Biochemical Pharmacology, Vol. 39, No. 11, pp. 1657–1664, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

GLUCOSYL PHOSPHOTRIESTERS OF NUCLEOSIDES: EXCHANGE MECHANISM OF TRANSMEMBRANE TRANSPORT AND APPLICATION TO 5-FLUORO-DEOXYURIDINE

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(*Received* 6 *July* 1989; *accepted* 15 *December* 1989)

Abstract—The structure and membrane interactions of lipophilic glucosyl phosphotriester derivatives of thymidine and 5-fluoro-deoxy thymidine are investigated by NMR spectroscopy. The self-association of these molecules, found in different solvents, presents a diastereoisomeric effect which is also observed in the transmembrane transport inside large unilamellar vesicles. The influence of the hydrophobic chain and the nature of the nucleoside in the water-membrane exchange process is discussed.

In the design of medically useful drugs, it is commonly assumed that the ability of a compound to penetrate a membrane is correlated to its lipophilic properties. A significant part of research is therefore devoted to phospholipid derivatives because of their structural relationship to membrane components [1, 2]. In our program of synthesis of biological phosphate molecules and studies of membrane–drug interactions, we were interested by the lipidic carrier glycosyl dolichol phosphate which served as the basis of phosphotriester derivative of thymidine, glucose and hexadecanol, **1** (TGC) which could be considered as an original non-ionic drug carrier (Scheme 1).

A previous paper [3] described the synthesis of such a molecule as well as its phosphodiester precursors and the NMR study of the interactions between these molecules and model membranes. A first result showed that four TGC molecules Xα, Xβ, Yα and Yβ are simultaneously synthetized: X and Y refer to the diastereoisomeric molecules R and S (the exact configuration of X and Y are undetermined) and α, β label the anomeric glucose moiety. These four molecules are especially discernible in a phosphorus NMR spectrum where the diastereoisomeric shift δX-δY is equal to 0.54 ppm for both anomeric species. The main result of our work, obtained by proton, phosphorus and carbon NMR spectroscopy, is that, in the presence of model membranes, TGC molecules are transported through the lipid bilayer. However, we observed that the two diastereoisomeric species exhibit quite different NMR relaxation parameters in the presence of membranes, which reveal a difference in the interaction process with the phospholipids.

In the first part of this paper, we present several results which contribute to explain the mechanism of TGC–membrane interactions and the difference observed between the diastereoisomers. A second part is devoted to the synthesis and the NMR study, in the presence of model membranes, of two new phosphotriester derivatives in which the thymidine nucleotide is replaced by the active antiviral drug 5-fluoro-deoxyuridine (Scheme 1). The alkyl chain of the first glucosyl phosphotriester of 5-fluoro-deoxyuridine, **2** (dU^FGC_{16}), is a hexadecanol unit as in TGC whereas the second analog, **3** (dU^FGC_6), contains a shorter alkyl chain (hexanol) in order to monitor the role of the alkyl chain length in the drug–membrane interaction process.

MATERIALS AND METHODS

Synthesis of **2** *dU^FGC_{16} and* **3** *dU^FGC_6*

1,2,3,4 Tetra-*O*-acetyl 6-D-glucopyranosyl cyanoethyl 5′-(2′-deoxy 5 fluoro) uridinyl phosphate was prepared according to published procedures [3] from 200 mg (0.81 mmole) of 2′-deoxy 5-fluoro uridine (dU^F) and 469 mg (0.97 mmole) of 1,2,3,4 tetra-*O*-acetyl 6-D-glucose cyanoethyl phosphate in 4 mL of anhydrous pyridine and 616 mg of (2,4,6-tri-isopropyl-benzenesulfonyl)-3 nitro-1,2,4 triazole (TPSNT). After 3 hr at room temperature, dichloromethane was added and the organic layer washed with a saturated $NaHCO_3$ solution, then with water and evaporated *in vacuo*. The residue was purified by chromatography on a column of silica gel (Merck 9385) with dichloromethane-methanol as the eluent to give 271 mg (47%). R_f:0.42 CH_2Cl_2-MeOH 9/1.

