Synthesis and Transmembrane Transport Studies by NMR of a Glucosyl Phospholipid of Thymidine†

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Abstract: A phosphotriester derivative of thymidine, glucose, and hexadecanol was prepared by the nucleophilic displacement of 1-bromohexadecane by 6-glucopyranosyl 5′-thymidinyl phosphate. This compound was synthesized as a model of a transport molecule related to dolichyl 1-glucose phosphate. Its structure and interaction with large unilamellar vesicles were studied by ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy: the detection of the phosphotriester of thymidine inside the vesicles implied its transfer across the lipid bilayer, in contrast with its phosphodiester precursors which either did not interact with the membrane or alter the lipid layer.

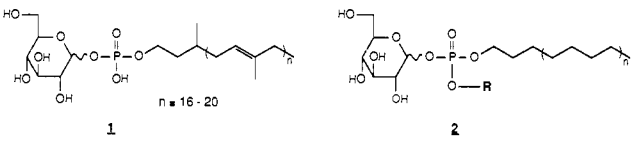
The penetration of a drug into cells is the first step necessary for its biological activity. Since most cell walls are permeable primarily to lipid-soluble materials, lipophilic derivatives are often based on phospholipid compounds because of their structural relationship to membranes.[1,2]

Quite surprisingly, investigations of membrane-permeable compounds have not focused on the dolichyl glucose 1-phosphate (**1**) (Chart I) although many studies have clearly demonstrated that the glycosyl moiety of proteins is assembled by a transmembrane movement mediated by the dolichol-linked intermediates.[3-5] In this work we examine the development of a phosphotriester derivative related to the dolichyl phosphate **1** and its potential to act as a transport system. We anticipated that if the third acid group of **1** could be esterified with a drug (R), such a phosphate compound **2** carrying a sugar moiety (S), a fatty chain (C), and the drug (R) should present the following characteristics: (1) hydrophilic solubility given by the carbohydrate. It is interesting to point out that the carbohydrate could be tailored to meet the specificity requirements of the receptors of properly targeted cells; (2) hydrophobic character, due to the fatty hydrocarbon chain and the absence of charge, which is likely to allow the absorption and the transport across membranes; (3) possibilities of active transfer by the glucose phosphate and the dolichol phosphate transport proteins; (4) among the different possibilities of in situ hydrolysis, one should obtain the monophosphate of the drug (R) which is often the biological active entity. This last point is of special interest with antiviral or antitumoral nucleosides which require intracellular kinasing into 5′-nucleotides. The nucleotides themselves are ineffective due to their charge and their rapid dephosphorylation by plasma enzymes. Therefore lipophilic derivatives, all belonging to the phosphodiester series, have already been synthesized in order to improve their pharmacological properties.[7,8]

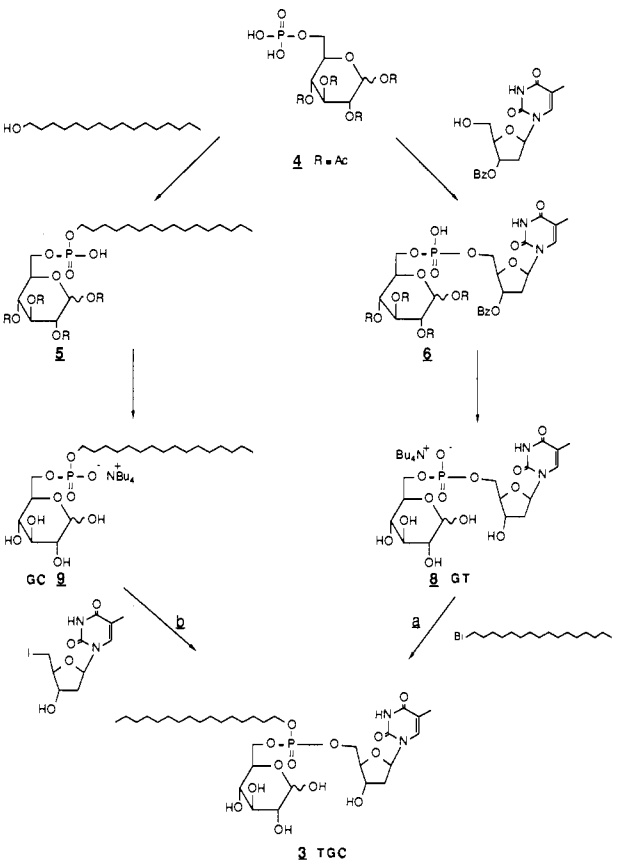
A careful survey of the literature has shown that phosphotriester derivatives of biological compounds are not well-known[9,10] and that synthetic procedures were available only for alkyl and aryl phosphotriesters[11] which were generally described as temporary protective groups for the isolation of the phosphodiester component.

In order to assess the validity of the concept of a glucosyl phospholipid transport molecule, we have synthesized a model phosphotriester **3** of thymidine. In a second section, the ^{1}H-NMR structural analysis of the phosphotriester molecule is described.

Chart I



Scheme I



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(2) *Targeting of Drugs in Synthetic Systems*; Gregorakis, J., Senior, J., Poste, G., Eds.; Plenum Press: New York, 1985.

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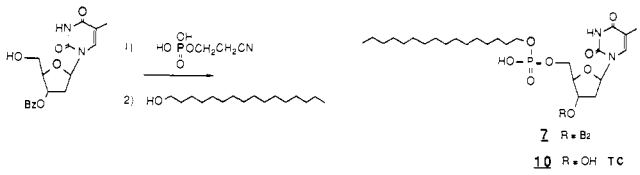
Scheme II



A third section reports the study of the interaction between model membranes (large unilamellar vesicles) and the phosphotriester or the phosphodiester precursors by ^{31}P, ^{13}C, and ^{1}H NMR.

