Journal of Chromatography, 645 (1993) 161–167
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 287

Rapid synthesis of isoprenoid diphosphates and their isolation in one step using either thin layer or flash chromatography

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(First received April 1st, 1993; revised manuscript received May 10th, 1993)

ABSTRACT

A rapid procedure for the preparation of short-chain (C_5–C_{20}) isoprenoid diphosphates is described. It is based on the method of Cornforth and Popjak [*Methods Enzymol.*, 15 (1969) 359–390] which utilizes bis-triethylammonium phosphate in trichloroacetonitrile as the phosphorylating reagent. The reaction takes place in 15 min, and product isolation, previously requiring several steps, is done in a single step using either preparative thin-layer chromatography or flash chromatography on silica. From a single TLC plate, up to 50 μmol of pure farnesyl diphosphate (*i.e.*, *ca.* 20 mg) can be isolated, while up to 1200 μmol can be isolated using a standard flash chromatography column.

INTRODUCTION

Isoprenoid diphosphates [*e.g.*, isopentenyl diphosphate (IPP), dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP)] are key intermediates in the branched pathway of mevalonate metabolism [1]. This pathway includes the biosynthesis of cholesterol, dolichol, ubiquinone, prenylated proteins, heme A, and isopentenyl tRNA. Accordingly, isoprenoid diphosphates are required in both radioactive and non-radioactive forms in order to carry out *in vitro* studies. Of the various procedures described for the preparation of these compounds [2–4] all require considerable time for synthesis; further, several steps are involved in product isolation. In the present report, we describe a procedure for the rapid preparation of pure isoprenoid diphosphates. The original procedure of Cornforth and Popjak [2] has been modified such that the reaction is much more rapid; in addition, product isolation is carried out in a single step using either preparative silica TLC for reactions in which up to 200 μmol of alcohol are phosphorylated, or flash chromatography for large-scale reactions using up to 4000 μmol of alcohol.

EXPERIMENTAL

[^{3}H]Farnesol was prepared according to Adair *et al.* [5]. Orthophosphoric acid (HPLC grade) was from Fisher Chemical (Pittsburgh, PA, USA). Triethylamine was from Sigma (St. Louis

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0021-9673/93/$06.00

MO, USA). Trichloroacetonitrile, 3-methyl-3-buten-1-ol (isopentenol), 3-methyl-2-buten-1-ol (dimethylallyl alcohol), geraniol, and farnesol [95% *trans* (*E,E*), 5% *cis* (*Z,E*)] were from Aldrich (Milwaukee, WI, USA). Geranylgeraniol was from TCI Chemicals (Seattle, WA, USA). All solvents were HPLC grade from Fisher Chemical. Solution A is prepared by diluting 25 ml of concentrated phosphoric acid (HPLC grade) into 94 ml acetonitrile. Solution B is prepared by diluting 110 ml triethylamine into 100 ml acetonitrile. Mobile phase 1 is isopropanol–conc. NH_4OH–H_2O (6:2.5:0.5). Mobile phase 2 is isopropanol–conc. NH_4OH–H_2O (6:3:1). All commercial chemicals were used without further purification. All glassware was treated with Sigmacote (Sigma) prior to use.

HPLC

HPLC was carried out on a Waters 501 instrument equipped with on-line UV monitoring (210 nm). The stationary phase was a 22 cm silica (5 μm) column from Brownlee Labs (obtained through Alltech, Deerfield, IL, USA). The mobile phase was 2% reagent alcohol in hexane and the flow-rate was 1 ml/min. "Reagent alcohol" is a mixture of 5% methanol, 90% ethanol, 5% 2-propanol from Fisher Chemical.

TLC

Analytical TLC was carried out using plastic-backed silica gel 60 plates from E. Merck (cat. No. 5735-7, obtained from Alltech). Preparative TLC was carried out using linear K plates (see below). Detection was achieved by spraying with either water (see below) anisaldehyde solution or molybdenum reagent. Anisaldehyde solution is 90 volumes ethanol, 5 volumes *p*-anisaldehyde (Sigma), and 5 volumes concentrated H_2SO_4. Molybdenum spray reagent was from Sigma.