The phosphodiester was deacylated by treatment with 1% sodium methoxide in methanol. After 15 mm at room temperature, the solution was neutralized with Dowex 50W cation-exchange resin (pyridinium form); the resin was filtered off, washed well with methanol and the filtrates were evaporated to dryness. The residue was dissolved in water and

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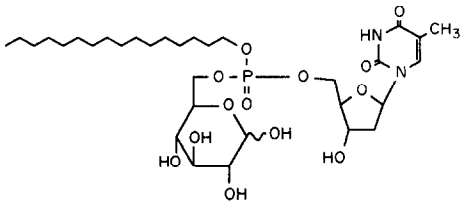
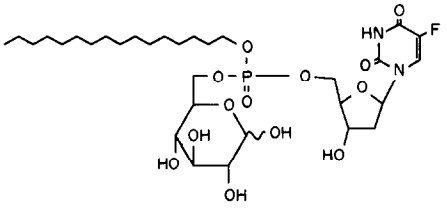
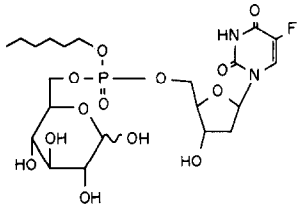


1 TGC



2 dU^FGC_{16}



3 dU^FGC_6

Scheme 1.

chromatographed on a column of Biogel P_2 with water to give 80% of phosphodiester 6-D-glucosyl 5'-(2'-deoxy 5-fluoro) uridinyl phosphate. R_f: 0.46 (isopropanol-NH_4OH-H_2O 6/1/2). Mass spectra (FAB+): 498(M+1).

The tetrabutylammonium salt of the previous phosphodiester (200 mg) was dissolved in 20 mL of anhydrous acetonitrile and 1-bromo hexadecane (2 mL) was added. The reaction was heated for 18 h at 80°. After evaporation to dryness, the residue was triturated with ether dissolved in water and purified on a column of reverse-phase silicagel (Lichroprep RP18 Merck 9303) with a gradient of water–methanol (0 to 100%) to yield 55% of the hexadecyl phosphotriester **2** dU^FGC_{16}. R_f: 0.53 CH_2Cl_2MeOH 8/2. Mass spectra (FAB+) 735 ($M + Na^+$).

The same tetrabutylammonium salt was alkylated with 1-iodohexane overnight to yield 29% of hexyl phosphotriester **3** dU^FGC_6 after purification. R_f: 0.56 (isopropanol-NH_4OH-H_2O 6/1/2). Mass spectra (FAB+) 573 (MH+).

NMR spectra of TGC in various solvents

TGC was dissolved in a given solvent at a concentration of 4 mM. Phosphorus NMR spectra (121 MHz) were recorded on a Bruker MSL 300 spectrometer and referenced relative to external trimethyl phosphate. Two levels of broadband proton decoupling were applied for all experiments. The transverse relaxation time of TGC was measured by using a Hahn spin echo sequence.

^{13}C NMR spectrum of TGC (4 mM) in aqueous solution was recorded at 150 MHz on a Bruker AM 600 spectrometer.

NMR structural analysis of dU^FGC_{16} and dU^FGC_6

The phosphotriester molecules were dissolved in DMSO at a concentration of 4 mM. Proton spectra (500 MHz) were recorded on a Bruker WM 500 spectrometer and referenced to internal tetramethylsilane. Structural analysis was achieved by recording two dimensional COSY and relayed coherence transfer COSY spectra and using the procedure described in a previous paper [3].

NMR study of TGC, dU^FGC_{16} and dU^FGC_6 in the presence of model membranes (large unilamellar vesicles)

Vesicles preparation. Large unilamellar vesicles (LUV) of defined size were prepared by reverse phase evaporation [4] using a mixture of egg phosphatidylcholine and phosphatidic acid (mole ratio 9:1). Phosphatidylcholine was extracted from egg yolk according to the method of Singleton *et al.* [5]. Phosphatidic acid was prepared from the former as described by Allgyer and Wells [6]. Buffers used were 50 mM pipes-KOH pH 7.2 supplemented with 100 mM potassium sulfate. The vesicle suspension was sequentially extruded through 200 nm polycarbonate membranes (Nuclepore) in order to obtain a uniform size distribution [7]. The final lipid concentration was about 25 mM. For each experiment, LUV integrity was analysed by recording a phosphorus NMR spectrum of a blank sample of vesicles.

NMR spectra. Small amounts of 20 mM stock solution of each phosphotriester analog was injected into NMR tubes containing a buffered aqueous solution with or without LUV. The final concentration of the phosphotriester molecules was 1.1 mM. NMR phosphorus spectra (121 MHz) were recorded by using a Hahn spin echo sequence with a refocusing delay of 1 msec in order to cancel the signal of membrane phospholipids whose transverse relaxation time is less than 0.5 msec. Phosphorus spectra (202 MHz) were recorded in the same conditions on a Bruker WM 500.

