papers and notes on methodology

Antiviral nucleoside diphosphate diglycerides: improved synthesis and facilitated purification

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Abstract Cytidine diphosphate diglyceride and its analogs have previously been synthesized by condensing phosphatidic acid with the monophosphomorpholidates of the various nucleosides. Yields have been low and purification of the product has been difficult. We report here an improved method for the synthesis of nucleoside diphosphate diglycerides with potential antiviral activity. Phosphatidic acid was activated with morpholine in the presence of dicyclohexylcarbodiimide to phosphatidic acid morpholidate. This compound was condensed with the 5'-monophosphate of the anti-HIV agents 3'-azido-3'-deoxythymidine, 3'-deoxythymidine or 2',3'-dideoxycytidine, and the monophosphate of the anti-HSV agent acyclovir. The resulting nucleoside diphosphate diglycerides are potential candidates for improved antiviral action when compared to the parent nucleoside analogs. Compared to the older method for the preparation of cytidine diphosphate diglyceride and analogs thereof, the new method has several advantages: reaction times are reduced from several days to several hours and the yield of the reactions is generally increased from 20–40% to between 50 and 80%. In addition, the purification of the compounds is greatly facilitated due to the small amount of phosphatidic acid remaining in the reaction mixture.—**van Wijk, G. M. T., K. Y. Hostetler, and H. van den Bosch**. Antiviral nucleoside diphosphate diglycerides: improved synthesis and facilitated purification. *J. Lipid Res.* 1992. **33:** 1211–1219.

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The human immunodeficiency virus (HIV) is the etiologic agent that causes the acquired immune deficiency syndrome (AIDS) (1, 2). Zidovudine (3'-azido-3'-deoxythymidine, AZT) inhibits the HIV reverse transcriptase after anabolic phosphorylation to its triphosphate by host cell kinases (3) and is the only agent currently approved for treating HIV infection (4). Macrophages and related cells involved in phagocytosis and antigen presentation have been implicated as a major HIV reservoir in AIDS (5). To direct large amounts of antiretroviral dideoxynucleosides to this reservoir, we synthesized phospholipid conjugates having dideoxynucleosides such as 3'-azido-3'-deoxythymidine and 2',3'-dideoxycytidine (ddC) as their polar head groups (6). Among those were analogs of the naturally occurring phospholipid cytidine diphosphate diglyceride (CDP-DG). Administration of antiviral nucleosides as nucleoside diphosphate diglycerides potentially has several advantages, e.g., unique metabolic pathways that would release monophosphorylated anti-HIV agents intracellularly, thereby bypassing essential nucleoside kinase activities (6). Those activities have been reported to be relatively weak in certain HIV target cells (7).

Acyclovir, an acyclic nucleoside analog of guanosine, is an antiviral drug with therapeutic usefulness in humans for the treatment of herpes simplex virus (HSV) infections (8, 9). The drug is selectively monophosphorylated by the viral thymidine kinase (10) and subsequently converted to the active antiviral acyclovir triphosphate by host cell kinases (11). As a result of this activation mechanism, acyclovir is not effective as an agent for the treatment of thymidine kinase-deficient HSV strains (12). Surprisingly, the CDP-DG analog of acyclovir, i.e., acyclovir

Abbreviations: CDP-DG, cytidine-5'-diphosphate diglyceride; AZTDP-DG, 3'-azido-3'-deoxythymidine-5'-diphosphate diglyceride; 3dTDP-DG, 3'-deoxythymidine-5'-diphosphate diglyceride; ddCDP-DG, 2',3'-dideoxycytidine-5'-diphosphate diglyceride; ACVDP-DG, acyclovir diphosphate diglyceride; MP, monophosphate; HIV, human immunodeficiency virus; HSV, herpes simplex virus; TLC, thin-layer chromatography; HPTLC, high performance thin-layer chromatography; HPLC, high performance liquid chromatography.

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[2]3'-Deoxythymidine is sometimes referred to as 2',3'-dideoxythymidine.

[3]Acyclovir does not contain a "true" 5'-hydroxyl function, as it contains an acyclic ribose analog.

diphosphate diglyceride, has been shown to retain its antiviral properties in thymidine kinase-deficient HSV strains (K. Y. Hostetler, unpublished observations). These results suggest a mechanism of drug release that would bypass the deficient viral thymidine kinase.

The biological importance of cytidine diphosphate diglyceride (CDP-DG) in cellular lipid metabolism has been well documented since the early 1960s (13–17). In mammals the compound is an obligatory intermediate in the biosynthesis of phosphatidylinositol (PI), phosphatidylglycerol (PG), and diphosphatidylglycerol (DPG, cardiolipin) (14–19). During PI, PG, and DPG biosynthesis, cytidine-5'-monophosphate (CMP) is released (20). However, the specificity of the enzymes involved in these conversions is not restricted to the CDP-DG substrate. We showed previously that the 2'-deoxycytidine, adenosine, guanosine, and uridine analogs of CDP-DG can also serve as the activated phosphatidic acid donor in the biosynthesis of PI, PG, and DPG (21, 22).

The first chemical synthesis of CDP-DG with a reasonable yield was described by Agranoff and Suomi (13), and was based on the condensation of phosphatidic acid (PA) with cytidine-5'-monophosphate morpholidate. However, this approach to preparing CDP-DG and analogous compounds has a number of problems including long reaction times, a rather troublesome purification due to the presence of large amounts of unreacted phosphatidic acid, and low yields of pure product (6, 13, 15, 23–27).

