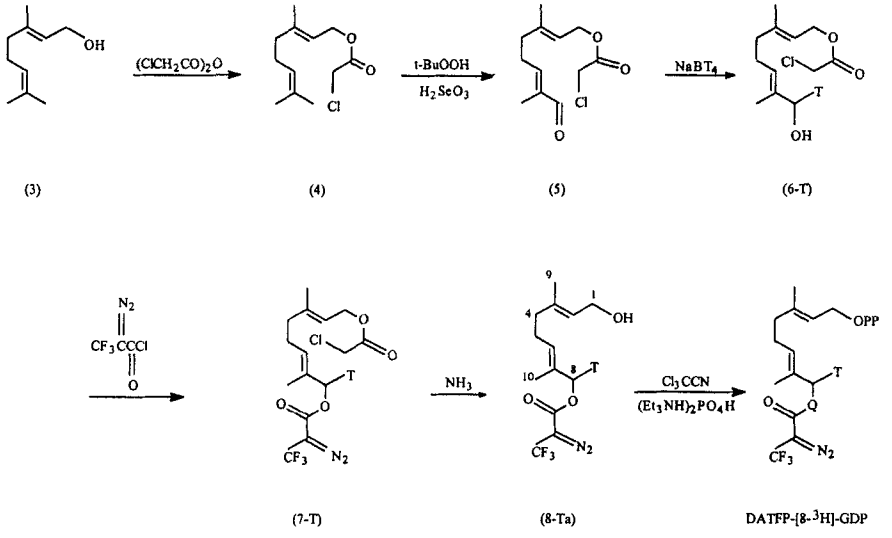
(2)
δ-Cadinene

RESULTS AND DISCUSSION

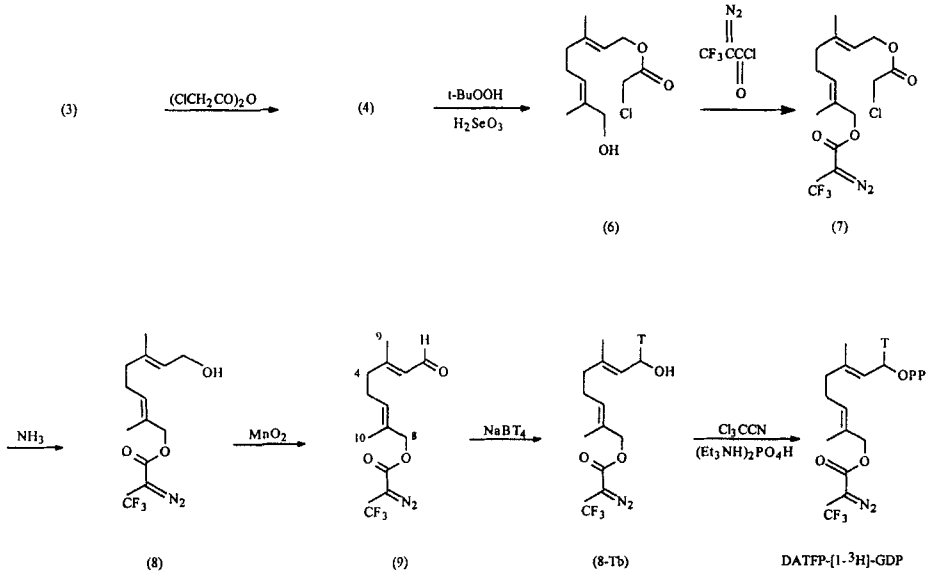
Baba and Allen reported the synthesis of DATFP-GDP in which tritium is introduced before the DATFP-group is added (Scheme I). We found that the addition of tritium could be delayed until the step before conversion of the alcohol to the diphosphate group with a much higher yield (Scheme II). In each sequence, geraniol **3** is converted to the α-chloroester **4**. Baba and Allen oxidize this to the aldehyde **5** (2). A slight modification of this procedure provides the alcohol **6** (Scheme II) (1). Baba and Allen introduce the tritium at C-8 by reduction of the aldehyde **5** to the tritiated alcohol **6-T**. However, the α-chloroester group of **5** is also very easily reduced under the reduction condition, and the reaction gives a moderate to low yield of the desired alcohol **6** (yield varied from 23% to 50% from **5** to **6** in our hands). The alcohols **6** or **6-T** are converted to the DATFP product **7** or **7-T**. The esters (**7** or **7-T**) are hydrolyzed to provide the alcohols **8** or **8-Ta**. Compound **8** is oxidized to the aldehyde **9** with activated MnO_2 (Scheme II). The resulting aldehyde provides the opportunity to introduce tritium at C-1 to give the alcohol **8-Tb**. The DATFP ester group of aldehyde **9** is relatively stable toward the reduction conditions, providing the desired alcohol **8-Tb** in higher yield compared to Allen's method (89% from **9** to **8-Tb**). The alcohols (**8-Ta** or **8-Tb**) are converted to the diphosphate by the method of Popjak, et al. (8).

