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The Chemical Synthesis and Antiviral Properties of an Acyclovir-phospholipid Conjugate

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The synthesis of acyclovir-phospholipid conjugate (*2*) is reported through an unambiguous one-step preparation of L-α-dimyristoyl phosphatidic acid triethylammonium salt (*5*). The biological activity of *2* as an antiviral drug has also been investigated.

Increased efficacy of an antiviral agent may be effected by converting it to a prodrug. For example, the methoxyacetate ester of cyclaridine (carbocyclic ara-A) was topically more active than the parent compound in the treatment of HSV-2-genital lesions in quinea pigs[1]. Ribavirin triacetate, a lipophilic derivative of ribavirin, was superior to ribavirin in the treatment of influenza infected mice[2] and dengue virus infections in mice.[3] Similarly acyclovir, 9-(2-hydroxyethoxymethyl)-guanine (*1*), which is a selective antiherpes agent both *in vitro* and *in vivo*, and currently under clinical and toxicological evaluation, has been derivatised to water-soluble esters for the treatment of herpes keratitis[4]. These esters are active in cell culture against herpes simplex virus (HSV) types 1 and 2 indicating that they may be rapidly hydrolysed to the parent compound. The efficacy of the nucleoside analogue 1-β-D-arabinofuranosyl cytosine (ara-C) as an antitumor agent was vastly improved by its conversion to prodrugs such as ara-CMP-L-dipalmitin or ara-CDP-L-dipalmitin[5]. These phospholipid-ara-C conjugates have three potential advantages over ara-C: (1) increased bioavailability, enabling the use of lower dosages; (2) targeting to specific tissues, thus lowering toxic effects; (3) activity against ara-C resistant cell lines which are unable to phosphorylate the nucleoside to the 5′-triphosphate.

In view of the above studies in the literature, we now report a stereospecific synthesis of an acyclovir-phospholipid conjugate (*2*) and its properties as an antiviral agent in comparison with the parent compound.

CHEMICAL SYNTHESIS

An unambiguous chemical route to the target compound (*2*) warrants a high-yielding regiospecific preparation of pure L-α-phosphatidic acid (*3*) and an appropriately *N*-2-

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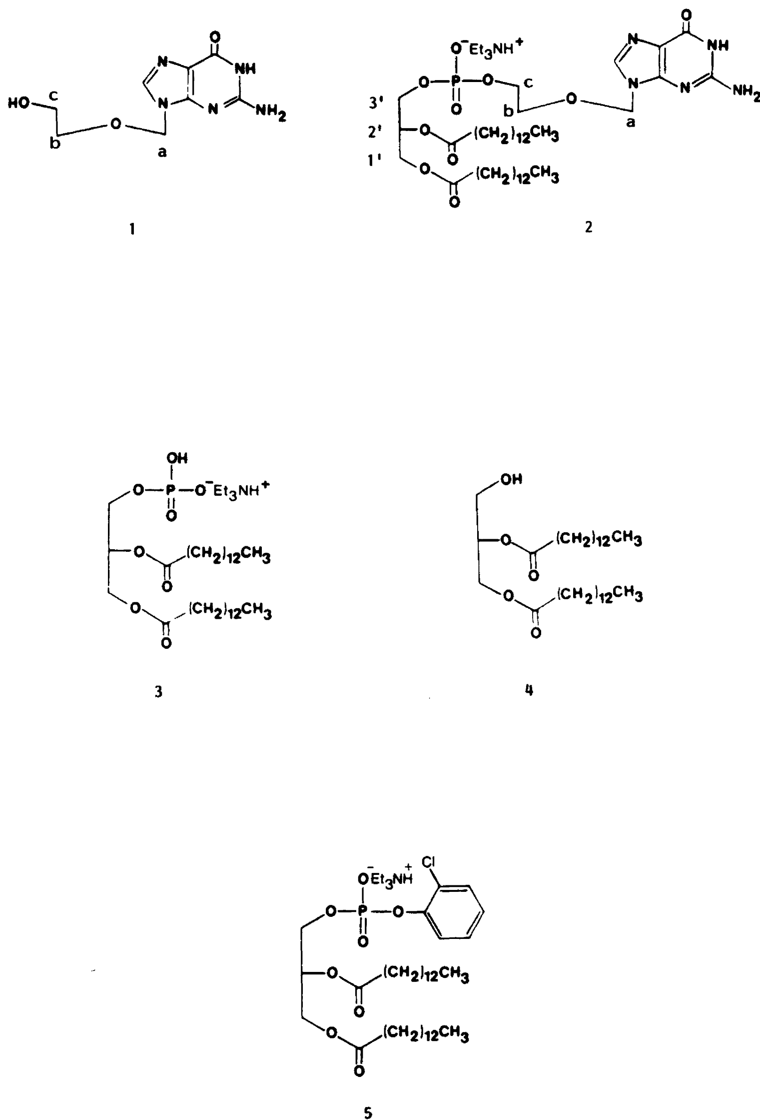
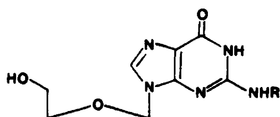
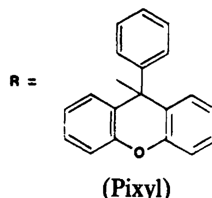