RESULTS AND DISCUSSION

Since TGC contains an hydrophobic chain and an aromatic ring, it may exhibit strong self association

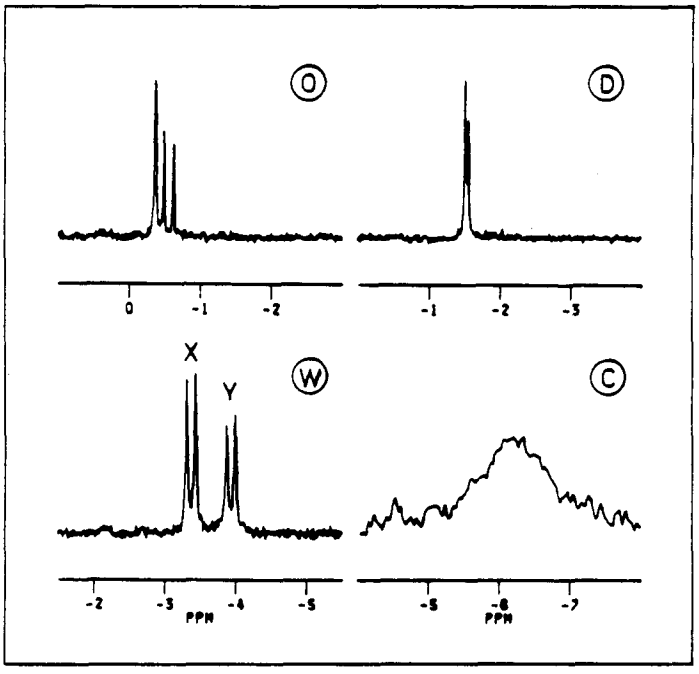


Fig. 1. Phosphorus spectrum (121 MHz) of TGC in various solvents at room temperature. The signals of the two diastereoisomers species are labeled X and Y. Each of them contains two resonances (α, β) relative to the two anomeric glucose moieties. W, O, D and C correspond to the following solvents: water, octanol, dimethylsulfoxide and chloroform.

properties which can be implied in the mechanism of the drug–membrane interactions. Furthermore, one can expect that the two diastereoisomers X and Y exhibit different intermolecular interactions: the presence of the two diastereoisomeric molecules TGC is not only revealed on a phosphorus NMR spectrum but also by the chemical shift difference observed between the corresponding resonances of the H6 thymidine proton (in DMSO) which cannot be related to a change of the nucleotide conformation [3]. For these reasons we have investigated the self association properties of TGC by analysing the phosphorus spectrum of TGC in various solvents. Indeed, the largest diastereoisomeric shift is observed on the phosphorus spectrum and the phosphorus transverse relaxation rate is an efficient probe of the molecular size. Proton and carbon NMR experiments are also described.

Self association properties of TGC

The effects of TGC self association are clearly illustrated by the solvent dependence of the TGC linewidths (Fig. 1). One can notice that the linewidth and the chemical shift difference between the resonances of the four synthetized TGC molecules Xα, Xβ, Yα and Yβ decrease when an octanol or a dimethylsulfoxide solution is used instead of water whereas a very broad signal is observed for TGC solubilized in chloroform. The correlation between the linewidth and the chemical shift variation indicates that the diastereoisomeric shift is related to the self association properties of TGC.

A more precise analysis of TGC self association was achieved by measuring the transverse relaxation rate R_2 of the TGC phosphorus signal in the following solvents: water; dimethylsulfoxide; methanol; pyridine; octanol; and chloroform. In Fig. 2, the various R_2 values are plotted versus E the dielectric constant of the solvent. Examination of these data leads to the following remarks: (i) the R_2 values are ranged between 0.75 and 220 sec^{-1}, i.e. two orders of magnitude; (ii) the minimum value corresponds to the transverse relaxation rate expected for a TGC molecule in a monomeric state (taking into account the TGC molecular size) and is obtained for E values intermediate between that of the water and of the apolar solvent chloroform; this feature reflects the equilibrium between the hydrophobic and hydrophilic properties of TGC; and (iii) when the four TGC resonances (Xα, Xβ, Yα, Yβ) are discernible, they exhibit the same R_2. The last remark means that both diastereoisomers exhibit the same degree of self association, i.e. the same aggregation number.

The self association of TGC is also clearly revealed by the temperature dependence of the TGC phosphorus chemical shifts in aqueous solution (Fig. 3): on increasing the temperature, the TGC chemical shifts increase. This variation reflects a fast exchange (on the NMR time scale) between an associated form and a monomeric state exhibiting a higher chemical shift value (as shown in Fig. 1, a decrease of TGC linewidth is correlated with an increase of the chemical shift). In contrast with the temperature dependence, the TGC chemical shifts are poorly affected by the concentration: a ten-fold dilution (from 5 to 0.5 mM) at 25° leads to a chemical shift variation less than 0.05 ppm and does not affect the linewidth. This means that, in the concentration range used in this