Starting from 97.4 μmol of geraniol we obtained 5.87 μmol of DATFP-GDP with a specific activity of 48.6 μCi/μmol utilizing $NaBT_4$ (25 mCi, 1000 mCi/mmol).

SCHEME I



SCHEME II



EXPERIMENTAL

Materials.

Sodium borotritide (25 mCi, 1000 mCi/mmol) was purchased from New England Nuclear. High purity solvent (HPLC grade) toluene, hexane, methanol are products of Burdick & Jackson. A.C.S. grade ethyl acetate and diethyl ether were obtained from EM Science. Silica gel TLC (F-254 nm) plates were purchased from Aldrich (Z12-272-6).

Instrumentation. ^{1}H-(300 MHz) and ^{13}C-(75 MHz) NMR spectra were recorded on a Bruker ARX-300 instrument in $CDCl_3$ with residual $CHCl_3$ as internal standard unless specified otherwise. Spectrophotometric measurements were performed on a Beckman DU 70 spectrophotometer with 1-cm quartz cuvettes.

Pretreatment of Amberlight XAD-2 resin and DEAE cellulose (0.85 meq/g, medium mesh, D-8382). Amberlight XAD-2 resin (Sigma Chemical) (50 ml, dry volume) was soaked in 200 ml of deionized water for 10 min and the supernatant was decanted. This operation was repeated several times until the supernatant became neutral. The resin was then washed alternately with aqueous 0.01 N NH_3 and 0.01 N NH_3 in methanol four times in a sintered glass funnel mounted on a suction flask. When fresh wash solution was introduced, the resulting suspension was allowed to settle for about 15 min before it was filtered off. The resin was washed further with aqueous 1 mM NH_3 and 1 mM NH_3 in MeOH. Finally, it was washed with aqueous 1 mM NH_3 twice and stored in aqueous 1 mM NH_3 in a refrigerator.

DEAE-Cellulose (Sigma Chemical) (5 g) was gently stirred in 100 ml of deionized water and allowed to settle for 1 - 2 min. The supernatant was decanted to remove fines. This operation was repeated 3 times. The anion exchanger was then stirred in 100 ml of 0.5 N HCl and allowed to stand for 30 min. The supernatant was decanted and the anion exchanger was washed with

deionized water until the wash was at pH ≥ 4. The ion exchanger was then stirred in 100 ml of 0.5 N NaOH and left for a further 30 min. The supernatant was decanted. This operation was repeated and the anion exchanger was washed until the wash was near neutral. The anion exchanger was soaked in 100 ml of 800 mM ammonium formate in methanol with occasional stirring for 10 min, and the suspension was allowed to settle for 1 - 2 min. The supernatant containing fines was decanted. Finally, the anion exchanger was soaked in 100 ml of 80 mM ammonium formate in methanol and filtered. It was stored in 80 mM ammonium formate in methanol at room temperature. Storage at these conditions should not exceed 2 days. It is ideal to prepare the anion exchanger immediately before use.