protected acyclovir moiety (*6*). The availability of pure L-α-phosphatidic acids has in the past been a major problem as their preparations have involved multistep procedures giving poor overall yields.[5a-f] The problem of migration of the acyl groups during the phosphorylation reaction has further reduced the availability of pure L-α-phosphatidic acids. Earlier workers [5a-d] have therefore, developed enzymatic preparations of phosphatidic acids from egg lecithin using phospholiphase D. The work described herein constitutes the first report of an unambiguous one-step preparation of pure, suitably protected, L-α-dimyristoyl phosphatidic acid triethylammonium salt (*5*) in 95 % field. This was conveniently carried out (experimental section) by the treatment of dimyristoyl-*Sn*-glycerol[6] with an excess of *o*-chlorophenylphosphorobis(1,2,4-triazolide)[7] in dry pyridine solution at room temperature

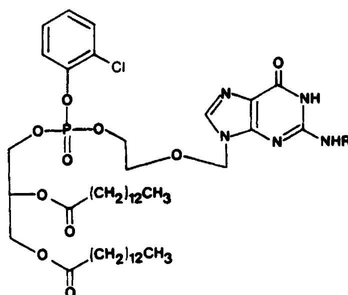


6. An *N*-2 protected acyclovir.

7. R=Pixyl.



(Pixyl)



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for 30 min. The ^{1}H and ^{31}P NMR data unequivocally established that the *o*-chlorophenyl phosphate function had been regiospecifically introduced at the C-3′position of dimyristoyl-*Sn*-glycerol without any concomitant migration of the acyl groups.

We then required an acyclovir derivative with a suitable protection of the exocyclic amino function (*6*). In view of our studies with 9-phenylxanthen-9-yl-(pixyl) as a hydroxyl protective group, which has been employed by us for the syntheses of DNA,[7] RNA[8] and A2′p5′A2′p5′A,[9] we decided to use this group for *N*-2 protection of the guanine residue of the acyclovir (*1*). 9-Hydroxyethoxymethyl)-2-*N*-(9-phenylxanthen-9-yl)-guanine (*7*) was therefore prepared in 98 % yield by transient protection of the hydroxyl function of the acyclovir (*1*) with the trimethylsilyl group (TMS) followed by a reaction with 9-chloro-9-phenylxanthene and then the hydrolysis of the TMS group. The spectroscopic data (UV and NMR) again substantiated the structure 7. The half-life of removal of the pixyl group was estimated to be in the order of 20 seconds at 20 °C in 2 % toluenesulfonic acid monohydrate in dichloromethane−methanol (7:3 v/v).

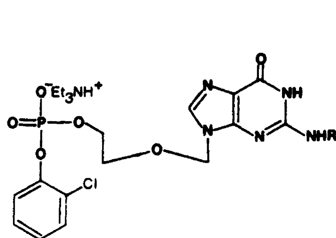
The pixylacyclovir (*7*) was coupled to L-α-dimyristoyl phosphatidic acid (*5*) in dry pyridine solution using 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MS-NT) (5 equiv.) as an activating agent under a condition that we have reported in the literature for DNA and RNA syntheses[6,7,8]. Thus the fully protected acyclovir-phospholipid conjugate (*8*) was obtained in 91 % yield as a glass. ^{1}H and ^{31}P NMR support our assignment of this structure (Table 1).