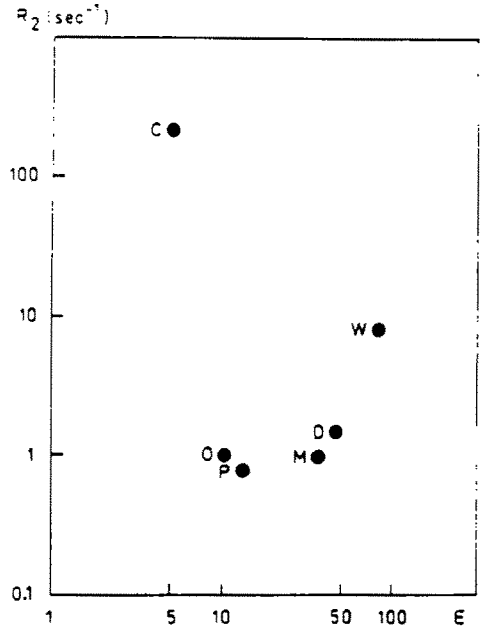


Fig. 2. Log-log plot of various couples (R_2, E) where R_2 and E are, respectively, the phosphorus transverse relaxation rate of TGC in a given solvent and the dielectric constant of the solvent used. The R_2 values are normalized with respect to the water viscosity. W, C, D, M, O and P correspond to the following solvents: water, chloroform, dimethylsulfoxide, methanol, octanol and pyridine.

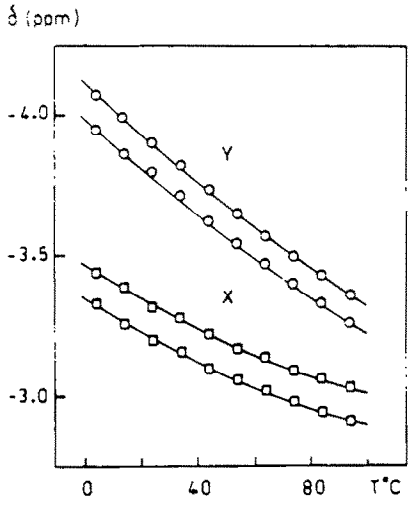


Fig. 3. Temperature dependence of the TGC Xα, Xβ, Yα and Yβ chemical shifts in aqueous solution.

study and at room temperature, the proportion of TGC monomers is small. A complete quantitative analysis of the TGC phosphorus relaxation parameters (longitudinal and transverse relaxation times, proton-phosphorus Overhauser effect, measured at two different frequencies) described in the appendix, gives the following information about the nature of TGC self-association: the mean radius of TGC aggregates (considered as spheres) is about 21 Å and the aggregation number is about 20. Moreover, proton NMR spectra of TGC recorded in the presence of increasing amounts of external paramagnetic Mn ions show that the signals of glucose and thymidine protons are rapidly broadened at low Mn concentrations whereas higher ion concentrations are required to similarly affect the alkyl chain protons and in particular the terminal methyl group; this means that both glucose and nucleoside moieties are exposed to the aqueous milieu whereas the alkyl chains form a hydrophobic core. Therefore, TGC aggregates can be considered as micelles having a mean radius of 21 Å. Such a result is in agreement with the work of Israelachvili *et al.* [8] which indicates that single chained lipids with large head group area, such as TGC, form small micelles in aqueous solution. In addition to the hydrophobic chains, the thymidine aromatic cycles of TGC may play a role in the self-association process. However, the base proton NMR signals (H6 and methyl protons) are not significantly sensitive to temperature and concentration variations. Nevertheless, the ^{13}C-NMR spectrum of TGC in aqueous solution (Fig. 4) shows that the X and Y resonances corresponding to each of the four TGC aromatic carbons exhibit different chemical shifts whereas no diastereoisomeric effect is observed on the other signals (glucose and chain carbons). Since such a difference cannot be explained at the monomer level, this could indicate that base–base stacking interactions differ between the X and Y molecules and thus play a role in the self-association process.

It has to be pointed out that for each diastereoisomeric species, the phosphorus chemical shift of α or β resonances exhibits the same temperature dependence whereas the phosphorus chemical shift difference between the X and the Y signals decreases when the temperature increases. It is thus clear that the diastereoisomeric shift $\delta X - \delta Y$ is directly related to the self association properties of TGC molecules. The question is now to know if the two molecules X and Y are associated in the same aggregated form or if we observe separately a self association of X molecules and one of Y molecules. It is important to notice that the X and the Y molecules may be associated as an enantiomeric aggregate (pair of homochiral enantiomers) or as a racemic form (heterochiral racemic pair). It is known that, in some cases, the interactions between two diastereoisomers are as strong as a covalent binding [9]. At the present time, no experiment (in both chemistry and spectroscopy domains) gives a decisive result. In order to facilitate the discussion, the X and the Y species are still called diastereoisomers in the following. At any rate, the results of the experiments described in the next section show that the molecules corresponding to the X signal and those corresponding to the Y signal form distinct aggregates.