Results and Discussion

I. **Synthesis of the Phosphotriester.** For a preliminary model, we decided to start with the glucose 6-phosphate series, instead of the more labile glucose 1-phosphate series. The fatty chain is provided by *n*-saturated hexadecanol, and the radical (R) is simply thymidine because, (i) its UV absorption facilitates its visualization and partition coefficient measurements, (ii) apart from its 5′-OH, it does not present other relatively reactive centers, and (iii) it may be considered as the precursor of several pharmacologically important nucleosides. Starting from the 1,2,3,4-tetra-*O*-acetyl-D-glucopyranose 6-phosphate (**4**), two different phosphodiesters could be obtained by esterification with hexadecanol into **5** or with 3′-*O*-benzoylthymidine into **6** (Scheme I). The main methods previously described for the preparation of carbohydrate-containing phosphodiesters are the condensation of protected sugar monophosphate with an alcohol in the presence of either dicyclohexyl carbodiimide (DCC), or triisopropylbenzenesulfonyl chloride (TPSCL)[12] or the activation by trichloroacetonitrile of the alcohol[13] and the phosphate.[14,15] In our hands, the most convenient method in gram quantities was the use of trichloroacetonitrile as the phosphate activating reagent:[14] the heating of a pyridine solution of the alcohol and the phosphate at 55–75 °C under inert atmosphere in order to avoid colored degradation compounds, followed by the evaporation of the solvent, gave, after simple silica gel chromatography, the crystalline phosphodiesters **5** (65%) and **6** (82%). The same procedure was used for the preparation of the hexadecyl 5′-thymidinyl phosphate **10** (Scheme II).

Surprisingly, the phosphodiester **6** could not be esterified with hexadecanol into the expected triester derivative by using the classical activations (TPSCL, TPSNT) described for the phosphotriester chemistry of oligonucleotides.[16,17] We then decided to deprotect the phosphodiester **6** into **8** and to apply a direct nucleophilic displacement by the tetrabutylammonium salt of **8**[18] of 1-bromohexadecane in acetonitrile at 80 °C (a). The phosphotriester **3** was isolated with 45% yield as a mixture of four isomers (α, β, X, and Y). The triester compound was also obtained (b) by nucleophilic displacement by phosphodiester **9** of 5′-iodothymidine with a lower yield (20%) (Scheme I).

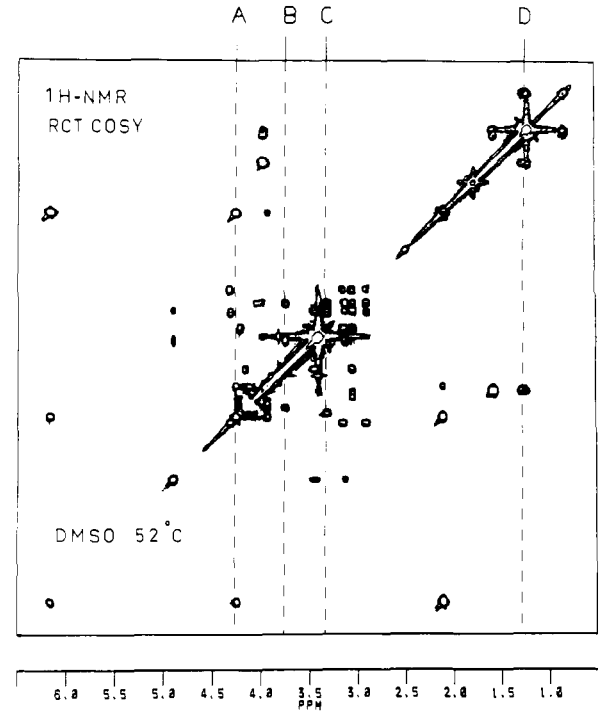


Figure 1. 500 MHz proton RCT COSY spectrum of the phosphotriester molecule (5 mM, Me_2SO, 52 °C). Columns labeled A, B, C, and D indicate the cross sections displayed in Figure 2 (column D), Figure 3 (columns B and C), Figure 4 (column A).

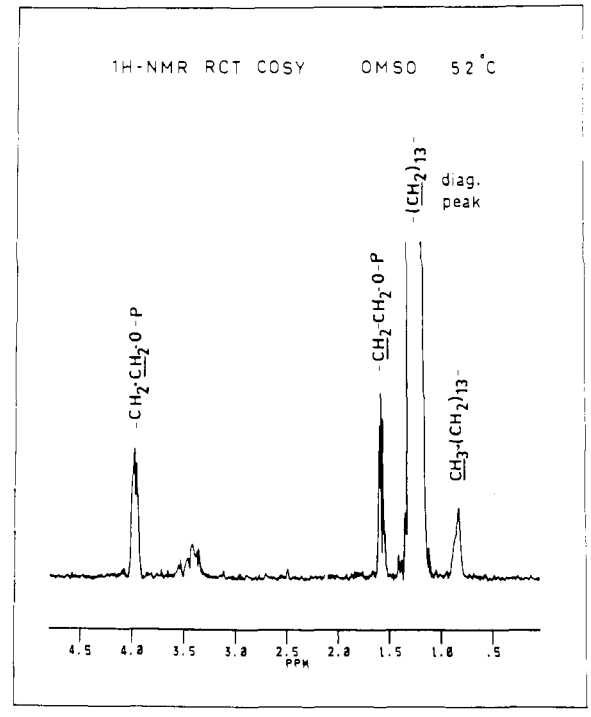


Figure 2. RCT COSY cross section relative to the bulk methylene protons of the hexadecanol unit.

The same scheme (a) was used for the preparation of the ^{13}C labeled phosphotriester **3** from D-[1-^{13}C]glucose for NMR studies.