Using the method of Agranoff and Suomi (13), we recently described the chemical synthesis, purification, and biological activity of azidothymidine diphosphate diglyceride, a CDP-DG analog with antiretroviral activity; this compound was found to be a potent inhibitor of the human immunodeficiency virus (HIV) in U937 and CEM cells, in vitro (6).

We now report the synthesis of several analogs of CDP-DG in which the cytidine was replaced by 3'-azido-3'-deoxythymidine (AZT), 3'-deoxythymidine (3dT), 2',3'-dideoxycytidine (ddC), and acyclovir using a new synthetic route. The reaction is based on the condensation of a nucleoside-monophosphate with a phosphatidic acid morpholidate in dry pyridine. The method is much more rapid and provides a substantially improved yield of product. Because of the nature of the reactants, the purification of the compounds is facilitated considerably due in large part to the substantial reduction of phosphatidic acid in the crude reaction mixture.

MATERIALS AND METHODS

Materials

Dilauroyl- and dimyristoyl phosphatidic acids, disodium salts were obtained from Avanti Polar Lipids (Pelham, AL) and dipalmitoyl phosphatic acid, disodium salt was a product from Genzyme (Boston, MA). Dowex 50W (50 × 2–200, 100–200 mesh, H^+ form), 2',3'-dideoxycytidine, and 3'-deoxythymidine were purchased from Sigma Chemical Co. (St. Louis, MO). 3'-Azido-3'-deoxythymidine and 3'-azido-3'-deoxythymidine-5'-monophosphate were generous gifts from Burroughs Wellcome Co. (Research Triangle Park, NC) and [^{3}H]-3'-deoxythymidine-5'-monophosphate (6 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). Acyclovir, morpholine, dicyclohexylcarbodiimide (DCC), and tertiary butylalcohol (2-methyl-2-propanol, tBuOH) were the highest grade available from Aldrich Chemical Co. (Milwaukee, WI). Phosphorus oxychloride, trimethylphosphate, silica 60 F 254 HPTLC plates, silica 60 F254 aluminum-plates, HPLC grade solvents (Lichrosolv), and all other chemicals were obtained from Merck (Darmstadt, FRG), unless stated otherwise.

Methods

Initially, the synthesis of nucleoside diphosphate diglycerides was done as described previously (6), essentially following the procedure reported by Agranoff and Suomi (13) (Method I). Using this method, a nucleoside-5'-monophosphomorpholidate is condensed with phosphatidic acid under anhydrous conditions in pyridine for 3–5 days. **Fig. 1** shows the overall reaction scheme of the newly developed synthesis, starting from nucleoside and phosphatidic acid (Method II). This procedure utilizes the morpholidate of phosphatidic acid and the nucleoside-5'-monophosphate[3] and will be described in more detail below. The purified compounds were analyzed for fatty acid ester (28) and phosphorus content (29). Ultraviolet and ^{1}H-NMR spectra were recorded using a Hitachi 150-20 spectrophotometer and a Brucker 360 spectrometer, respectively. Infrared absorption spectra (KBr disc method) were obtained using a Perkin-Elmer 1600 FT infrared spectrophotometer.

Synthesis of nucleoside-5'-monophosphates. Nucleoside-5'-monophosphates were prepared as described by Yoshikawa, Kato, and Takenishi (30, 31). Briefly, an unprotected nucleoside is phosphorylated in trimethylphosphate with $POCl_3$. After careful hydrolysis (ice cold water) and neutralization with NaOH to pH 7, the product is purified by anion exchange chromatography using a Mono Q HR 5/5 (HPLC) or a Q-Sepharose fast flow (open) column (both from Pharmacia, Uppsala, Sweden). The reaction products were injected onto the HPLC column and separated using the following elution scheme: 0–5 min, water; 5–12 min, 0.1 M NH_4HCO_3 (pH 7.2); 12–20 min, linear gradient of 0.1–0.6 M NH_4HCO_3 (pH 7.2). The flow rate was 1 ml/min and detection was at 254 nm. Nucleoside-5'-monophosphates were eluted in the isocratic interval between 5 and 12 min. This procedure was used to follow the progress of the