8-DATFP-Geraniol. It was prepared according to the general procedures of Allen and Baba (9), except for the purification of one of the intermediates, 8-DATFP-geranyl chloroacetate 7. Thus, after the normal work up, the crude product 7 (0.73 g) was separated on a silica gel (Baker, 40-140 mesh 60Å) column (toluene slurry packed, 2.2 x 16 cm). The column was eluted with a gradient of EtOAc and toluene at a rate of 2.4 ml/min (18 ml/fraction). The gradient started from 0% EtOAc in toluene and increased 1% in EtOAc per 50 ml increment of eluent. A total of 28 fractions were collected and all the odd numbered fractions were analyzed by silica gel TLC (toluene/EtOAc, 9/1). The presence of product was detected under UV light (dark spot). The product in fractions 5-14 (Rf = 0.70) were pooled and concentrated to give the desired intermediate 7 as an oil (0.631 g, 79% starting from 0.512 g of 8-hydroxy-geranyl chloroacetate). Hydrolysis of the chloroacetate was accomplished according to the method of Allen and Baba (9) to give 8-DATFP-geraniol. NMR data for 8-DATFP-geraniol: ^{1}H-NMR ($CDCl_3$) δ 5.435 (1H, tq, $J_{5,6}$ = 7.0 Hz, $J_{6,10}$ = 0.9 Hz, H6), 5.368 (1H, tq, $J_{1,2}$ = 6.8 Hz, $J_{2,9}$ = 0.9 Hz, H2), 4.590 (2H, s, H8), 4.108 (2H, d, H1), 2.152 (2H, dt, $J_{4,5}$ = 7.2 Hz, H5), 2.038 (2H, t, H4), 1.634 (3H, d, H9 or H10), 1.616 (3H, d, H10 or H9); ^{13}C-NMR ($CDCl_3$) δ 160.82 (C11), 138.74 (C3), 130.02 (C6), 129.38 (C7), 123.98 (C2), 122.63 (C13, q, $^1J_{CF}$ = 269.2 Hz), 71.54 (C8), 61.34 (C12, broad q, $^2J_{CF}$ = 44.2 Hz), 59.27 (C1), 38.66 (C4), 25.80 (C5), 16.12 (C9), 13.73 (C10); UV (absolute EtOH) λ_{max} (ε) = 233 nm (11650).

8-DATFP-Geranial (**9**). 8-DATFP-Geraniol (0.362 g, 1.18 mmol) dissolved in 26 ml of hexane was cooled in an ice-water bath, and activated manganese dioxide (10) (6.3 g, 72.5 mmol) was added in 3 portions during a 20 min period under nitrogen while stirring. The mixture was stirred for an additional 2.5 hr. at 0°C. The reaction mixture was filtered by vacuum and the solid was washed thoroughly with hexane (150 ml). The filtrate was concentrated to give the title compound as a pale oil (0.282 g, 78%). ^{1}H-NMR ($CDCl_3$) δ 9.973 (1H, d, $J_{1,2}$ = 8.0 Hz, H1), 5.849 (1H, dq, $J_{1,2}$ = 8.0 Hz, $J_{2,9}$ = 0.9 Hz, H2), 5.430 (1H, m, H6), 4.602 (2H, s, H8), 2.263 (2H, s, H5 or H4), 2.252 (2H, s, H4 or H5), 2.151 (3H, d, H9) 1.641 (3H, s, H10); ^{13}C-NMR ($CDCl_3$) δ191.14 (C1), 162.70 (C3), 160.74 (C11), 130.62 (C7), 128.37 (C2 or C6), 127.54 (C6 or C2), 122.61 (C13, q, $^1J_{CF}$ = 270.2 Hz), 71.08 (C8), 39.71 (C4), 25.28 (C5), 17.59 (C9), 13.83 (C10).

[1-^{3}H]-8-DATFP-Geraniol (**8-Tb**). The title compound was prepared according to the general method of Baba et al. (2). All operations were conducted in a well ventilated hood (Caution: tritium gas is released in the reaction). In a 25 ml, round-bottomed flask was placed 8-DATFP-geranial (12 mg, 0.0395 mmol) dissolved in 0.5 ml of absolute ethanol. The flask was cooled to 0°C by an ice-water bath under nitrogen. Sodium borotritide (25 mCi, 1000.0 mCi/mmol, 0.025 mmol, slightly pink crystals) was dissolved in 0.8 ml of absolute ethanol and added to the flask dropwise while stirring. The wall of the NaB^3H_4 vial was washed with absolute ethanol (2 x 0.8 ml) and the two washes were also slowly added to the reaction mixture. The reaction mixture was stirred for an additional 20 min at 0°C under nitrogen. HCl (0.1N) was added to the reaction mixture until the pH of the reaction mixture reached about 3 (~ 1 ml). The reaction mixture was stirred for 2 min and 0.5 ml of 1 M $NaHCO_3$ along with 2 ml of water were added. The reaction mixture was extracted with ether (3 x 15 ml). The combined ether solution was washed with 10 ml of distilled water. The ether phase was dried over Na_2SO_4 (anhydrous) and filtered. The filtrate was concentrated at reduced pressure to give 37.2 mg of crude product.