The phosphotriester **3** obtained from glucose 6-phosphate is quite stable in solution: heating at 65 °C overnight or at 37 °C more than 24 h gave no noticeable degradation products as checked by HPLC. The physico-chemical properties of **3** are quite interesting as compared to thymidine itself: the phosphotriester of thymidine is more soluble in water (10.6%) than thymidine (4.6%). Its partition coefficient in dichloromethane (0.15)– and octanol (1.47)–water mixtures shows that **3** is more lipophilic than the

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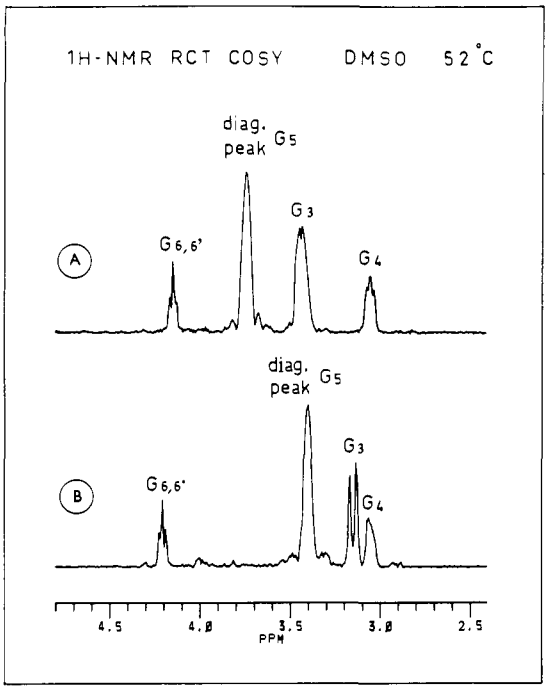

Figure 3. RCT COSY cross sections relative to (A) the glucose H5α proton and (B) the glucose H5β proton.

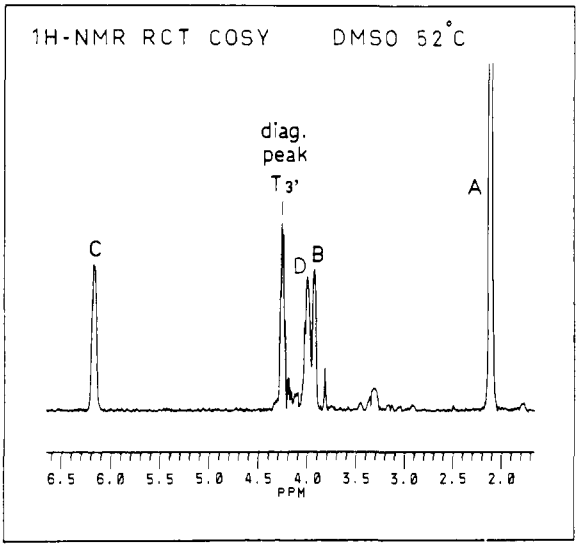

Figure 4. RCT COSY cross section relative to the thymidine H3′ signal. The cross peaks labeled A, B, C, and D correspond to the $H_{2',2''}$, $H_{4'}$, $H_{1'}$, and $H_{5',5''}$ protons.

nucleoside alone (0.08 and 0.17, respectively).[19]

II. ^{1}H NMR Structural Analysis of Phosphotriester TGC in Me_2SO. The structural analysis of TGC **3** (5 mM in Me_2SO-d_6, 52 °C) was achieved as follows: (i) Proton resonances were assigned by recording COSY and relayed coherence transfer (RCT) COSY spectra. Figure 1 shows the 2D-matrix of the latter experiment and indicates the columns used to edit cross section spectra of each TGC **3** building unit, i.e., hexadecanol, glucose, and thymidine (Figures 2, 3, and 4). (ii) The existence of a phosphotriester linkage was confirmed by ^{1}H-(^{31}P) experiments and by comparing, for each unit, the chemical shift of the methylene protons scalar-coupled to the phosphorus with those corresponding to the three phosphodiester precursors, GT **8**, GC **9**, and TC **10** (assignment not described).

The detailed analysis of each building block is given in the following section, and all the proton resonances of GT **8**, GC **9**, TC **10**, and TGC **3** are listed in Table I.

Table I. Proton Chemical Shifts (in ppm from TMS) of the Phosphotriester (TGC) and Its Three Phosphodiester Precursors (GC, TC, GT) (5 mM, Me_2SO, 52 °C)

compd	GC **9**	TC **10**	GT **8**	TGC **3**
	Hexadecanol Protons			
CH_3-	0.85	0.85		0.83
CH_2	1.24	1.23		1.21
CH_2-CH_2-OP	1.46	1.52		1.58
CH_2-OP	3.60	3.78		3.98
	α-Glucose Protons			
G1	4.90		4.89	4.90
G2	3.13		3.13	3.13
G3	3.42		3.42	3.43
G4	3.15		3.20	3.04
G5	3.54		3.55	3.74
G6	3.95		3.96	4.15
G6′	3.93		3.94	4.03
	β-Glucose Protons			
G1	4.24		4.26	4.30
G2	2.91		2.92	2.91
G3	3.24		3.24	3.15
G4	3.15		3.20	3.04
G5	3.02		3.07	3.31
G6	3.97		3.95	4.20
G6′	3.89		3.90	3.99
	Thymidine Protons			
H6		7.61	7.78	7.45
				7.43
H1′		6.19	6.19	6.18
				6.16
H2′, 2″		2.09	2.13	2.10
H3′		4.26	4.31	4.24
H4′		3.89	3.83	3.92
H5′		3.95	3.80	4.12
H5″		3.94	3.78	4.09
CH_3		1.79	1.80	1.78

(1) Hexadecanol Protons. Figure 2 shows the RCT COSY cross section at the frequency of bulk methylene protons (δ = 1.21 ppm) (Figure 1). This spectrum displays all the resolved hexadecanol proton resonances: the terminal methyl protons, the CH_2–CH_2–O–P and CH_2–O–P protons. The chemical shift value of the latter signal (δ = 3.98 ppm, Table I) is quite larger than that observed for the phosphodiester analogues containing a hexadecanol unit (GC **9** and TC **10**, δ < 3.8 ppm) and confirms the existence of a phosphotriester linkage.