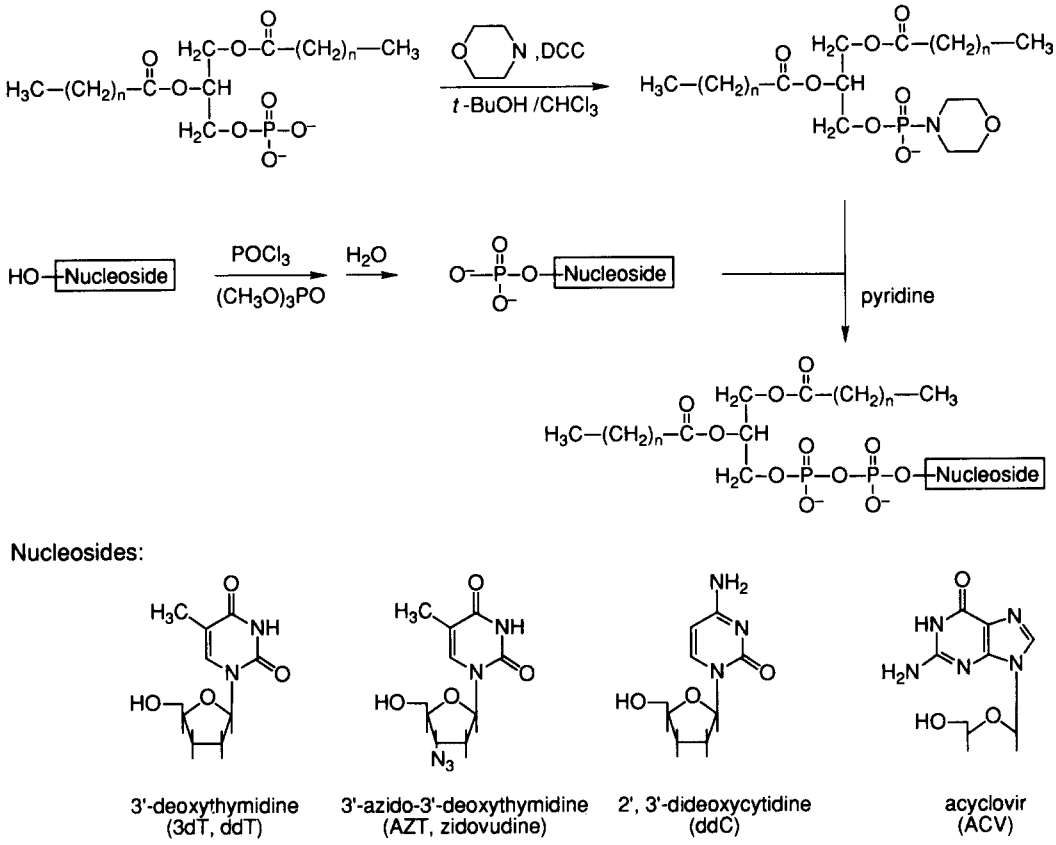

Fig. 1. Chemical synthesis of nucleoside-5'-diphosphate-3-*sn*-1,2-diacylglycerols. Compounds prepared via this new route were as follows: Compound 1: Nucleoside = AZT; n = 12; AZT-diphosphate dimyristoylglycerol (AZTDP-DG diC14). Compound 2: Nucleoside = 3dT; n = 10; 3dT-diphosphate dilauroylglycerol (3dTDP-DG diC12). Compound 3: Nucleoside = 3dT; n = 12; 3dT-diphosphate dimyristoylglycerol (3dTDP-DG diC14). Compound 4: Nucleoside = ddC; n = 10; ddC-diphosphate dilauroylglycerol (ddCDP-DG diC12). Compound 5: Nucleoside = ddC; n = 12; ddC-diphosphate dimyristoylglycerol (ddCDP-DG di 14). Compound 6: Nucleoside = ACV; n = 14; ACV-diphosphate dipalmitoylglycerol (ACVDP-DG diC16).

phosphorylation reaction and to purify small amounts (5 to 20 mg) of nucleoside-5'-monophosphates. The phosphorylations were generally completed in less than 2 h as judged by the disappearance of free nucleosides eluting in the 0–5 min interval. Larger amounts of products (up to 300 mg) were purified on an open Q-Sepharose fast flow column (16 × 5.4 cm) which was first eluted with 500 ml water and then with 0.1 M NH_4HCO_3 (pH 7.2) at a flow rate of about 10 ml/min. The product was desalted by repeated lyophilization. The purified product was analyzed by thin-layer chromatography (HPTLC) using silica 60 F 254 plates developed with 1-propanol–25% ammonia–water 20:20:3 (v/v) and gave single UV- and phosphorus (P_i)-positive spots: AZT-5'-monophosphate, R_f = 0.63; 3dT-5'-monophosphate, R_f = 0.61 and ddC-5'-monophosphate, R_f = 0.51. Yields were between 70 and 98%, depending upon the nucleoside. Acyclovir-5'-monophosphate was also prepared using essentially similar procedures, but triethylphosphate was used as solvent rather than trimethylphosphate (L. M. Stuhmiller and K. Y. Hostetler, personal communication, 1991).

Preparation of pyridine-soluble form of acyclovir monophosphate (ACVMP). To a suspension of 1.8 mmol ACV-monophosphate (as free acid) in 20 ml methanol, 3.6 mmol tributylamine (TBA) was added (32, 33). The mixture was vigorously stirred at room temperature and after 15 to 30 min a clear solution was obtained. After evaporation of methanol and lyophilization, the TBA-salt of ACVMP was readily soluble in pyridine.

Conversion of phosphatidic acid salts to the free acid form. Phosphatidic acids, disodium salts, were acidified by application of an extraction procedure according to Bligh and Dyer (34). For example, 1 mmol of phosphatidic acid (disodium salt) was dissolved in a homogeneous mixture of 100 ml chloroform, 200 ml methanol, 100 ml 0.1 M HCl and stirred at room temperature for 1 h. Then 100 ml water and 100 ml chloroform were added, the separated chloroform layer was isolated, and the aqueous phase was extracted twice with 200 ml chloroform. The combined chloroform extracts were evaporated to dryness and lyophilized. Yield: 95–100% phosphatidic acid (free acid).