Flash chromatography

CAUTION! Due to the noxious nature of the mobile phase, all steps must be carried out in a well ventilated hood.

Chromatography was carried out using a Chromaflex chromatography column (48 cm × 5.5 cm O.D.) from Kontes Glass, Vineland, NJ, USA. Seven hundred cm^3 of silica resin (40 μm "Flash", Universal Scientific, Atlanta, GA, USA) was heated in a vacuum oven for 3–4 h at 100°C, allowed to cool, and suspended in mobile phase 1 to a final volume of 1000 ml. The bottom of the column was filled with approximately 1 cm of 50–70 mesh sand (Aldrich) and wetted with the mobile phase. The suspended silica was poured into the column via a funnel and allowed to settle with the stopcock closed. After settling, the stopcock was opened and solvent was applied with house air pressure to give a flow-rate of 10 ml/min. When the resin was finished packing (5–10 min), air pressure was applied until the mobile phase just reached the top of the column bed. The top of the column was opened and 1 cm of sand was applied to the stationary phase. The mobile phase was pumped using air pressure until the sand was packed evenly over the top of the resin. Air pressure was then applied until the mobile phase reached the top of the silica bed. The column was then opened again and the entire reaction mixture (40 ml, see below) was directly applied. Air pressure was applied until the entire sample had been absorbed into the silica. The sample was washed onto the column with a small amount (5–10 ml) of isopropanol. The mobile phase then was begun at a flow-rate of 10 ml/min. Generally, 150 fractions of 16–18 ml each were collected.

Small-scale preparation of isoprenoid diphosphates

The reaction is carried out in a fume hood. Approximately 200 μmol of the neat alcohol (*e.g.*, 52 μl farnesol for preparation of FPP) is transferred to a 75 × 12 mm polypropylene tube. Trichloroacetonitrile (0.5 ml, *ca.* 5 mmol) is then added followed by the addition of 0.5 ml of a solution of bis-triethylammonium phosphate (TEAP) in acetonitrile. The TEAP solution is prepared by slowly mixing 0.91 ml of solution A with 1.5 ml solution B while stirring. The sample is placed in a 37°C bath for 5 min. Two more additions of TEAP are added as before with 5 min incubations after each addition. The entire reaction is then streaked onto the application zone (about 2 cm from the bottom of the plate) of a 20 × 20 cm silica preparative TLC plate (Whatman Linear K, 1000 μm, with fluorescent indicator, obtained through Fisher Chemical). A 2-ml "Pi-Pump" (Fisher Chemical) connected to