(2) Glucose Protons. As expected, two anomers of the glucose moiety-1α and -1β are present since two signals of equal intensity can be assigned to the glucose H1 proton: the H1α resonance characterized by a J(H1–H2) coupling constant of 4 Hz is located at 4.9 ppm, while the H1β 8 Hz doublet is found at 4.3 ppm.

The other glucose resonances of each anomer are assigned by analyzing the COSY and RCT COSY correlation peaks. Figure 3 (parts A and B) shows the RCT COSY cross sections at the H5α and H5β resonance frequencies, respectively (Figure 1) which contain the fingerprint of the H3, H4, and H6,6′ signals. The chemical shift values of the latter protons scalar-coupled to the phosphorus are at least 0.1 ppm larger than the corresponding values observed for the phosphodiester analogues containing a glucose moiety (GT **8** and GC **9**) (Table I) and thus confirm the existence of a phosphotriester linkage.

(3) Thymidine Protons. The signals of the H6 and CH_3 base protons as well as that of the deoxyribose H1′ proton are easily assigned in a 1D spectrum. All the deoxyribose proton resonances are detected in the RCT COSY cross section at the H3′ proton frequency (Figure 4). The chemical shift values of the H5′ and H5″ resonances (Table I) as well as the ^{1}H-(^{31}P) decoupling spectrum confirm the phosphorylation at the C5′ position.

As indicated in Table I the thymidine H6 and H1′ signals are split into two distinct resonances separated by 9.5 (H6) and 6.5 Hz (H1′). In both cases the intensity ratio between the split

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Table II. Chemical Shift (in ppm from External TMP) and Line Width (in Hz) of ^{31}P Resonances of TGC and Its Phosphodiester Precursors—GT, TC, and GC—in Aqueous Solution at 27 °C

TGC **3**	
$\delta_1 = -3.34$	$\Delta\nu = 4$ Hz
$\delta_2 = -3.46$	
$\delta_3 = -3.89$	
$\delta_4 = -4.00$	
GT **8**	
$\delta_1 = -2.74$	$\Delta\nu < 2$ Hz
$\delta_2 = -2.80$	
TC **10**	
$\delta = -2.82$	$\Delta\nu = 15$ Hz
GC **9**	
$\delta = -2.21$	$\Delta\nu = 45$ Hz

resonances is 57:43. No other resonance of the phosphotriester molecule exhibits such a splitting. The total intensity of a thymidine resonance is equal to that of a proton of the alkyl chain and is two times greater than the intensity of an α (or β) glucose resonance.

The explanation of the splitting observed for the H6 and H1′ resonances is given by the ^{31}P NMR spectrum of **3** (in aqueous solution) shown in Figure 5A. The phosphorus spectrum displays four resonances $\delta1$, $\delta2$, $\delta3$, and $\delta4$ (numbered with respect to the decreasing chemical shifts, Table II) which exhibit the same line width and can be gathered into two couples containing the neighboring peaks of equal intensity: ($\delta1$, $\delta2$) and ($\delta3$, $\delta4$). The ($\delta1 - \delta2$) and ($\delta3 - \delta4$) differences are identical (= 0.12 ppm), and the intensity ratio between the two sets is equal to 57:43, i.e., the same ratio as that observed between the split thymidine resonances. Since two distinct diastereoisomers (*R*,*S*) are created during the formation of the phosphotriester linkage, as well as two glucose anomers (α,β 50:50), the four phosphorus resonances are assigned to the four following molecules: Xα, Xβ, Yα, and Yβ (the exact configuration of X and Y is undetermined). Thus the only sign of the presence of two diastereoisomers in the ^1H NMR spectrum is the splitting of the thymidine H6 and H1′ resonances.

In order to identify the two diastereoisomers in the ^1H NMR spectrum, we recorded a 2D NOESY spectrum of TGC in Me_2SO (the splitting of the H6 and H1′ resonances is not observed in aqueous solution): one can imagine that each diastereoisomer exhibits particular NOE contacts between the corresponding H6 proton and some protons of the glucose and/or the alkyl chain moieties. Unfortunately, both H6 resonances exhibit dipolar correlation peaks related only to intranucleotide contacts (H6–CH_3, H6–H1′, H6–H2′,2″, and H6–H3′); moreover, examination of the relative intensities of these intranucleotide NOEs does not lead to any significant differentiation between the two diastereoisomers. The NOE correlation peaks relative to the H1′ resonance do not provide any additional information.

III. Interaction of TGC and Its Phosphodiester Precursors with Large Unilamellar Vesicles (LUV). ^{31}P, ^{13}C, and ^1H NMR Studies. (1) Phosphodi- and Phosphotriesters in Aqueous Solution. Figures 5A, 6A, 7A, and 8A show the ^{31}P NMR spectrum of TGC **3** and those of the three corresponding phosphodiester precursors GT **8**, GC **9**, and TC **10**, respectively. Table II gives the chemical shift and the line width (at half-height) of the phosphorus resonances. The simple comparison between the line widths ($\delta\nu$) observed for the various molecules provides interesting information about their physico-chemical properties. First, it is important to note that the main relaxation factor at 121 MHz is the chemical shift anisotropy (CSA), and the transverse relaxation rate ($R_2 = \pi\delta\nu$) is directly related to the correlation times t_c and independent of the ^1H–^{31}P distances:[21]

$$\delta\nu = (\omega^2/45\pi)\delta\sigma^2 f(\omega,t_c) \quad (1)$$

where ω is the Larmor frequency, $\delta\sigma$ the CSA factor and $f(\omega,t_c)$