TGC exchange process in the presence of water/chloroform interface. Interpretation of the diastereoisomeric effect observed in the presence of membranes

The understanding of the interaction of TGC with membranes in aqueous solution is difficult since in

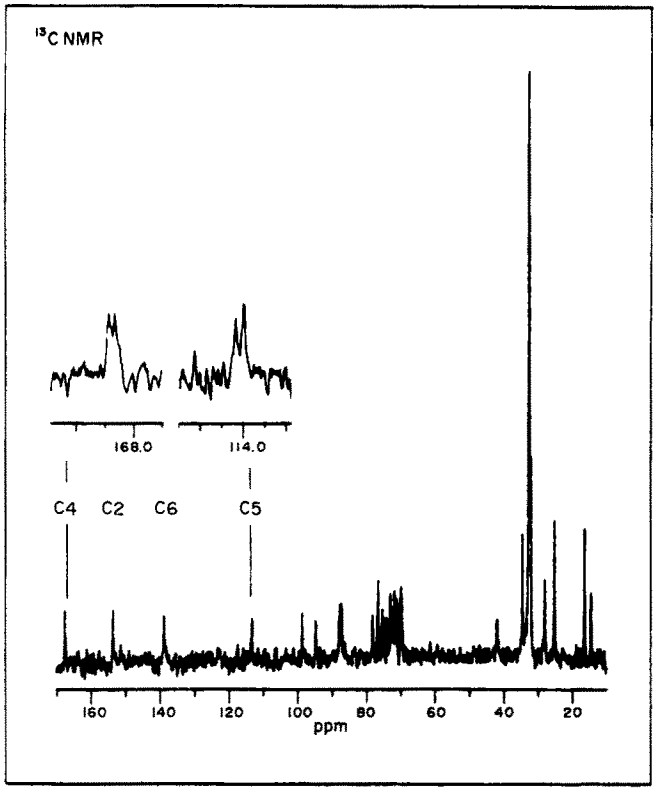


Fig. 4. ^{13}C-NMR spectrum of TGC (4 mM) in aqueous solution at room temperature. Inserts display expanded spectral regions containing thymidine aromatic carbons and show the chemical shift difference between X and Y resonances.

the corresponding phosphorus spectra, we only observe the signal of TGC in the aqueous millieu, the interactions with membranes being revealed by a broadening effect [3]. Fortunately, the following experiment which mimics the exchange of TGC between two phases enables us to distinguish one TGC resonance per medium.

TGC was first dissolved in chloroform and the corresponding phosphorus resonance appears as a broad signal centered at −6 ppm similar to that shown in Fig. 1. Pure water was then added in the NMR tube and a spectrum was recorded every 20 min (the water and chloroform volumes are identical and calculated in order to localize the water/chloroform interface at the center of the NMR receiving coil). In the first spectrum obtained (Fig. 5), the broad resonance centered at −6 ppm is no longer detected whereas two distinct signals appear: a new signal located at 0 ppm and the usual resonances of TGC (Xα, Xβ, Yα, Yβ) in aqueous solution centered at −3.5 ppm. In the successive recorded data, the intensity of the latter resonances continuously decreases while, in parallel, the new signal is narrowed and its intensity increases. The unknown signal is assigned to TGC in the intermedium region since, at the end of the experiment a thin opalescent layer is observed at the water/chloroform interface in the sample tube and the unknown signal disappears when the interface is moved out of the receiver

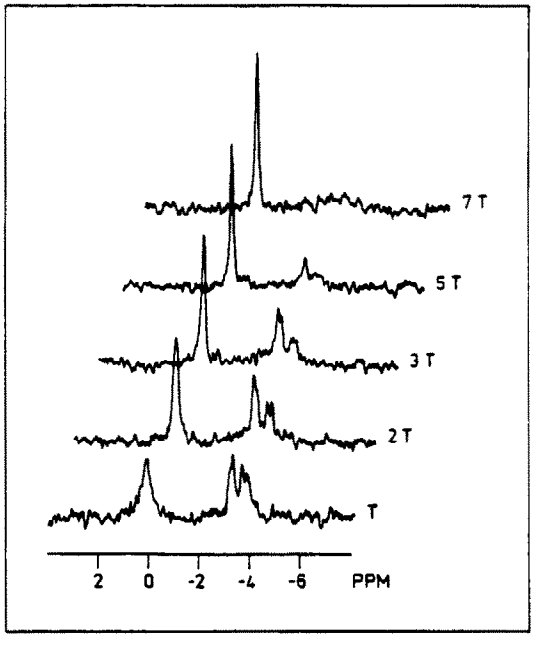


Fig. 5. Time evolution of the 121 MHz phosphorus spectrum of TGC, initially solubilized in chloroform, after addition of a pure water solution (see text). The time interval T is equal to 20 min.