a Pasteur pipette is ideal for streaking the sample. The plate is allowed to dry (*ca.* 15 min) and then placed in a TLC tank containing 125 ml mobile phase 2. Two plates can be run in a single tank if desired. A quick check of the reaction can be carried out by diluting 1 μl into 10 μl of methanol and streaking 3 μl on a 8 × 2 cm analytical TLC plate (silica). This plate, which can be developed in a small TLC tank in about 20 min, is stained with anisaldehyde solution (see Fig. 1). Development of the preparative plates requires about 8 h, but plates can be left overnight without deleterious effects. TLC takes place in a well ventilated hood. Following removal of the preparative plates, they are placed flat in the hood and allowed to dry until the odor of ammonia is no longer present (*ca.* 1 hour). Upon viewing with an ultraviolet light, fluorescent bands are seen at 4, 6, 8, 13.5, and 15 cm from the bottom of the plate (the chemical nature of these bands is not known). In addition, a yellow band is seen about 12 cm from the bottom of the plate. Plates containing TEAP reactions of geraniol, farnesol, or geranylgeraniol are sprayed liberally with water using a typical TLC sprayer. About 10 ml of water are required to soak a single plate. During spraying, the monophosphate (close to the solvent front), diphosphate and triphosphate appear as thick white bands, with the diphosphate appearing around 9–11 cm from the origin (R_F = 0.5). The diphosphate band is scraped into a 50 ml graduated screw-capped polypropylene centrifuge tube. A plastic "cell lifter" (cat. No. 3008, Costar Scientific, Cambridge, MA, USA) is ideal for scraping. The sample is treated with 20 ml of 10 m*M* ammonium hydrogen carbonate (pH 8), the tube capped, and vortexed vigorously for 1 min. Following centrifugation in swinging bucket rotor for 10 min at 3000 *g*, the supernate, which still contains fine particles of silica, is passed through a 50-ml disposable sterilization filter (0.2 μm) connected to a 50-ml polypropylene centrifuge tube (Costar No. 8301) under reduced pressure. The pellet is washed with 10 ml of the same buffer, centrifuged and the supernate filtered through the same filter as before. The concentration is determined on 10- and 25-μl aliquots of the pooled filtrate by assaying for phosphate according to the method of Ames [6] using 1 m*M* adenosine diphosphate (molar absorbance 15.4×10^3 l mol^{-1} cm^{-1} at 259 nm) as standard. The yield at this point should be 30–70 μmol, depending on the isoprenoid diphosphate (see below). If a more concentrated solution is desired, samples can be flash evaporated or lyophylized. Care must be taken to keep solutions of the allylic diphosphates alkaline during these steps. The final product is filtered again if necessary and stored in 10 m*M* ammonium hydrogencarbonate–methanol (1:1). Such samples are stable for months when kept at − 20°C. Samples of FPP and GGPP prepared using the above procedure can be concentrated by applying them to a C_{18} sorbent extraction column (*e.g.*, Bond Elut, Analytichem International, Harbor City, CA, USA or Sep-Pak, Waters/Millipore, Milford, MA, USA) and eluting with 2 ml methanol. Such samples are diluted with an equal volume of 10 m*M* ammonium hydrogencarbonate and stored at − 20°C.

Large-scale preparation of isoprenoid diphosphates

The phosphorylation reaction is carried out exactly as described for TLC except that it is scaled up 20-fold (*i.e.*, 4000 μmol of starting material is used). A 50-ml screw-capped centrifuge tube is a convenient reaction vessel. After completion of the reaction, the entire sample is subjected to flash chromatography as described above. Aliquots (2 μl) of every fifth fraction are analyzed by silica TLC using mobile phase 2. Appropriate fractions are pooled and concentrated to a volume of *ca.* 25 ml on a standard rotary evaporator (bath temperature 30°C) connected to a water aspirator pumping at 20 mmHg (1 mmHg = 133.322 Pa). The sample is then diluted to 50 ml with 10 m*M* ammonium hydrogencarbonate and an equal volume of methanol is added. Samples are stored at − 20°C in 50 ml polypropylene centrifuge tubes. A small amount silica may precipitate upon storage; it is easily removed by centrifugation or filtration.

RESULTS

Rapid small-scale synthesis of isoprenoid diphosphates

In preliminary studies, several different con-

centrations of TEAP and ratios of TEAP–trichloroacetonitrile were tested. The effect of reaction temperature was also investigated. The conditions chosen, which involve three additions of relatively concentrated TEAP and 5 min incubations at 37°C, yield *ca.* 30% diphosphate product when farnesol is reacted, as judged by anisaldehyde spray and by radioscanning when [^{3}H]farnesol is used as substrate (Fig. 1). We found no advantage to crystallizing the TEAP prior to reaction, as described by Cornforth and Popjak [2]. The pattern observed in Fig. 1 is reproducible: it was consistently observed in over 100 reactions during the course of a year. In addition, under the conditions described, phosphodiesters of farnesol (farnesol–phosphate–farnesol; farnesol–pyrophosphate–farnesol) are not observed, as evidenced by a lack of side products on TLC and by the complete hydrolysis of the isolated diphosphates by alkaline phosphatase (see below).