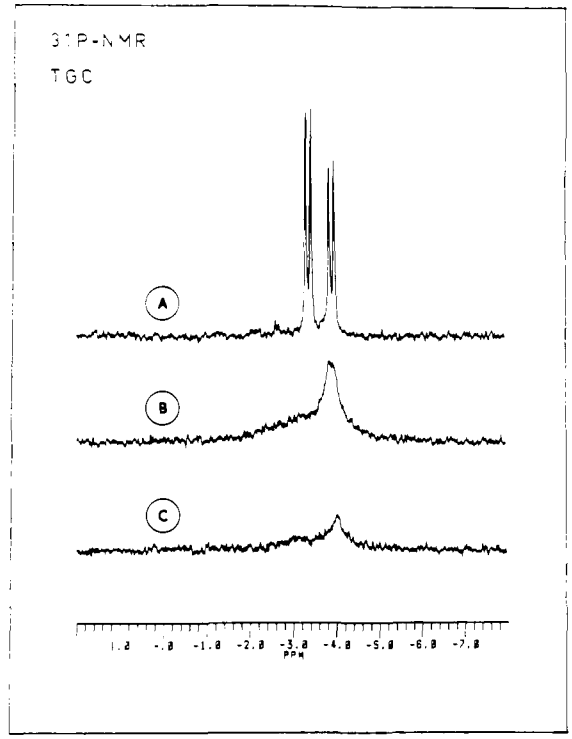

Figure 5. ^{31}P spectrum of the phosphotriester (1.8 mM) in aqueous solution at 27 °C in the absence (A) and presence (B) of LUV (lipid concentration: 30 mM) and after addition of Mn ions (C).

$= 7t_c$ if $\omega^2t_c^2 \ll 1$ or $= 4\ t_c$ if $\omega^2t_c^2 \gg 1$. By using the Stockes–Einstein relation, t_c is proportional to the volume of the molecule considered as a sphere. Therefore, by comparison with the GT molecule **8** ($\delta\nu < 2$ Hz) it is clear that the TC **10** and GC **9** molecules ($\delta\nu = 15$ and 45 Hz, respectively) which contain an alkyl chain, are in a large aggregated form in aqueous solution.[22] In contrast with the TC and GC analogues, the line width observed for the phosphotriester TGC **3** ($\delta\nu = 4$ Hz) resonances is in agreement with its remarkable hydrosolubility previously detected by the measurement of partition coefficients.

(2) Interaction with Large Unilamellar Vesicles (LUV). (i) Phosphodiester Compounds. Figures 6, 7, and 8 show the ^{31}P NMR spectra of the phosphodiester precursors in the presence of LUV (B) and their modifications after addition of paramagnetic ions (C). In the case of GT **8** molecules (Figure 6) no interaction with the model membranes is detected: the GT (α and β) phosphorus resonances are not modified in the presence of vesicles and vanish after addition of paramagnetic ions.

In contrast, when GC **9** and TC **10** molecules are in the presence of LUV (Figures 7B and 8B), one can observe the disappearance of the resonances of the phosphodiester analogues observed in the absence of LUV and the appearance of a broad signal centered at −4 ppm the area of which is two (Figure 7B) and seven times (Figure 8B) larger than the signal intensity measured in the absence of LUV. This indicates that the observed signal includes resonances of membrane phospholipids. As mentioned in the Experimental Section, phosphorus spectra were recorded by using a spin-echo experiment with a refocusing delay of 1 ms in order to cancel the signal of LUV phospholipids whose transverse relaxation time (T_2) is less than 500 μs. We can conclude therefore that the membrane phospholipid resonances detected in Figure 7B and 8B exhibit T_2 values considerably longer than their initial value in LUV. This means that at least a significant proportion of vesicles are altered by the presence of GC **9** and TC **10** molecules. Since our study specifically concerns the transmembrane transport of molecules, a precise investigation of the LUV alteration induced by the phosphodiester precursors was not attempted. After addition of Mn^{2+} ions, the signal is broadened beyond detection (Figures 7C and 8C). In Figure 7B (8B) the absence of a signal whose chemical shift corresponds to that of

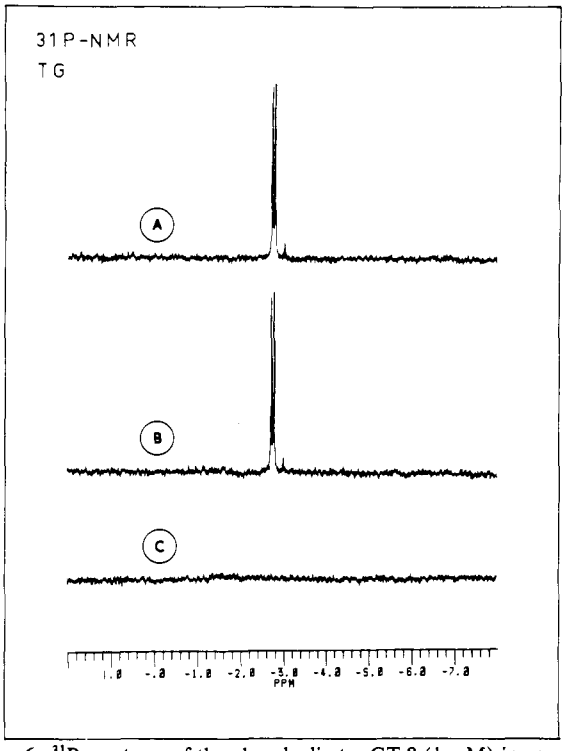

Figure 6. ^{31}P spectrum of the phosphodiester GT **8** (1 mM) in aqueous solution at 27 °C in the absence (A) and presence (B) of LUV (lipid concentration: 30 mM) and after addition of Mn ions (C).