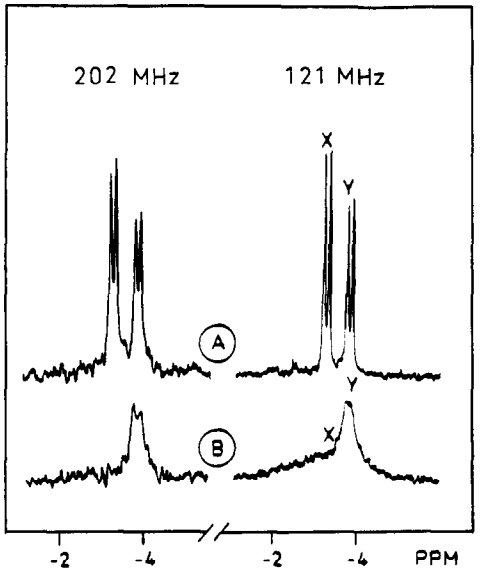


Fig. 6. Phosphorus spectra (121 MHz and 202 MHz) of TGC in aqueous solution at room temperature in the absence (A) and presence (B) of model membranes.

Table 1. Proton chemical shifts (in ppm from TMS) of dU^FGC_{16} and dU^FGC_6 (4 mM, DMSO, 27°)

	dU^FGC_{16}		dU^FGC_6	
	α	β	α	β
Acyl chain				
CH_3	0.86		0.86	
$(CH_2)_n$	1.28		1.28	
CH_2-CH_2-O-P	1.59		1.60	
$\overline{CH_2}$-O-P	3.99		4.00	
Glucose				
H1	4.91	4.32	4.92	4.32
H2	3.12	2.91	3.12	2.91
H3	3.43	3.15	3.43	3.15
H4	3.04	3.04	3.04	3.04
H5	3.75	3.32	3.75	3.33
H6	4.00	3.98	4.04	4.00
H6′	4.16	4.20	4.16	4.21
Nucleotide				
H6 Y	7.88		7.90	
X	7.85		7.88	
H1′	6.16		6.17	
H2′2″	2.13		2.14	
H3′	4.24		4.23	
H4′	3.98		3.93	
H5′5″	4.00		3.99	

coil. The phosphorus spectra simply reflect a slow equilibrium (on the NMR time scale) between TGC in aqueous solution and TGC in the intermedium region.

Indeed, the most interesting result of this experiment is the significant difference observed between the exchange kinetics of TGC X(α, β) and Y(α, β) species: as shown in Fig. 5, the Y signals disappear before the X signals. Such a result suggests that the X and the Y species form distinct aggregates by self association and enables us to examine the difference observed between the two diastereoisomers in the presence of membranes in terms of a difference in exchange kinetics.

Figure 6 shows the phosphorus NMR spectra of TGC in the absence (Fig. 6A) and in the presence (Fig. 6B) of model membranes in aqueous solution, recorded at 121 and 202 MHz. In the presence of membranes, both X and Y signals are broadened and the linebroadening of the X signal is much larger than that of the Y signal. Moreover, when the spectrometery frequency is increased from 121 to 202 MHz, the X linewidth increases so that the corresponding signal is broadened under detection whereas the Y linewidth is not significantly affected by the frequency change. It is well known that, in the presence of a chemical equilibrium (e.g. the exchange process of molecules between two different phases) the linewidth of the corresponding NMR signals can depend on the exchange rate [10, 11]. In the case of a slow exchange on the NMR time scale (i.e. the transverse relaxation rate as well as the difference between the resonance frequencies associated with the molecule in each phase are greater than the exchange rate) the linebroadening effect is independent of the spectrometer frequency; by contrast, such an effect can become frequency dependent when the exchange rate increases [10, 11]. Thus, the phosphorus spectra of TGC in the presence of membranes indicate that, as in the case of the water–chloroform interface, the water–membrane exchange kinetics of the X and Y species are different and that the exchange process of Y molecules is slower (on the NMR time scale) than that of X molecules.

It has to be pointed out that the main result of our previous paper [3] is the detection, after external addition of paramagnetic ions, of a signal with a reduced intensity but exhibiting the same linewidth as in the absence of paramagnetic effect and corresponding to the TGC molecules in the intravesicular water–membrane interface. This means that the exchange process includes the equilibrium of TGC molecules inside the lipid bilayer, for example a flip-flop mechanism. However, taking into account the chemical structure of TGC, one can assume that such a mechanism is very slow on the NMR time scale and in any case, slower than the water–membrane exchange and thus does not influence the above interpretation. Nevertheless, the complexity of the mechanisms involved in the complete exchange process experienced by the TGC molecules precludes a quantitative approach.

The next section describes the study of the two new phosphotriester analogs dU^FGC_{16} and dU^FGC_6. As mentioned above, these molecules differ from TGC by the presence of a 5-fluoro-deoxyuridine instead of a thymidine and dU^FGC_6 contains a hexanol unit as the alkyl chain. One can expect that the change of the fatty chain length modifies the exchange mechanism inside the bilayer.