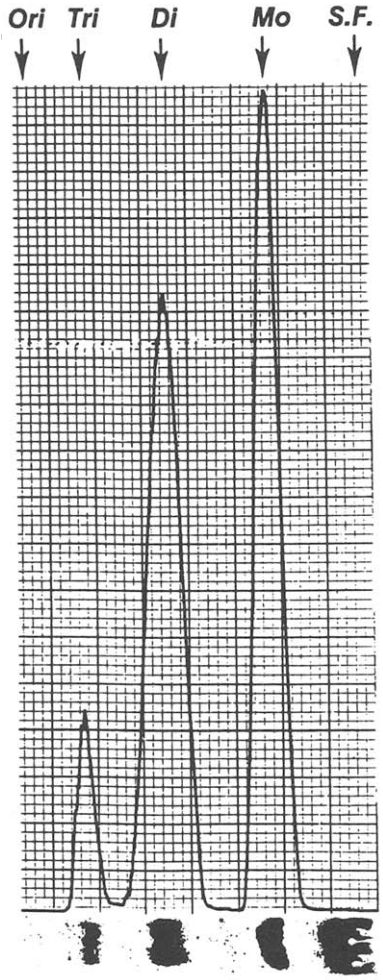

Fig. 1. Analytical TLC of reaction products. Farnesol (2 μmol) was mixed with 10^7 dpm [^{3}H]farnesol and reacted under the standard conditions scaled down 100-fold (*i.e.*, 20 μl total volume). After reaction, 1 μl was diluted into 10 μl methanol and 3 μl spotted onto a TLC plate and chromatographed using mobile phase 2. After development, the plate was radioscanned (top) and then sprayed with anisaldehyde (bottom). Positions of the origin (Ori), triphosphate (Tri), diphosphate (Di), monophosphate (Mo), and solvent front (S.F.) are indicated.

The procedure of Danilov *et al.* [4] using tetrabutylammonium phosphate was also investigated. These workers reported that, if the concentration of trichloroacetonitrile–alcohol is greater than 5, the main products of moraprenol phosphorylation are the mono- and diphosphates in about 45% yield for each. Fig. 2 shows the

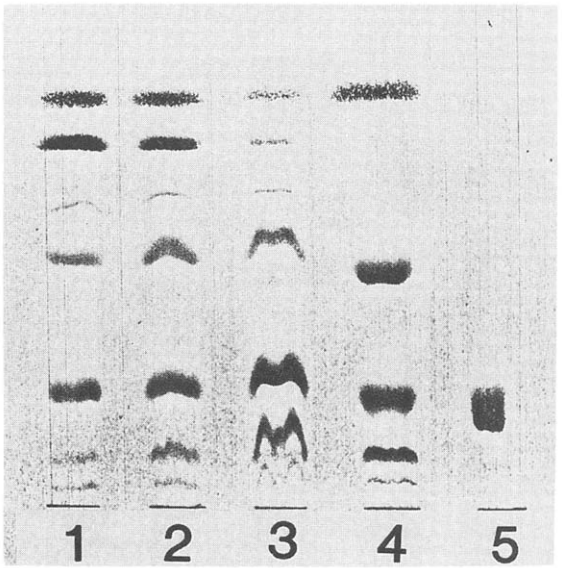

Fig. 2. Comparison of procedure of Danilov *et al.* and current method. Lanes 1, 2, and 3, reactions carried out according to Danilov *et al.* [4]. Farnesol (12.7 μmol) was treated with 50 μl trichloroacetonitrile and either 70 (lane 1), 140 (lane 2) or 280 μl (lane 3) of 360 m*M* tetrabutylammonium phosphate in acetonitrile. Aliquots of 5, 8, and 12 μl (representing 0.5 μmol of original farnesol) were diluted into methanol to give a final volume of 30 μl. Aliquots (3 μl) were streaked on an analytical TLC and chromatographed as described in Experimental. Lane 4: 12.7 μmol of farnesol was reacted under the standard conditions with 33 μl trichloroacetonitrile and three portions of 33 μl TEAP in acetonitrile (see Experimental). An aliquot (5 μl, representing *ca.* 0.5 μmol of farnesol) of this reaction was diluted with 25 μl of methanol and 3 μl were streaked on the TLC plate. Lane 5: FPP (25 nmol).