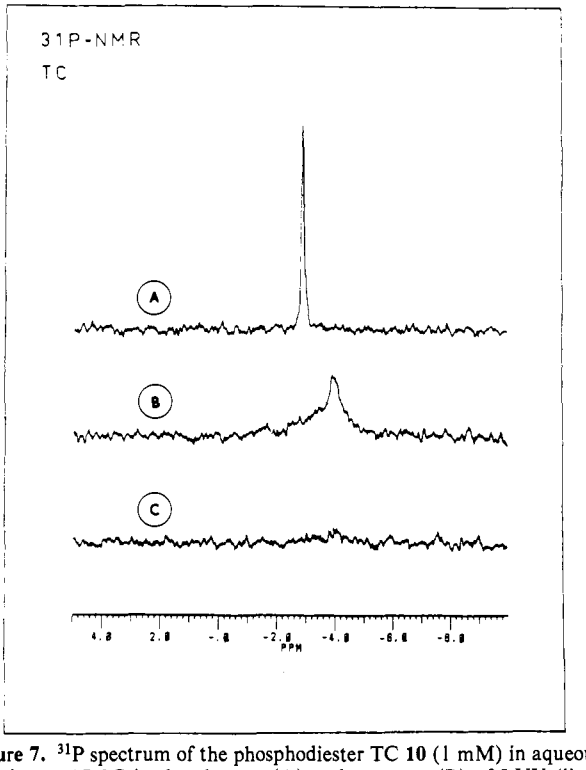

Figure 7. ^{31}P spectrum of the phosphodiester TC **10** (1 mM) in aqueous solution at 27 °C in the absence (A) and presence (B) of LUV (lipid concentration: 30 mM) and after addition of Mn ions (C).

the TC (GC) phosphate resonance observed in Figure 7A (8A) and the disappearance of any resonance after addition of paramagnetic ions preclude a transmembrane transport of TC (GC) molecules.

(ii) **Phosphotriester Molecule.** In contrast with the TC and GC phosphodiester precursors, Figure 5B shows that the phosphotriester resonances are still observed in the presence of LUV but are broadened (the α and β anomers become undiscernable); the broadening effect is much more intense for Xα, Xβ diastereoi-

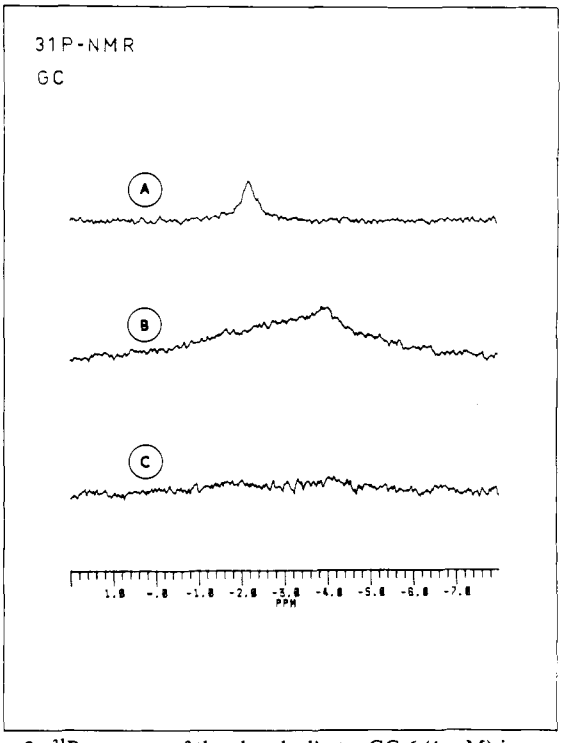

Figure 8. ^{31}P spectrum of the phosphodiester GC **6** (1 mM) in aqueous solution at 27 °C in the absence (A) and presence (B) of LUV (lipid concentration: 30 mM) and after addition of Mn ions (C).

somers: the line widths of the X and Y diastereoisomers signals are, respectively, 15 and three times larger than in the absence of LUV. Furthermore, the total area of the resonances of spectrum 5B as well as the intensity ratio between the X and Y isomers (57:43, measured after deconvolution) are identical with that measured in the absence of LUV (spectrum 5A). The most interesting result is that the addition of paramagnetic ions (Figure 5C) does not induce the total disappearance of phosphorus resonances but leads to a decrease of the signal intensities by a factor 3. Therefore one can conclude that phosphotriester **3** molecules are present in the internal milieu of the vesicles. However, even if the chemical shift and area values are conserved between the spectra 5A and 5B, the above interpretation could be ambiguous since the average chemical shift of the membrane phospholipid resonances (about −4 ppm, see above) is close to that which is relative to the phosphotriester resonances. Thus we decided to use a phosphotriester molecule containing (1-^{13}C)glucose (see Materials and Methods) in order to simultaneously and unambiguously observe both membrane phospholipid and phosphotriester resonances by ^{13}C NMR since the C1 glucose resonance is well separated from the various LUV lipid resonances.

Figure 9 shows the ^{13}C NMR spectra of (1-^{13}C)TGC **3** in the absence (A) and presence of LUV (B) and after addition of paramagnetic ions (C) (the experimental conditions are identical with those used for the ^{31}P NMR experiments). The two intense resonances observed in Figure 9A were assigned to the C1α (low field resonance) carbons via the H1α and H1β proton resonances by a ^{1}H–^{13}C magnetization transfer experiment. As in the case of the glucose proton resonances, the X and Y isomers are not distinguishable. In the presence of LUV (Figure 9B) each resonance (α and β) appears as the superposition of two signals exhibiting very different line widths, the largest peak giving the shoulders observed at the edges of the narrow peak; the line width of the narrow peak is about three times larger than in the Figure 9A (the β signal is slightly more broadened that the α signal). Thus the ^{13}C NMR experiment confirms the interpretation of the ^{31}P NMR spectrum: the narrow α and β peaks correspond to the Y (α, β) isomers. Furthermore, the subtraction of the spectrum shown in Figure 9B from a spectrum of LUV in the absence of TGC (recorded under the same conditions) displays only the TGC resonances. This confirms that the LUV are not altered by the