*Synthesis of 1,2-diacyl-*sn*-glycero-3-phosphomorpholidate.* Method A: From free phosphatidic acid. This synthesis was adapted from investigations on the activation of hydrophilic phosphorus compounds reported previously by Moffatt and Khorana (33). Free phosphatidic acid (1 mmol) was dissolved in 20 ml chloroform and this solution was transferred to a two-necked round-bottom flask, which contained 20 ml t-BuOH, 4 mmol morpholine, and at least 4 mmol H_2O. This mixture was gently refluxed and a solution of 4 mmol dicyclohexylcarbodiimide (DCC) in 20 ml t-BuOH was added stepwise from a dropping funnel over a period of 2 h. The reaction was monitored by thin-layer chromatography using silica 60 F254 HPTLC plates developed in chloroform–methanol–25% ammonia–water 70:38:8:2 (v/v). The reaction was judged to be completed by the appearance of a major P_i-positive spot (R_f = 0.9) and the disappearance of the P_i-positive spot of the phosphatidic acid at R_f = 0.1. The reaction mixture was evaporated to dryness, suspended in 50 ml water, and transferred to a dropping funnel. The suspension was extracted three times with chloroform or diethyl ether, evaporated to dryness, and lyophilized. The estimated yield was 70–95% of 1,2-diacyl-*sn*-glycero-3-phosphomorpholidate as the 4'-morpholine-N,N' dicyclohexylcarboxamidinium salt. The product contained 90–98% of the theoretical amount of phosphate (29) and was used without further purification for the synthesis of the nucleoside diphosphate diglycerides.

Method B: From phosphatic acid, disodium salt. This reaction was performed essentially as described above. Sometimes, however, the reaction mixture had to be clarified by the addition of a small amount of methanol–water 1:1 (v/v). The aqueous phase was extracted with chloroform or diethyl ether, evaporated, lyophilized, and used in the condensation reaction without further purification.

Synthesis of nucleoside diphosphate diglycerides. Lyophilized mixtures of phosphatidic acid morpholidates (prepared by either method A or B) and nucleoside-5'-monophosphates (H^+-form, 13), (the disodium salt of AZT-5'-monophosphate was also used successfully) were dissolved in pyridine and evaporated to dryness in vacuo. For the preparation of acyclovir diphosphate diglyceride, the tributylamine salt was used for reasons outlined in the next section. This procedure was repeated several times taking care to allow nitrogen to enter the rotavapor. A final amount of pyridine, equivalent to about 15 to 20 ml per mmol starting material, was added to give a clear solution. About 50% of the pyridine was removed in vacuo and the reaction vessel was sealed under nitrogen. The reaction was allowed to proceed at room temperature (except for ACV diphosphate diglyceride which was heated at 60°C in a water bath) and was checked every 30 min by means of TLC using chloroform–methanol–25% ammonia–water 70:38:8:2 (v/v) as the developing system. The reaction was generally completed within 1–20 h as judged by the appearance of a major UV- and P_i-positive spot at R_f values between 0.1 and 0.3, depending on the nucleoside.

RESULTS AND DISCUSSION

Synthesis

The influence of the molar ratio of the reactants on the yield in the synthesis of AZT-diphosphate dimyristoylglycerol (AZTDP-DG diC14) by the new procedure was initially determined by condensing 0.14 mmol of dimyristoyl phosphatidic acid morpholidate with AZT-5'-monophosphate in a molar ratio of 1:0.5, 1:1, and 1:2, respectively. The yields after final purification (see below) based on weight, phosphate and UV analysis amounted to 81%, 80%, and 60%, respectively, of the theoretical yield. From then on a molar ratio of phosphatidic acid morpholidate to nucleoside-5'-monophosphate between 1:0.5 and 1:1 was used routinely.

In the following experiments compounds 2–5 (Fig. 1) were synthesized with a 1:1 molar ratio of reactants at a scale of 0.1–0.2 mmol. The yields were 57%, 37%, 52%, and 61%, respectively. The somewhat lower yields in preparing compounds 2–5 may have been caused by some residual ammonium bicarbonate introduced during the purification of the respective nucleoside monophosphates. This phenomenon was also observed when the compounds were initially synthesized by Method I (13) and can be overcome by a more thorough desalting of the purified nucleoside-5'-monophosphates.

Fig. 2 shows an HPTLC separation in which the difference between Methods I and II concerning reaction time and composition of the reaction mixture is depicted. In this example, AZT-diphosphate dimyristoylglycerol was prepared by both methods on a 0.05 mmolar scale in 0.5–1.0 ml anhydrous pyridine. Aliquots of the reaction mixtures were withdrawn at the indicated reaction times and used for HPTLC analysis. It is obvious from Fig. 2 that the newly developed Method II (lanes 1 and 2) gives much simpler mixtures than Method I (lanes 3 and 4), both by phosphate (Fig. 2A) and UV (Fig. 2B) analysis. The mixtures produced by Method II contain mainly AZTDP-DG diC14 after relatively short reaction times of 5 and 10 h. In fact, we observed that the product can already be detected in high yield as a UV- and phosphate-positive spot as early as 2 min after the addition of dry pyridine. Notably, after 5 and 10 h, the reaction mixtures contain very little phosphatidic acid. By contrast, the reaction mixtures obtained by Method I contain, even after 5 days, large amounts of residual phosphatidic acid as well as other UV-positive spots that have not been further identified. The virtual absence of residual phosphatidic