Application to the phosphotriester derivatives containing the active drug 5-fluoro-deoxyuridine: dU^FGC_{16} and dU^FGC_6

The structures of dU^FGC_{16} and dU^FGC_6 were investigated by using the proton NMR procedure

Table 2. Phosphorus chemical shifts (in ppm from external TMP) of dU^FGC_{16} and dU^FGC_6 in aqueous solution and in DMSO at 27°

	dU^FGC_{16}		dU^FGC_6	
	α	β	α	β
H_2O				
X	−3.40	−3.52	−3.60	−3.67
Y	−3.78	−3.86	−3.67	−3.78
δX-δY	0.36		0.09	
DMSO				
X	−1.41	−1.45	−1.38	−1.42
Y	−1.45	−1.47	−1.42	−1.46
δX-δY	0.03		0.04	

described for TGC in our previous paper [3]. The corresponding proton and phosphorus chemical shifts are listed in Tables 1 and 2, respectively. As in the case of TGC, the four molecules Xα, Xβ, Yα and Yβ are synthetized and one can notice several significant differences between dU^FGC_{16} and dU^FGC_6: (i) the dU^FGC_6 X:Y ratio is 70:30 instead of 55:45 for dU^FGC_{16} (and 57:43 for TGC); (ii) the diastereoisomeric shift δX–δY observed in aqueous solution between the dU^FGC_6 phosphorus resonances (0.09 ppm, Table 2) is much smaller than that obtained for dU^FGC_{16} (0.36 ppm) or TGC (0.56 ppm) under the same experimental conditions; and (iii) the transverse relaxation rate of dU^FGC_6 measured in aqueous solution is two times smaller than the value obtained for dU^FGC_{16} which is close to that found for TGC (8 sec^{-1}). The aggregation number of dU^FGC_6 is thus smaller than those of dU^FGC_{16} and TGC. In contrast, dU^FGC_6, dU^FGC_{16} and TGC in DMSO solution exhibit similar phosphorus NMR parameters (Table 2). These results corroborate the conclusion mentioned in the study of TGC: the diastereoisometric shift is correlated to the self association properties of the phosphotriester molecules.

In the presence of LUV membranes, dU^FGC_{16} and dU^FGC_6 phosphorus resonances are broadened and in both cases, a single signal is observed (Fig. 7), the intensity of which is identical to the total area of the phosphotriester resonances measured in the absence of membranes. Therefore, the difference observed between the linebroadening of the X and Y species of TGC in the presence of membranes is not detected for dU^FGC_{16} and dU^FGC_6. Addition of paramagnetic ions (Fig. 7) leads to a decrease of the dU^FGC_{16} signal by a factor of three and to the complete disappearance of the dU^FGC_6 signal. The first effect previously observed and analysed with TGC (see above and Ref. 3) indicates the presence of dU^FGC_{16} in the intravesicular water membrane interface whereas the second result suggests that dU^FGC_6 only interacts with the external phospholipid layer.

The fact that dU^FGC_6 does not cross the membrane bilayer confirms that the transmembrane transport of phosphotriester derivatives implies a flip-flop process from the external to the internal layer: such a mechanism requires the presence of an alkyl chain

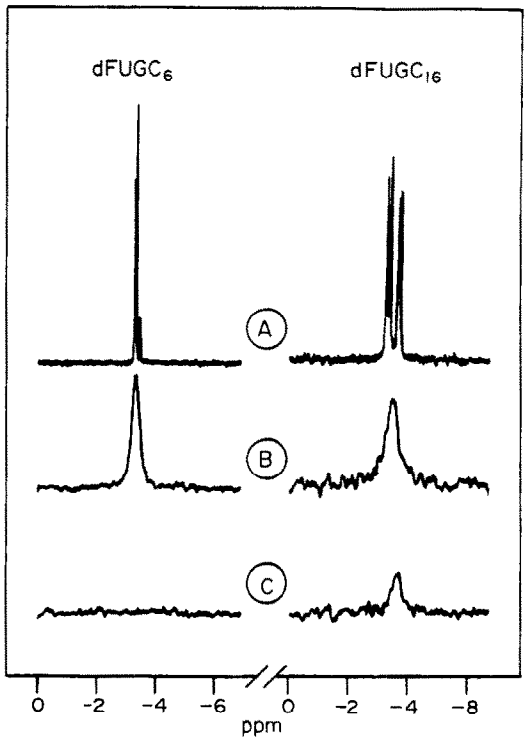


Fig. 7. Phosphorus spectra (121 MHz) of dU^FGC_6, dU^FGC_{16} in aqueous solution at room temperature in the absence (A) and presence (B) of model membranes and after addition of paramagnetic Mn ions (C).

the length of which is close to that of the phospholipid molecules and the short chain of dU^FGC_6 does not satisfy this condition.