The acyclovir-phospholipid conjugate, (*8*), was alternatively prepared in the following way: *N*-2-pixyl acyclovir (*6*) was phosphorylated by an excess of *o*-chlorophenylphosphoro-bis(1,2,4-triazolide), using the same condition described for the preparation of compound (*5*), to obtain the phosphodiester salt of *N*-2-pixylacyclovir (*9*) in 95 % yield. This was

Table 1. NMR Chemical shifts (in δ scale) of compounds: (2) to (11).

Compounds	Solvent	^{1}H NMR absorptions with TMS as internal standard: H-8 and other aromatic protons	H_a	H_b	H_c	H-1′	H-2′	H-3′	^{31}P NMR absorptions (85 % H_3PO_4 as external standard).
(*4*)	$CDCl_3$	–	–	–	–	4.28	5.09	3.73	–
(*5*)	$CDCl_3$	7.66–6.94 (4H)	–	–	–	4.32–4.05	5.30	4.32	–5.92
(*7*)	DMSO-d_6	8.08 (1H); 7.67–7.22 (13H)	4.83	3.35	3.35	–	–	–	–
(*8*)	$CDCl_3$	8.01 (1H); 7.56–7.17 (17H)	4.88	3.29	4.38	4.38	5.34	4.38	–7.63;–7.75
(*9*)	DMSO-d_6	8.14 (1H); 7.65–7.20 (17H)	5.36	3.47	4.79	–	–	–	–6.72
(*10*)	$CDCl_3$	7.90 (1H) 7.58–7.17 (13H)	5.30	3.50	4.35	4.35	5.30	4.35	–1.21
(*11*)	Me_3CO-d_6	7.87 (1H) 7.52–7.20 (4H)	5.52	3.88	4.40	4.40	5.31	4.40	–7.45
(*2*)	$CDCl_3$	7.67 (1H)	5.41	3.81	4.35	4.05	5.30	4.05	–1.19

subsequently condensed with dimyristoyl-*Sn*-glycerol in dry pyridine solution, using the previously described condition, to obtain the fully protected acyclovir-phospholipid conjugate (*8*) in 75 % yield. The TLC and spectroscopic properties of compound (*8*) from both of these preparations were identical. However, it is clear that the first synthetic route is superior to and less ambiguous than the second approach. This is apparent due to the possibility of the formation of a small amount of the isomeric by-product formed by the migration of the myristoyl group during the condensation reaction, in the second approach. Formation of these by-products cannot be ruled out *a priori*, despite the availability of spectroscopic and chromatographic data, without the performance of enzymatic digestion of the material with the phospholipase A_2 and C. In the present synthetic route, the protective group for the exocyclic amino function of the guanine and the phosphate residues were so chosen as to be able to deprotect the molecule selectively either at the *N*-2 position to generate the free exocyclic amino function or at the phosphotriester moiety to give the biologically occuring phosphodiester function. Such a flexibility for the removal of the protective groups from a fully-protected acyclovir-phospholipid conjugate is clearly important to be able to delineate the nature of the molecular interaction between the conjugate and the thymidine kinase that would be subsequently expressed through the inhibition profile of a particular compound as an antiviral agent. Thus, the fully-protected acyclovir-phospholipid conjugate (*8*) was converted to *10* by selective removal of the *o*-chlorophenyl group from the phosphotriester moiety using a 0.05 M solution of n-tetrabutylammonium fluoride (3 equiv.) in tetrahydro-furan–pyridine–water mixture (8:1:1 v/v/v) for 6 h. at 20 °C. The usual work-up and purification by column chromatography on silica gel gave a gum in 98 % yield. A detailed NMR analysis (Table 1) confirmed the structure of the compound to be *10*. We then converted *8* to *11* in 95 % yield by the selective removal of the pixyl group by treatment with an excess of 4-toluenesulfonic acid monohydrate in methylene chloride-methanol mixture (7:3 v/v; 0.1 M) for 3 min followed by saturated sodium bicarbonate work-up and purification by chromatography. NMR spectroscopy of *11* (Table 1) again support the structure. Finally, the fully-deprotected