results of a phosphorylation reaction of farnesol according to the conditions described by Danilov *et al.* [4] using varying amounts of tetrabutylammonium phosphate. A standard reaction as described in the Experimental was added for comparison. It is clear that the procedure of Danilov *et al.* [4] also yields the tri- and tetraphosphate species. In addition, the yield of diphosphate is not significantly greater than that obtained using the procedure described in Experimental. Since the workup of the reaction described by Danilov *et al.* is considerably more lengthy than that described below, this procedure was deemed unsuitable for the rapid preparation of small or large amounts of the isoprenoid diphosphates.

Isolation of isoprenoid diphosphates using preparative TLC

As described in the Experimental section, the entire TEAP reaction can be applied to a preparative thin-layer plate. After overnight chromatography, the desired products can be detected by subjecting a lateral portion of the plate to either anisaldehyde or molybdate spray solutions. However, since we desired maximum yield, we attempted to detect the products by simply spraying with water. As shown in Fig. 3, water spray yields strong white bands corresponding to the mono-, di-, and triphosphates.

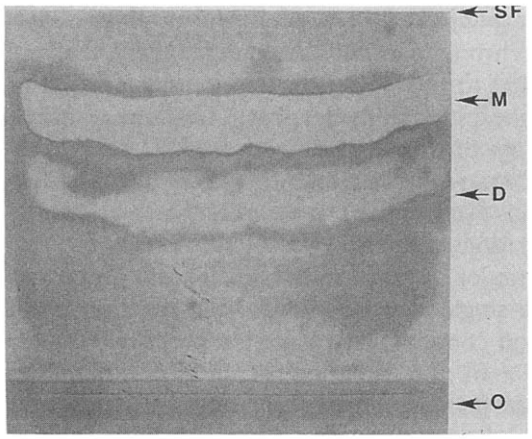

Fig. 3. Preparative TLC of reaction products. A typical reaction mixture containing 200 μmol farnesol was applied to preparative TLC and developed overnight in mobile phase 2. After drying 1 h, the plate was sprayed with *ca.* 10 ml water. Bands representing the monophosphate (M) and diphosphate (D) are indicated.

Each of these can be subsequently eluted using the procedure described in Experimental. Yield of farnesyl diphosphate from the plate is around 80%, giving an overall yield of *ca.* 24% from the alcohol. It is to be noted that the water spray technique is only applicable to the phosphorylated derivatives longer than C_{10}; isopentenol and dimethylallyl alcohol phosphates can only be detected by spraying a "guide lane" with either anisaldehyde or molybdenum spray (note: the plate must be free of ammonia in order for these sprays to work).

Large-scale preparation of isoprenoid diphosphates

For the isolation of larger quantities of isoprenoid diphosphates, the same reaction conditions described for the isolation on TLC are employed except that the volumes are scaled up 20-fold. Following incubation, the entire reaction is subjected to flash chromatography as described in Experimental. Fig. 4 shows that the mono-, di-, and triphosphates are well resolved using this procedure. Following localization of the desired product, appropriate fractions are pooled and concentrated by rotary evaporation using an aspirator pump.