The crude product was dissolved in 0.6 ml of toluene and loaded onto a silica gel column (1 x 17 cm, slurry packed in toluene). The sample container was washed with toluene (3 x 0.8 ml)

and each was loaded onto the column. The column was eluted at a rate of 1 ml/min (5 ml/fractions). A gradient of toluene/EtOAc was used beginning with 1% EtOAc and increasing in 1% increments to 8%, in 2% increments to 10%, and in 3% increments to 37% EtOAc; 10 ml of eluent was utilized at each increment. The fractions were monitored by TLC (silica gel, toluene/EtOAc, 9:1). Fractions 14 to 20 containing desired product (Rf = .282) were pooled and concentrated at reduced pressure to give [1-^{3}H]-8-DATFP-geraniol (3.59 mCi, 0.036 mmol, specific activity 101 mCi/mmol, 89% yield). The amount of product in moles was determined spectrophotometrically using ε = 11650 at 233 nm. ^{1}H-NMR ($CDCl_3$) and UV spectra of the product were identical to that of the corresponding cold material, 8-DATFP-geraniol (**8**).

A corresponding deuterated material, [1-^{2}H]-8-DATFP-geraniol was prepared in an identical way (11.2 mg, 0.0365 mmol) with an identical UV spectrum in absolute ethanol. ^{1}H-NMR ($CDCl_3$) δ 5.473 (1H, t, $J_{5,6}$ = 6.5 Hz, H6), 5.408 (1H, d, $J_{1,2}$ = 6.7 Hz, H2), 4.630 (2H, s, H8), 4.134 (1H, d, H1), 2.190 (2H, dt, $J_{4,5}$ = 7.2 Hz, H5), 2.081 (2H, t, H4), 1.679 (3H, s, H9 or H10), 1.656 (3H, s, H10 or H9). This material was mixed with the above hot [1-^{3}H]-8-DATFP-geraniol and the resulting mixture is referred to as [1-^{3}H, ^{2}H]-8-DATFP-geraniol (0.0718 mmol, 49.9 mCi/mmol, [^{2}H]/[^{3}H] = 1.03).

[1-^{3}H,^{2}H]-8-DATFP-Geranyl Diphosphate. This material was prepared according to the procedures of Popjak et al. (8) and Halloway et al. (11) with modification. [1-^{3}H,^{2}H]-8-DATFP-Geraniol (3.59 mCi, 0.0718 mmol, 49.9 mCi/mmol) and trichloroacetonitrile (65 μl, 0.65 mmol) were placed in an oven-dried 10 ml pear-shaped flask equipped with a magnetic stirrer and a pressure equalizing dropping funnel. Bis(triethylammonium) hydrogen phosphate (66 mg, 0.22 mmol) dissolved in dry acetonitrile (1.3 ml) was introduced through the funnel over a period of 20 min under nitrogen at room temperature with stirring. The reaction mixture was stirred overnight at room temperature under nitrogen. The reaction mixture was extracted with 0.1 N NH_3 (3 x 3 ml) after mixing with 9 ml of EtOAc/hexane (1:3). The aqueous phase was washed with EtOAc/hexane (1:3) (3 x 6 ml) and then shell frozen on the surface of a 25-ml freeze-drying

bottle. Lyophilization afforded 37.2 mg of crude product as a flocculent yellowish solid (1.97 mCi). Purification of this crude product was accomplished by Amerlight XAD-2 and DEAE cellulose column chromatography.

The pretreated Amberlight XAD-2 was slurry packed in 1 mM NH_3 (1 x 19.5 cm). The column was washed with 200 ml of 1 mM NH_3 in methanol (at this point, no more column material eluted as evidenced by UV analysis). After equilibrating with 200 ml 1 mM NH_3, the column was loaded with 4 μmol of nonradioactive 8-DATFP-geranyl diphosphate and eluted first with 75 ml of 1 mM NH_3, then with 50 ml of 1 mM NH_3 in methanol at a flow rate of 1 ml/min (5 ml/fractions). At the end of the elution, no more 8-DATFP-geranyl diphosphate was detected (UV monitored). The column was then equilibrated with 200 ml of 1 mM NH_3. The radioactive crude [1-^{3}H,^{2}H]-8-DATFP-geranyl diphosphate (1.97 mCi) dissolved in 0.7 ml of 1 mM NH_3 was loaded onto the column. The wall of the container was washed three times (3 x 0.5 ml) and combined washes were loaded onto the column. The column was eluted first with 1 mM NH_3 at a flow rate of 0.8 ml/min (82 ml) and then with 1 mM NH_3 in methanol at a flow rate of 1 ml/min; 2-ml fractions were collected for the first 4 fractions, 3-ml for 5th and 6th, 4 ml for 7th and 8th, 5 ml for 9th and 10th, and 20-ml for the rest of the fractions up to the 24th fraction. Each fraction was monitored with a liquid scintillation counter and UV spectrophotometer. Though radioactivity was detected as early as the 5th fraction, a good UV spectrum with a characteristic absorption maxima at 238 nm was not shown until the 10th fraction. Fractions 10 - 24 were pooled and evaporated to dryness by rotary evaporator at reduced pressure to give a crude product as a white solid.