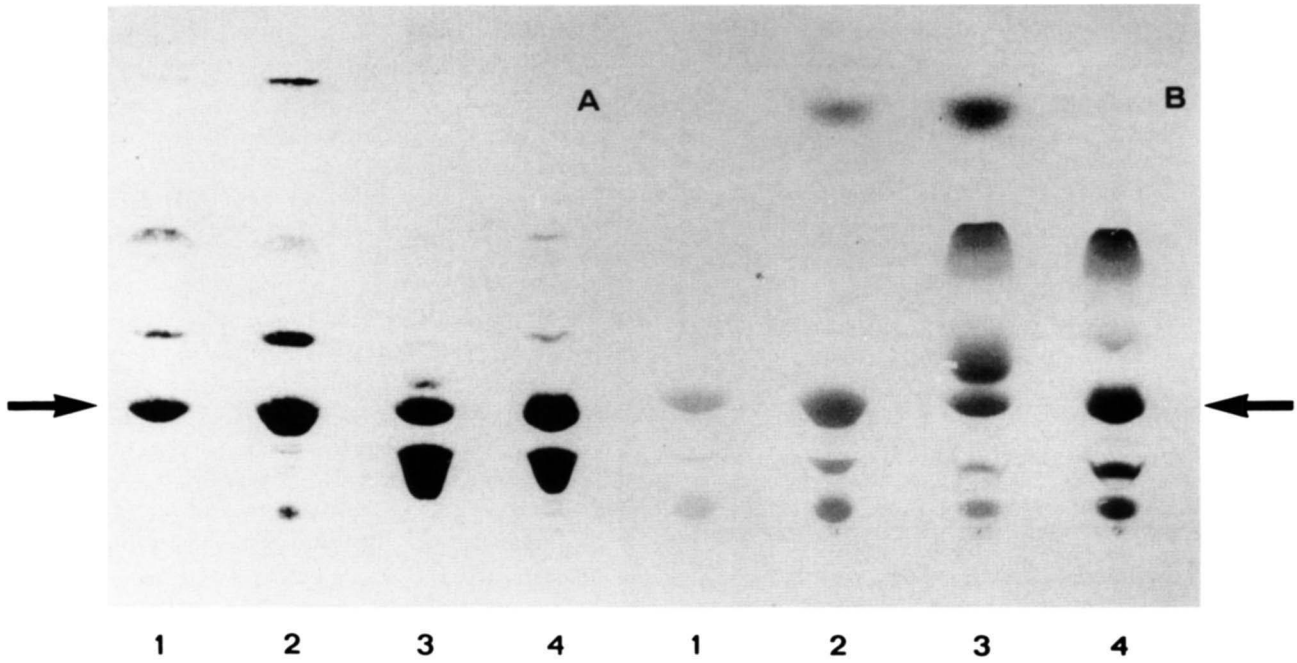

Fig. 2. HPTLC showing the difference in reaction time and composition of the reaction mixture when comparing Methods I and II for the synthesis of AZTDP-DG diC14. Visualization of the products was performed by (A) phosphate detection (36) and (B) ultraviolet absorption (254 nm). Developing solvent: chloroform–methanol–25% ammonia–water 70:38:8:2 (v/v). Lanes 1 and 2, reaction mixture obtained by Method II after t = 5 and 10 h, respectively; lanes 3 and 4, reaction mixture obtained by Method I after t = 10 h and t = 5 days, respectively. Arrows point at the product, at R_f = 0.3. Lanes 3 and 4 show the abundant presence of PA at R_f = 0.1.

acid in the reaction mixtures obtained by Method II suggests a facilitated purification of the product.

In additional experiments it appeared possible to use AZT-5'-monophosphate directly as its sodium salt, rather than as the free acid, in the preparation of AZT-diphosphate dimyristoylglycerol. This eliminates the need to use the Dowex(H^+) ion exchange procedure to prepare the free acid. In some cases it appeared necessary to heat the reaction mixture for 5 to 10 min in a water bath at 40°C to obtain a clear solution. The composition of the reaction mixture (**Fig. 3**) and the yield of the product were very similar to those obtained with AZT-5'-monophosphoric acid. Preliminary investigations also suggest that the conversion of disodium phosphatidate to its free acid form for the preparation of phosphatidic acid morpholidate is not essential, and that the disodium salt of phosphatidic acid may also be used. The small scale synthesis of AZTDP-DG suggests that Method II is also suitable for the preparation of radioactive compounds. The result of such a synthesis is depicted in Fig. 4 for [^{3}H]-3dTDP-DG.

In another experiment we performed a detailed analysis of the reaction course of a synthesis of 3dTDP-DG diC14. In this particular case 0.65 mmol PAdiC14 morpholidate was condensed with 0.35 mmol 3dT-monophosphate (**Fig. 5**). It can be concluded that the