This study shows that a phosphotriester analog of the antiviral drug 5-fluoro-deoxyuridine, containing a hexadecanol unit (dU^FGC_{16}), is able to cross the membrane bilayer. The removal of the charge from a monoanionic phosphodiester in order to give a neutral phosphotriester is not enough to confer transmembrane transport properties to a nucleoside and one should ascertain whether the hydrophobic chain has the proper length to allow a flip-flop movement. Furthermore, the comparison with the phosphotriester derivative of thymidine (TGC) indicates that, when a methyl group is replaced by a fluorine atom, no diastereoisomeric effect is observed in the water–membrane exchange process. A possible explanation of this difference is suggested by the following observation: the presence of a methyl group instead of a fluorine increases the base–base stacking properties of the nucleotide [12, 13]. Thus, one can imagine that the presence of a methyl group could strengthen the molecular interactions between the phosphotriester analogs and that this effect is more efficient for one of the two diastereoisomers and thus modifies the corresponding water–membrane exchange kinetics.

Acknowledgements—This work was supported by an Institute Pasteur grant (CCAR 1988). We wish to thank Prof. J. Igolen for his support and encouragement.

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APPENDIX

Quantitative analysis of phosphorus NMR data of TGC in aqueous solution

The phosphorus transverse relaxation rate (R_2) as the other phosphorus relaxation parameters, the nuclear Overhauser enhancement (n) and the longitudinal relaxation rate (R_1), are mainly governed by the proton–phosphorus dipolar interactions (*dd*) and the chemical shift anisotropy (*csa*). They can be expressed as:

$$n = (\upsilon_H/\upsilon_P)(\sigma_{dd}/R_1) \quad (1)$$

$$R_1 = R_{1dd} + R_{1csa} \quad (2)$$

$$R_2 = R_{2dd} + R_{2csa} \quad (3)$$

where υ_H and υ_P are the proton and phosphorus gyromagnetic ratios and σ_{dd} the dipolar cross relaxation term. In the presence of a chemical exchange, the above relations remain valid if the exchange is fast on the NMR time scale and the proportion of one species is negligible. These conditions are verified in our case. Examination of the relations (1), (2) and (3) shows that a quantitative analysis of these data necessitates an evaluation of the *dd* and *csa* contributions. This can be achieved by using a procedure previously described [14] which requires the measurement of n and R_1 at two different frequencies.

Longitudinal relaxation data. The nuclear Overhauser enhancement and the longitudinal relaxation rate of TGC phosphorus signals were measured at 36.5 and 121.5 MHz. We obtained $n = 50\%$, $R_1 = 0.95\,\text{sec}^{-1}$ at 36.5 MHz and $n' = 3.5\%$, $R_1' = 0.83\,\text{sec}^{-1}$ at 121.5 MHz. No significant difference was detected between the four TGC signals. From the ratio $n'R_1'/nR_1$ we can deduce an effective correlation time which reflects the local molecular motion. A correlation time of 1.75 nsec was found and then used to identify the *dd* and *csa* contributions [14]: $R_{1dd} = 0.83\,\text{sec}^{-1}$, $R_{1csa} = 0.12\,\text{sec}^{-1}$ at 36.5 MHz and $R_{1dd}' = 0.15\,\text{sec}^{-1}$, $R_{1csa}' = 0.68\,\text{sec}^{-1}$ at 121.5 MHz. By using all these data and the theoretical expressions of R_{1dd} and R_{1csa} [15] the *csa* relaxation rates give a value of 115 ppm for $\delta\sigma$ (the *csa* factor) and by assuming that six protons (the CH_2-O-P protons of the three building blocks of TGC) are dipolar-coupled to the phosphorus, the *dd* relaxation rates give an average proton–phosphorus distance of 2.9 Å. This value is in agreement with the molecular structure of the phosphotriester linkage.

Transverse relaxation rate. By using the *csa* factor and the proton–phosphorus distances derived from the longitudinal relaxation data and the theoretical expression of the transverse relaxation rate [15], a correlation time t_c of 10 nsec is deduced from the experimental R_2 value ($8.3\,\text{sec}^{-1}$). This value characterizes the isotropic overall rotational diffusion of TGC and by using the Stockes–Einstein relation:

$$t_c = 4\pi R^3 \upsilon/3kT \quad (4)$$

(where υ is the water viscosity and T the absolute temperature) corresponds to a sphere having a mean radius of 21 Å. Taking into account the approximative volume of one TGC molecule (in agreement with the minimum R_2 value of TGC in pyridine) we find an aggregation number of 20 for TGC in aqueous solution. Thus we can conclude that TGC in aqueous solution exhibits an equilibrium $(TGC)_{20} \leftrightarrow 20$ TGC the exchange rates of which are fast on the NMR time scale.