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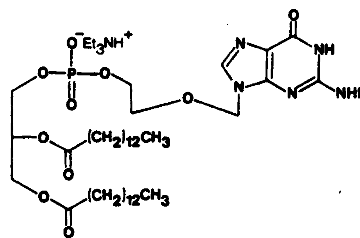

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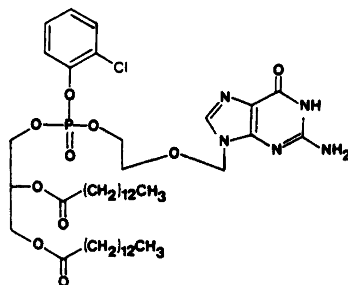

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acyclovir-phospholipid conjugate (*2*) was prepared in 95 % yield by treating *8* consecutively with n-tetrabutylammonium fluoride and then with 4-toluenesulfonic acid monohydrate followed by a short column chromatography. The ^{1}H and ^{31}P NMR absorptions of the fully deprotected conjugate (*2*) are recorded in Table 1 and support the structural assignment.

BIOLOGICAL RESULTS

The phospholipid conjugate of acyclovir (*2*) inhibited replication of Herpes simplex virus type 1 (HSV-1; strain C42) in Vero cells [10], the ID_{90} being 1 μM. At this concentration the conjugate did not inhibit growth of human fibroblasts. Cell growth [10] was, however, inhibited 80 % at 100 μM of *2*; acyclovir being non-toxic for the human fibroblasts at these concentrations.

Table 2. Biological activity of ACV and its phospholipid conjugate in Vero cells.

Parameter	Acyclovir (μM)	Acyclovir phospholipid conjugate (μM)
ID_{90}, HSV-1, (C42)	0.1	1.0
ID_{90}, HSV-1, TK^- (C915 TK)	30	22
ID_{90}, HSV-1, ACV^R	60	70
ID_{90}, HSV-1, (C42)-DNA-synthesis	0.25	2.5

The inhibition of herpes viral DNA synthesis was studied by using a recently developed spot-hybridisation technique.[11] The phospholipid conjugate inhibited viral DNA synthesis by 50 % at 0.25 μM and acyclovir gave the same inhibition at 0.1 μM.

The inhibition of viral DNA synthesis and the antiviral activity of acyclovir (*1*) depend on the phosphorylation of this guanosine analogue to its monophosphate by a virus-encoded thymidine-kinase.[14] Thus, using a thymidine kinase negative (TK^-) strain,[10] inhibition of virus multiplication was only achieved at high concentrations, 30 μM for 90 % inhibition instead of 0.1 μM for the same inhibition of the wild type strain (Table 2). The ID_{90} of the phospholipid conjugate was also higher when using TK^-strains, 22 μM compared with 1 μM for the wild-type (Table 2). In addition a strain of HSV-1 selected for acyclovir resistance (ACV^R) was also refractory to inhibition by the phospholipid conjugate of acyclovir (Table 2). This suggests that the phospholipid conjugate of acyclovir was hydrolysed, at least in part, to generate acyclovir. It cannot be excluded that some acyclovir monophosphate is formed as well: the ratio of the concentrations of drugs needed to inhibit the TK^-strain and the wild-type strain was higher for acyclovir than for the conjugate (Table 2). It should be noted that the phospholipid hydrolases are not present in the medium used to maintain the cells (result not shown), but are cell-bound, or secreted. Taken together these results show that the phospholipid conjugate of acyclovir is hydrolysed to a thymidine kinase-dependent inhibitor, probably acyclovir, of HSV- replication *in vitro*.

Antiviral activity *in vivo* depends on a suitable pharmaceutical formulation to achieve a sufficient drug level in the infected tissue. We have tested the phospholipid conjugate *in vivo* as follows:

(a) topically, 3 times daily for four days, against cutaneous HSV-1 infection in guinea pigs[12] (using either a 2 % suspension in DMSO, or a 100 mM suspension in water containing 20 % glycerol and 0.2 % Triton X-100).