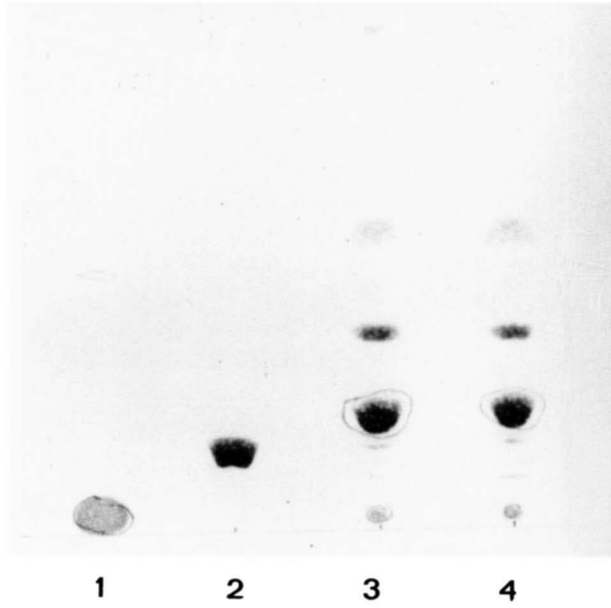

Fig. 3. HPTLC showing two reaction mixtures prepared by Method II. Lane 1, AZT-5'-monophosphate; lane 2, phosphatidic acid; lane 3, condensation products of PA morpholidate and AZT-5'-monophosphate (Dowex - H^+ treated); lane 4, condensation products of PA morpholidate with the sodium salt of AZT-5'-monophosphate. Compounds were detected by phosphate reagent (36). UV-positive spots are encircled. Developing solvent as in Fig. 2.

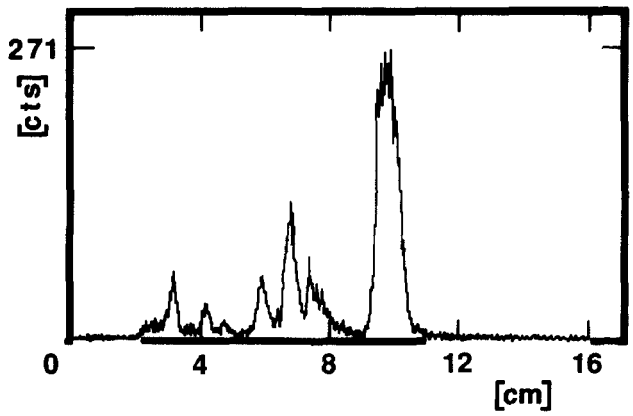

Fig. 4. Synthesis of [^{3}H]-3dT-diphosphate dimyristoylglycerol starting from 50 μmol [^{3}H]-3dT-5'-monophosphate (1.2 Ci/mol) and 100 μmol PA morpholidate. An aliquot of the reaction mixture was spotted on an HPTLC plate and eluted with chloroform-methanol-25% ammonia-water 70:58:8:8 (v/v). Radioactivity was localized using a Berthold LB 2842 automatic TLC linear analyzer. The main radioactive peak at R_f = 10 cm co-migrated with unlabeled 3dT-diphosphate dimyristoylglycerol. Quantitative analysis showed that 60–65% of the total amount of radioactivity was introduced into the desired product. Cts, radioactivity counts.

reaction is principally completed within 30 min as judged by the amount of 3dTDP-DG diC14 formed (curve A, yield 71%) and the sharp decrease in the amounts of 3dTMP (curve E) and dimyristoyl phosphatidic acid morpholidate (curve D).

Application of the newly developed procedure to the synthesis of acyclovir diphosphate diglyceride (compound 6, Fig. 1) was initially disappointing. Condensation of phosphatic acid morpholidate with acyclovir monophosphoric acid at room temperature in anhydrous pyridine resulted in turbid mixtures, which contained minor amounts (< 10%) of the desired product. This phenomenon, probably due to the insolubility of ACVMP in anhydrous pyridine (and in several other commonly used organic solvents), was overcome by preparation of the tributylamine salt of the acyclovir monophosphate, which can be dissolved in anhydrous pyridine. In one particular synthesis, 2.5 mmol of dipalmitoyl phosphatidic acid morpholidate in 10 ml of anhydrous pyridine was added to a solution of 1.8 mmol of ACVMP (as TBA salt) in 25 ml pyridine. The reaction mixture was evaporated to dryness three times and a final amount of 30 ml pyridine was added. After heating for 20 h at 60°C (water bath) the reaction was stopped by evaporation of pyridine and extraction of the crude mixture according to Bligh and Dyer (34) using 0.1 N HCl as aqueous phase. Aliquots of both the aqueous methanol and the chloroform layer were analyzed for ultraviolet-absorbing materials by means of HPTLC (silica 60 F254 plates, 10 × 20 cm), using chloroform-methanol-25% ammonia-water 70:58:8:8 (v/v) as the developing system. UV-positive spots were scraped from the plates, the silica was extracted with 2 ml chloroform-methanol-0.1 N HCl 1:2:1 (v/v) and the amount of material in both phases was determined spectrophotometrically at 256 nm. The ratio absorbance at 256 nm aqueous-chloroform was at least 45:55. Since there was only one UV-positive spot in the chloroform layer, the yield of ACVDP-DG was about 55%.

Purification

For the purification of AZT-, 3dT-, and ddC-diphosphate diglycerides, we developed a purification procedure as described below.