Yield and purity of isolated products

Table I shows the yield obtained of the isoprenoid diphosphates using the small-scale and large-scale procedures. Analytical TLC (Fig. 5)

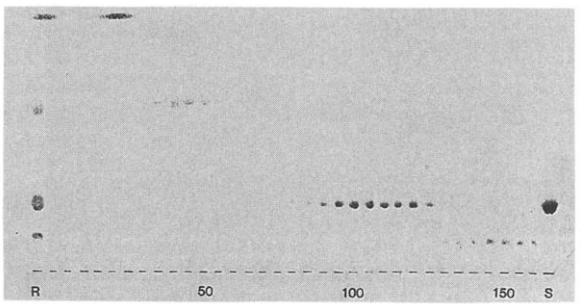

Fig. 4. Monitoring of flash chromatography column elution by analytical TLC. Farnesol (4000 μmol) was subjected to the TEAP reaction as described in Experimental and chromatographed using flash chromatography. Fractions (18 ml each) were collected and 2-μl aliquots of every fifth fraction were spotted for TLC. R: 0.3 μl of the reaction mixture was spotted; S: 15 nmol standard FPP.

TABLE I

YIELD OF ISOPRENOID DIPHOSPHATES PREPARED BY PREPARATIVE TLC AND FLASH CHROMATOGRAPHY

The indicated products were prepared and assayed for organic phosphate according to the procedure described in Experimental. Results are expressed as the mean ± S.D. with the number of preparations in parentheses.

Isoprenoid diphosphate	Yield (%)	
	TLC	Flash
IPP	35.6 ± 1.6 (n = 3)	25 (n = 1)
DMAPP	36 (n = 1)	
GPP	28.6 ± 1.7 (n = 3)	30 (n = 1)
FPP	24.0 ± 1.0 (n = 3)	31 ± 1 (n = 2)
GGPP	17.1 ± 2.5 (n = 3)	35 (n = 1)

shows the high degree of purity of the isoprenoid diphosphates prepared using the TLC procedure. To further test purity of the prepared products, we carried out enzymatic dephosphorylation of FPP and subjected the resultant extract to HPLC. Analysis by reversed-phase HPLC indicated a single peak coeluting in the position of farnesol (not shown). Analysis of the same sample by silica HPLC (Fig. 6) revealed the presence of a small amount of the α-*cis* species (*i.e.*, *Z,E*) which was present in the starting compound prior to phosphorylation.

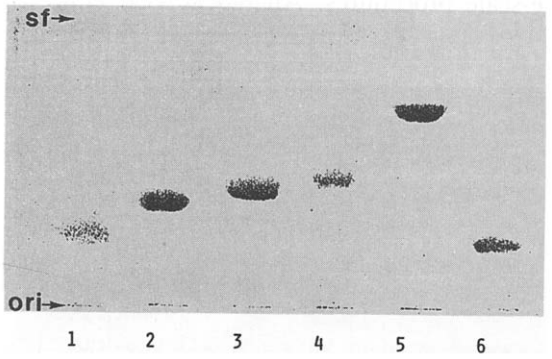

Fig. 5. Analytical TLC of purified isoprenoid phosphates. Isoprenoid phosphates were prepared by preparative TLC and *ca.* 20 nmol were applied to analytical TLC plates. Detection was with anisaldehyde. 1 = IPP; 2 = GPP; 3 = FPP; 4 = GGPP; 5 = farnesyl monophosphate; 6 = farnesyl triphosphate.

DISCUSSION

The present study was prompted by our desire to have available a rapid procedure for the preparation of both labeled and unlabeled isoprenoid diphosphates. Such a procedure would greatly facilitate our ongoing studies of mevalonate metabolism in rat liver. We found that the original procedure of Cornforth and Popjak [2] was both tedious and time consuming. The procedure of Danilov *et al.* [4] also involves several steps prior to resolution of the products on DEAE-cellulose using gradient elution. The procedure of Davisson *et al.* [3] has the advantage of generating only the diphosphate product. However, it requires preparation of an intermediate halide, and a low temperature reaction (−40°C) using dry solvents in an inert atmosphere. In addition, the diphosphorylating agent must be prepared using ion exchange chromatography. Following a 24-h reaction, isolation of the diphosphate involves ion-exchange chromatography, lyophylization, and flash chromatography on cellulose.