(b) intraperitoneally (56 μmol per kg), 2 times daily for 5 days, against a systemic HSV-2 infection in mice[13] (using a suspension of the phospholipid in phosphate-buffered saline containing 0.2 % Triton X-100).

In both studies the phospholipid-conjugate was without effects, whereas therapeutic effects were found for comparative concentrations of acyclovir as earlier published.[12–14] The lack of therapeutic efficacy of the acyclovir-phospholipid conjugate (*2*) could be due to its unknown pharmacokinetic properties. More work is needed to determine the rate of hydrolysis of the conjugate to acyclovir, and to compare the bioavailability of acyclovir and acyclovir derived from the phospholipid conjugate *in vivo* in order to understand their different efficacies.

EXPERIMENTAL

UV absorption spectra was measured with a Varian DMS 100 double beam scanning spectrometer. ^{1}H-NMR spectra were measured at 60 MHz with a Perkin-Elmer R 600 and 90 MHz with a Jeol FX 90 Q spectrometer. IR spectra were measured with a Perkin-Elmer 298 spectrometer. Merck Kieselgel 60 G was used for short column chromatography. Merck Silica gel 60 F_{254} pre-coated plates were used for TLC.

L-α-Dimyristoyl phosphatidic acid triethylammonium salt (5). Dimyristoyl *Sn*-glycerol[6] (*4*, 513 mg, 1 mmol), coevaporated with *dry* pyridine *in vacuo*, was dissolved in dry pyridine (10 ml), *o*-chlorophenylphosphorobis-(1,2,4-triazolide)[7] (8 ml, 0.25 M solution in dry acetonitrile) was added at room temperature and stirred for 30 min. The reaction was then quenched by the addition of triethylammonium bicarbonate (TEAB) buffer (pH 7.3; 0.5 M;

1 ml). After 10 min, the reaction mixture was poured to TEAB buffer (200 ml) and extracted with chloroform (3×100 ml). The organic phase was washed with water (200 ml) and then evaporated to dryness by several coevaporations with toluene *in vacuo*. The residue was finally dried at 0.05 mmHg to obtain *5* (765 mg, yield 95 %), ^1H NMR($CDCl_3$; TMS as internal standard): 7.7−7.0 (*m*, 4H), *o*-chlorophenyl group; 5.30 (*m*, 1H), H-2′ of glycerol; 4.32−4.05 (*m*, 4H), H)1′ and H-3′ protons of glycerol; 3.01 (*m*, 6H), CH_2- of triethylammonium ion; 2.25 (*t*, 4H), -CH_2- of myristoyl groups; 1.25 (br.*s*, 44H), CH_2 of myristoyl; 0.89 (*m*, 15H), CH_3 of myristoyl and triethyl ammonium groups. ^{31}P NMR (H_3PO_4 as external reference): −5.92 ppm (*s*, IP).

*9-(Hydroxyethoxymethyl)-2-*N*-(9-phenylxanthen-9-yl)-guanine* (7). A dry pyridine (10 ml/mmol of substrate) suspension of acyclovir (2.2 mmol) was treated with trimethylchlorosilane (3 equiv.) at room temperature; when the reaction mixture formed a clear solution, 9-chloro-9-phenylxanthene (1.2 equiv.) was added and stirred for 30 min at 20 °C. The mixture was poured into a solution of saturated sodium bicarbonate (100 ml) and extracted with chloroform (3×50 ml). The solvents were then removed *in vacuo* and the residue was dissolved in pyridine (10 ml/mmol) and methanol (2 ml) added. After 30 min the mixture was evaporated to dryness, dissolved in a small volume of dichloromethane and precipitated from diethyl ether to obtain pure *7* in 98 % yield (R_f=0.72 in 30 % ethanol−chloroform). The spectroscopic data (Table 1) substantiated the above structure.

ID_{90} was measured by the plaque-reduction assay using monolayers of Vero cells. The concentrations reported are those required to inhibit plaque formation by 90 percent. DNA synthesis was measured by nucleic acid spot hybridization procedure.[11] The ID_{90} concentration reported are those required for Viral DNA synthesis by 90 %.

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