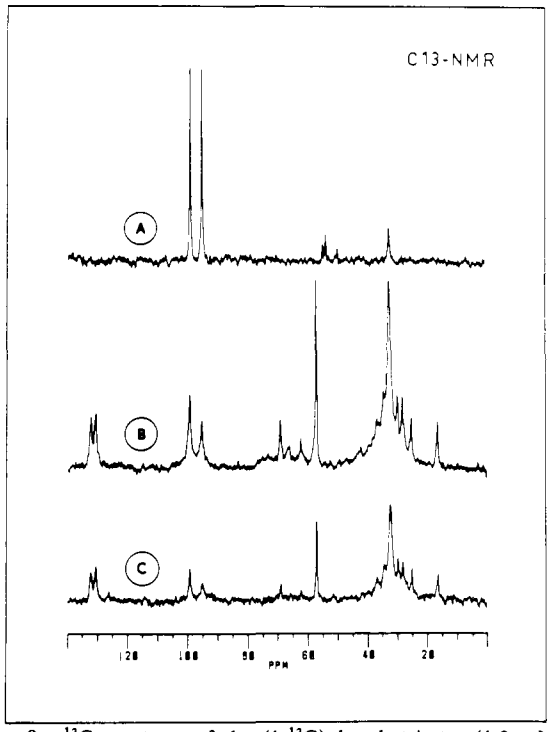

Figure 9. ^{13}C spectrum of the (1-^{13}C)phosphotriester (1.8 mM) in aqueous solution at 27 °C in the absence (A) and presence (B) of LUV (lipid concentration: 30 mM) and after addition of Mn ions (C).

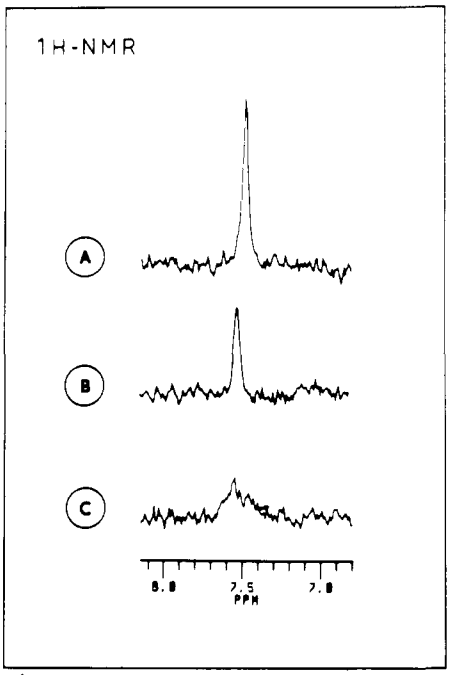

Figure 10. ^1H spectrum of the phosphotriester (1.8 mM) in aqueous solution (20% D_2O, 80% H_2O) at 27 °C in the absence (A) and presence (B) of LUV (lipid concentration: 30 mM) and after addition of Mn ions (C).

TGC molecules in the concentration range used for these experiments. After addition of paramagnetic ions (Figure 9C) the intensity of membrane phospholipid resonances is reduced by a factor of 2 since the signals relative to the LUV external layer are broadened beyond detection, while the intensity of TGC C1 resonances is reduced by a factor of 3, as in the case of the TGC ^{31}P resonances. Therefore, due to its singular physico-chemical properties (hydrophilic and lipophilic solubilities) it is clear that TGC is transported through the LUV membranes.

The meaning of the factor 3 observed between the intensities of the TGC signals before and after addition of paramagnetic ions (this factor is called θ in the following) requires an explanation. A model of the TGC transport across the LUV bilayers can be described by the following reaction:

$$w(TGC)^e \leftrightarrow m(TGC)^e \leftrightarrow m(TGC)^i \leftrightarrow w(TGC)^i \quad (2)$$

where w and m indicate a TGC molecule in aqueous solution and in a phospholipid layer respectively; e (i) indicates the external (internal) milieu or the external (internal) lipid layer. If the concentration of w(TGC) is negligible, the θ factor must be equal to 2 (as in the case of lipid resonances). Conversely, if the concentration of m(TGC) is negligible, the θ factor is equal to the ratio between the total volume of the sample and the LUV internal volume. This factor was estimated by preparing LUV in the presence of inorganic phosphate and by measuring the intensity of the corresponding phosphorus signal by ^{31}P NMR before and after addition of paramagnetic ions: θ is found to be equal to 5. Thus the factor 3 found experimentally for the TGC signal represents an intermediate value between the two extreme cases described above.

It is interesting to note that the ^{31}P and ^{13}C line widths in the presence of LUV are markedly different between the X and Y TGC isomers. This isomeric effect can result from a difference in the interaction process with the phospholipids.

This feature is also illustrated by the ^1H NMR spectra which were recorded under the same experimental conditions as the ^{31}P and ^{13}C NMR data; the thymidine H6 proton located far from the water resonance can easily be observed (Figure 10). As mentioned above, the X and Y isomers are not distinguishable in the H6 signal of TGC in aqueous solution (Figure 10A). In the presence of LUV (Figure 10B), about one half of the signal intensity is lost. In accordance with the ^{31}P and ^{13}C NMR results showing that the signal of the X isomer is dramatically broadened in the presence of LUV, it is clear that the part of the H6 resonance still observable is due to the Y isomer. After addition of paramagnetic ions (Figure 10C), the H6 resonance appears as a narrow peak overlapped by a broad signal. The latter is thus related to the TGC molecules ($^2/_3$) located in the external phosholipid layer and in the external milieu. One can note that the paramagnetic relaxation appears less efficient in the ^1H NMR experiment than in the ^{31}P and ^{13}C NMR spectra where, under the same conditions, the corresponding signal ($^2/_3$ of TGC molecules) is broadened beyond detection.

Our results demonstrate that a glucosyl phospholipid of thymidine can be prepared as a stable phosphotriester compound and is transported through model membranes, in contrast with its phosphodiester precursors. Furthermore, in the concentration range used, no alteration of the membrane structure due to this model drug carrier is detected. The phospholipid–phosphotriester interactions are stereospecific since the two diastereoisomers synthesized exhibit quite different NMR relaxation parameters in the presence of model membrane, whatever the observed nucleus (^{31}P, ^{13}C, ^1H). Ongoing experiments on the hydrolysis and pharmacological properties of glucosyl phospholipid derivatives of biologically active nucleosides are in progress and will be reported later.