In developing a rapid procedure to synthesize isoprenoid diphosphates, we reasoned that there was really no disadvantage to generating the mono- and triphosphate side products, since neither the cost nor availability of the starting alcohols was a limiting factor. In addition, it is often useful to have available the mono- and triphosphate derivatives for control experiments and chromatography standards. We therefore decided to use TEAP in trichloroacetonitrile as the phosphorylating reagent. After many modifications of the original procedure [2], the conditions chosen were found to generate the expected products in good yield after a 15-min incubation in the absence of side products.

A major advance in the small-scale procedure is the single step isolation of the reaction products on commercially available preparative TLC plates. In the isolation of FPP described by Cornforth and Popjak [2], several steps are required, including extraction with diethyl ether, absorption onto Amberlite XAD-2 (an overnight procedure) and DEAE-cellulose chromatography. In the procedure described here, the product can be used after elution from TLC

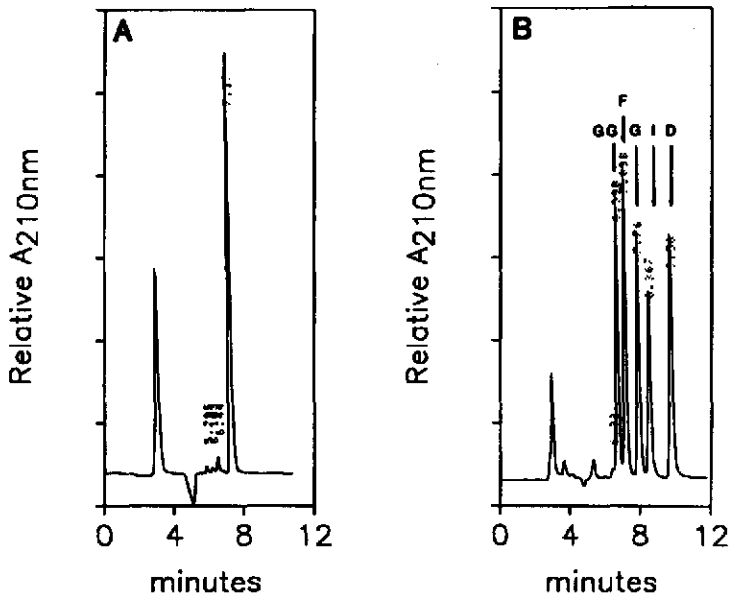

Fig. 6. (A) Silica HPLC of dephosphorylated FPP. FPP (50 nmol) was subjected to dephosphorylation in 1 ml 0.1 *M* sodium glycinate, 1 m*M* $ZnCl_2$, 1 m*M* $MgCl_2$, pH 10.7, using 1 unit bovine intestinal alkaline phosphatase (Sigma). After 1 h at 37°C, the reaction was extracted with 1 ml methylene chloride. An aliquot (100 μl) of the extract was mixed with 10 μl reagent alcohol and reduced to about 10 μl using a gentle stream of argon. Hexane (0.2 ml) was then added and the sample subjected to HPLC on a 22 cm silica column. Mobile phase: 2% reagent alcohol in hexane. Flow-rate: 1 ml/min. Detection: A_{210nm}. Right panel, standard alcohols: 1 μg each geranylgeraniol (GG), farnesol (F), and geraniol (G), 10 μg isopentenol (I), and 2 μg dimethylallyl alcohol (D).

without further purification. Product analysis indicates that the isoprenoid diphosphates are essentially pure (Fig. 5) and generate the expected alcohols after dephosphorylation (Fig. 6, and data not shown). In addition, they have been found to be biologically active in enzymatic assays (*e.g.*, squalene synthetase [7]).

For the preparation of isoprenoid diphosphates in amounts greater than 40 μmol, either multiple TLC plates or flash chromatography can be employed. Under the conditions described herein, flash chromatography is completed in about 3 h and yields 800–1200 μmol of pure material.

ACKNOWLEDGEMENTS

Supported in part by funds from the Division of Technology Transfer, University of South Florida.

The technical assistance of Fequiere Vilsaint, Alison Rhinehart, and Gary Sessums is gratefully acknowledged.

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