This material was dissolved in 0.5 ml of 80 mM ammonium formate in methanol and loaded onto a DEAE-cellulose column [1 x 7.7 cm, slurry packed in 80 mM ammonium formate in methanol (buffer A) and equilibrated by passing the same buffer through the column (200 ml)]. The container wall was washed with the same buffer five times (5 x 0.5 ml) and each was loaded onto

the column. The column was eluted first with buffer A (25.3 ml) then with a methanol solution containing 80 mM ammonium formate and 0.2 N ammonia (buffer B, 80 ml) at a flow rate of 0.58 ml/min (2.3 ml/fraction). All fractions were monitored with a liquid scintillation counter and TLC plates [silica gel, isopropanol/water/ammonia (conc), 6:3:1]. [1-^{3}H,^{2}H]-8-DATFP-Geranyl diphosphate was eluted between the 13th and 25th fractions and appeared as a dark spot on the TLC plate under UV light (Rf=0.09). The corresponding monophosphate had a Rf value of 0.49 (fractions 4 ~ 7). Fractions 13 ~ 25 were pooled and shell frozen in two 50-ml freeze-drying bottles. Lyophilization afforded 3.6 mg of a white flocculent solid (285.1 μCi, 8%). The amount of product was more accurately determined with a UV spectrophotometry using an ε value of 14000 at λ = 236 nm given by Allen et al. (1). The amount of material thus determined was 5.868 μmol which resulted in a specific activity of 48.6 μCi/μmol. Half the material was stored in its natural solid state and the other half was stored in 1 mM NH_3 in distilled deionized water (2.93 μmol/ml or 142 μCi/ml) under nitrogen at -78°C. Radiochemical purity of the final product is 94% based on silica gel TLC. ^{1}H-NMR (D_2O, DOH signal was taken as δ 4.839 ppm) δ 5.589 (1H, t, $J_{5,6}$ = 6.7 Hz, H6), 5.487 (1H, distorted t, $J_{1,2}$ = 5.5 Hz, H2), 4.689 (2H, s, H8), 4.488 (1.5H, distorted dd, $^{3}J_{1,P}$ = 5.5 Hz, H1), 2.250 (2H, dt, $J_{4,5}$ = 7.2 Hz, H5), 2.145 (2H, t, H4), 1.739 (3H, s, H9), 1.687 (3H, s, H10); UV (1 mM NH_3) λ_{max} (ε) = 236 nm (14000). The corresponding unlabeled material had identical UV and ^{1}H-NMR spectra except that for the unlabeled material the integration for H1 was exactly two and both H1 and H2 were sharp triplets. The ^{13}C-NMR and ^{31}P-NMR for the unlabeled material were as follows: ^{13}C-NMR (D_2O, CH_3OH signal taken as δ = 49.00 ppm) δ 163.33 (C11), 142.33 (C3), 130.23 (C6 or C7), 130.02 (C7 or C6), 122.86 (C13, q, $^{1}J_{CF}$ = 268.4 Hz), 120.33 (C2, d, $^{3}J_{C,P}$ = 9.1 Hz), 71.85 (C8), 62.56 (C1, d, $^{2}J_{C,P}$ = 5.0 Hz), 62.03 (C12, broad q, $^{2}J_{C,F}$ = 46.1 Hz), 38.45 (C4), 25.62 (C5), 15.74 (C9), 13.06 (C10); ^{31}P-NMR (D_2O, H_3PO_4 as external standard, 0.0 ppm) (^{1}H-decoupled) δ - 5.152 (d, J_{PP} = 22.2 Hz, external P), -9.062 (d, internal P); (^{1}H-coupled): -5.152 (d, J_{PP} = 22.2 Hz, external P), -9.062 (dd, $^{3}J_{H,P}$ = 6.0 Hz, internal P).

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