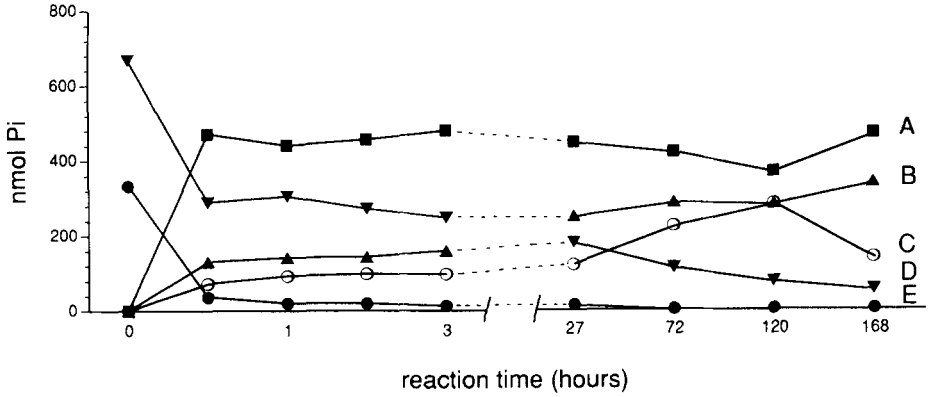

Fig. 5. Analysis of the reaction course of the synthesis of 3dTDP-DG diC14. At t_r = 0.5, 1.0, 1.5, 3, 27, 72, 120, and 168 h, the composition of the reaction mixture was analyzed using silica 60 F254 plates (10 × 20 cm, upside-down) and chloroform-methanol-25% ammonia-water 70:38:8:2 (v/v) as the developing system. At the indicated time intervals 250 μl of the reaction mixture was withdrawn and the pyridine was removed with a gentle N_2 stream. The residue was redissolved in chloroform-methanol 1:1 (v/v) and this solution was again dried under a N_2 stream. Finally 250 μl chloroform-methanol 1:1 (v/v) was added and the samples were stored at −20°C until analysis. Aliquots were analyzed simultaneously on HPTLC as described above. Reaction products were visualized by UV absorption and spraying with phosphorus reagent. The amount of phosphorus in every spot (A, B, C, D and E) was quantified using the method of Rouser, Fleischer, and Yamamoto (29). Standardization was performed by setting the absolute amount of totak Pi at 1000 nmol/analysis (recovery was > 80% at all time points). A, 3dTDP-DG diC14; B, unknown product; Pi positive (strong); C, unknown product, UV positive, Pi positive (weak); D, dimyristoyl phosphatidic acid morpholidate; and E, 3dTMP.

The crude reaction products were either purified directly without further processing, or were first converted to their di-ammonium salts as described elsewhere (6). In both cases the evaporated lyophilized reaction mixtures were dissolved in a minimum of elution solvent or chloroform–methanol 1:1 (v/v) and purified by means of HPLC, using a silica μ-Porasil column (Waters Associates Inc., Milford, MA); 19 mm (i.d.) × 30 cm (1)) and the solvent system hexane–2-propanol–25% ammonia–water 43:57:3:7 (v/v) (35). Detection was performed by UV absorption at 206 nm. Using this method, 50 to 100 mg of crude product could be purified in one half-hour run, with flow rates between 12 and 16 ml/min. The R_f values of the purified compounds with TLC using the developing system chloroform–methanol–25% ammonia–water 70:38:8:2 (v/v) were as follows: AZTDP-DG = 0.30; 3dTDP-DG = 0.29; and ddCDP-DG = 0.25. When stored in dry form or in chloroform–methanol 1:1 (v/v), the compounds were stable (> 95%) for at least 3 months at −20°C.

Representative HPLC purifications of AZT-diphosphate dimyristoylglycerol, obtained by either Method I or II, are shown in **Fig. 6**. When the column was loaded with only a few mg of crude product, good separation of AZT-diphosphate dimyristoylglycerol and the parent phosphatidic acid was obtained even with reaction mixtures prepared by Method I (Fig. 6B). However, when the semi-preparative column was loaded with 50 to 100 mg of crude product, a baseline separation between AZTDP-DG and PA was only obtained with mixtures prepared by Method II. As predicted by the results of Figs. 2 and 6, crude mixtures prepared by Method I produced a considerable contamination of the AZT-diphosphate dimyristoylglycerol peak with phosphatidic acid, resulting in low yields of pure product.

The above described isocratic elution procedure was not very suitable to purify ACVDP-DG (Dr. L. M. Stuhmiller, personal communication). An efficient purification procedure for this compound has yet to be developed, but it has been purified to homogeneity, using silica gel and anionexchange chromatography (data not shown). It shows a single UV- and phosphorus-positive spot on HPTLC. R_f = 0.1 and 0.3 with chloroform–methanol–25% ammonia–water 70:38:8:2 (v/v) and 70:58:8:8 (v/v) as developing system, respectively.

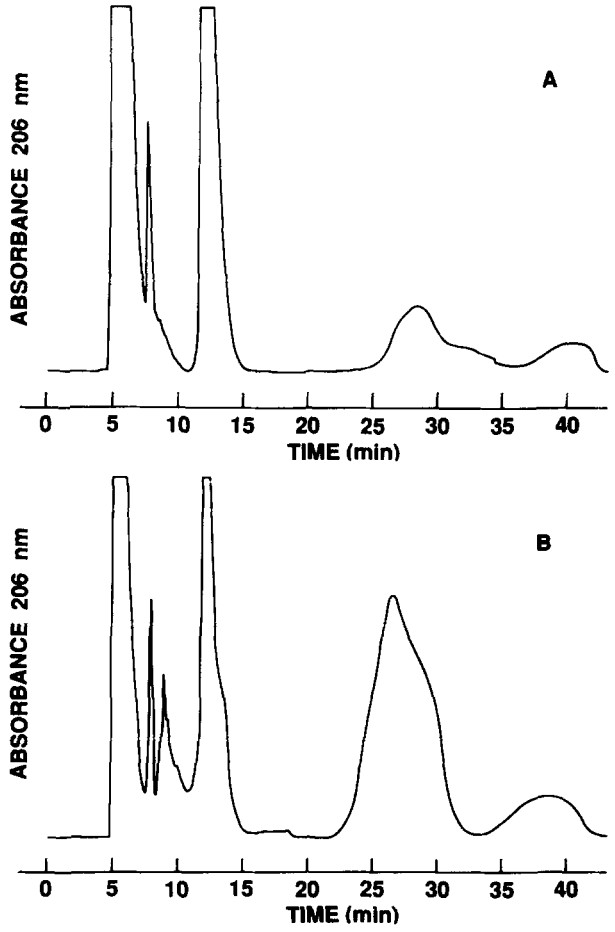

Fig. 6. HPLC purification of AZT-diphosphate dimyristoylglycerol prepared by Method II (A) or I (B), respectively. Conditions as described in the text, with a flow rate of 12 ml/min. About 3–5 mg of crude product in 0.5 ml chloroform–methanol 1:1 (v/v) was injected. Notice the difference in the amount of PA present (t_r = 26 min.). The product elutes after about 12 min.

Analyses

Proton NMR spectra of AZTDP-DG in $CDCl_3$–MeOD 1:1 (v/v) contained the following chemical shifts relative to tetramethylsilane: 0.87 (6H, bt, acylCH_3); 1.25 (40H, s, acylCH_2); 1.58 (4H, bs, βCH_2 acyl chains); 1.84 (3H, s, CH_3 thymine); 2.30 (4H, m, αCH_2 acyl chains); 2.48 (2H, m, ribose 2'H); 3.86 (2H, bd, ribose 5'H); 4.04 (2H, m, *sn*-3-CH_2 glycerol); 4.15 (1H, dd, *sn*-1-CH_2 glycerol); 4.53 (1H, m, ribose 4'H); 5.23 (1H, m, *sn*-2-CH glycerol); 7.35 (1H, s, H6 thymine). The ratio of acyl chains:glycerol:ribose:thymine as deduced from appropriate resonances amounted to 2.1:0.9:1.0:1.0. Chemical analysis gave a fatty acid ester:phosphate:nucleoside ratio of 2.1:2.0:1.0. Infrared analysis of AZTDP-DG showed the typical phosphorus vibrational region at 1246 cm^{-1} (P = 0), 1064 cm^{-1} (P–O–C), 952 cm^{-1} (P–O–P) and 519 cm^{-1} (P–O–P) and other characteristic absorptions at 2110 cm^{-1} (N_3), 1741 cm^{-1} (C = O, fatty acid) and 1704 cm^{-1} (C = O, thymidine). For ACVDP-DG the following infrared assignments were made: 1735 cm^{-1} (C = O, fatty acid), 1231 cm^{-1} (P = O), 1067 cm^{-1} (P–O–C), 957 cm^{-1} (P–O–P) and 522 cm^{-1} (P–O–P). Chemical analysis of ACVDP-DG yielded a fatty acid ester:phosphate ratio of 1.05.

In mammalian cells CDP-DG can be hydrolyzed by a pyrophosphatase activity that was first described by Rittenhouse et al. (37) in guinea pig brain. We have recently described a similar pyrophosphatase activity in rat liver

mitochondria. This enzyme hydrolyzed AZTDP-DG, 3dTDP-DG, and ddCDP-DG with the concomitant release of phosphatidic acid and the 5'-monophosphates of AZT, 3dT, and ddC, respectively (38). In addition, AZTDP-DG, 3dTDP-DG, and ddCDP-DG were able to substitute to varying extents for CDP-DG in the biosynthesis of PI, PG, and cardiolipin by rat liver subcellular fractions (39). Using similar techniques it was shown that ACVDP-DG was split by the pyrophosphatase activity into phosphatidic acid and ACVMP and that it substituted for CDP-DG in mitochondrial PG biosynthesis (G. M. T. van Wijk and H. van den Bosch, unpublished experiments). These enzymatic data confirm the structure of the compounds as nucleoside diphosphate diglycerides.

Conclusion

In summary, we synthesized selected nucleoside diphosphate diglycerides by a new method based on the condensation of a 1,2-diacyl-*sn*-glycero-3-phosphomorpholidate and a nucleoside-5'-monophosphate. These compounds are currently under evaluation for their anti-HIV activity (the AZT-, ddC-, and 3dT-analogs) and anti-HSV activity (the ACV analog). The method seems to be generally applicable and is independent of the nature of the nucleoside, provided it can be dissolved in anhydrous pyridine. In one particular case, i.e., acyclovir diphosphate diglyceride, the insolubility of acyclovir monophosphate initially resulted in a low yield, but this problem was overcome by preparing a pyridine-soluble amine salt of ACVMP and heating the reaction mixture at 60°C for 20 h. The other nucleoside diphosphate diglycerides that have been prepared, AZTDP-DG, 3dTDP-DG, and ddCDP-DG, are readily formed in high yield at room temperature within several hours. In comparison to methods used previously, the new synthetic method has considerably shorter reaction times, yields a simpler reaction mixture with fewer byproducts and less unreacted phosphatic acid, and provides much higher yields of the pure product. *

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