Experimental Section

Materials. 3′-*O*-Benzoylthymidine and TPSNT[17] were prepared according to published procedures. 1,2,3,4-Tetra-*O*-acetyl-D-glucopyranose 6-phosphate was prepared either in three steps from D-glucose[23,24] or by acetylation of commercial glucose 6-phosphate (Aldrich). DCC, cyanoethyl phosphate, trichloroacetonitrile, 1-bromohexadecane, and 1-hexadecanol were purchased from Aldrich. Pyridine was twice redistilled on calcium hydride and *p*-toluenesulfonyl chloride; acetonitrile was distilled on phosphorous pentoxide. Merck silica gel plates ($60F_{254}$) were

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used for analytical thin-layer chromatography. Chromatographic separations were carried out on 230–400 mesh Merck silica gel and Pharmacia Sephadex LH20 or Sephadex G10. Optical rotations were measured with a Perkin-Elmer 241 MC polarimeter. UV spectroscopy was carried out using a Perkin-Elmer 550 S spectrophotometer. Mass spectra were obtained using a VG70-250 instrument.

Methods. 1,2,3,4-Tetra-*O*-acetyl-6-D-glucopyranosyl 3′-*O*-Benzoyl-5′-thymidinyl Phosphate (6). 1,2,3,4-Tetra-*O*-acetyl-D-glucopyranose 6-phosphate (**4**) (2.5 g, 5.8 mmol) and 3′-*O*-benzoylthymidine (4.0 g, 11.7 mmol) were completely dehydrated by repeated additions and evaporations of freshly distilled pyridine (3 × 20 mL). The mixture was dissolved in pyridine (30 mL) and followed by flushing with a current of nitrogen for 1 h at room temperature. Trichloroacetonitrile (6 mL, 60 mmol) was added, and the reaction mixture was warmed at 55 °C overnight under a nitrogen atmosphere. Evaporation of the solution gave a solid which was dissolved in dichloromethane; the organic layer was washed with water, dried (Na_2SO_4), and evaporated in vacuo. The solid was fractionated on a column of silica gel eluting with dichloromethane–methanol and then chromatographed on a column of Sephadex LH 20 with 3:1 dichloromethane–hexane as the eluent. Recrystallization from dichloromethane–methanol gave 2.9 g (65%): mp 184–185 °C; r_f 0.45 (3:1 dichloromethane–methanol). Anal. Calcd for $C_{31}H_{37}N_2O_{18}P\cdot 4H_2O$: C, 44.93; H, 5.47; N, 3.38. Found: C, 45.01; H, 5.32; N, 3.54.

6-D-Glucopyranosyl 5′-Thymidinyl Phosphate 8 (GT). A fresh solution of 1% sodium methoxide in methanol (50 mL) was added to 2.5 g of phosphodiester **6**. The suspension was stirred at room temperature for 20 min; TLC indicated that all the starting material (r_f 0.45, 3:1 dichloromethane–methanol) had been converted into a new product (r_f 0, 3:1 dichloromethane–methanol; r_f 0.28, 13:5:1 dichloromethane–methanol–water). The mixture was brought to pH 5 with a Dowex 50W cation-exchange resin (H^+ form); the resin was filtered off and washed well with methanol, and the combined filtrates were evaporated to dryness. The resin was washed with water, and the filtrate was lyophilized to give a white solid which was pure according to TLC in two solvent systems. A small amount was chromatographed on a column of Sephadex G10 with water as the eluent for elemental analysis. Recrystallization from methanol–diethyl ether gave 1.6 g (100%): mp 176–177 °C; r_f 0.28 (13:5:1 dichloromethane–methanol–water). Anal. Calcd for $C_{16}H_{25}N_2O_{13}P\cdot 5H_2O$: C, 33.45; H, 6.14; N, 4.88. Found: C, 33.53; H, 5.95; N, 4.87.

1,2,3,4-Tetra-*O*-acetyl-6-D-glucopyranosyl Hexadecyl Phosphate 5. A mixture of phosphate **4** (2.5 g, 5.8 mmol) and hexadecanol (2.8 g, 11.6 mmol) was exhaustively dried by repeated additions and evaporations of freshly distilled pyridine (3 × 20 mL). Trichloroacetonitrile (6 mL, 60 mmol) and pyridine (30 mL) were added, and the reaction mixture was heated at 75 °C overnight under nitrogen atmosphere. Pyridine and trichloroacetonitrile were removed by evaporation, ethyl acetate was added, and the solution was washed with water, dried (Na_2SO_4), and evaporated in vacuo. The residue was fractionated by chromatography on column of silica gel with dichloromethane–methanol as the eluent. A viscous liquid was obtained, yield 3.1 g (82%): r_f 0.52 (4:1 dichloromethane–methanol).

6-D-Glucopyranosyl Hexadecyl Phosphate 9 (GC). The phosphodiester **5** (3.1 g) was deacylated by treatment with 1% sodium methoxide in methanol (60 mL). After 20 min at room temperature, TLC showed that all the starting material (r_f 0.52, 4:1 dichloromethane–methanol) had been converted into a new product (r_f 0.1, 4:1 dichloromethane–methanol; r_f 0.43, 13:5:1 dichloromethane–methanol–water). The solution was brought to pH 5 with a Dowex 50W cation-exchange resin (H^+ form); the resin was filtered off and washed well with methanol and water, and the combined filtrates were evaporated to dryness. The residue was dissolved in water and lyophilized to give a white solid which was pure according to TLC in two solvent systems. A small amount was chromatographed on a column of Sephadex G10 with water as eluent for analysis. Recrystallization from methanol–diethyl ether gave 2.1 g (91%): mp 172–174 °C; r_f 0.43 (13:5:1 dichloromethane–methanol–water); MS (FAB^+) = 507 (MNa^+), 485 (MH^+). Anal. Calcd for $C_{22}H_{45}O_9P\cdot 4H_2O$: C, 47.47; H, 9.60. Found: C, 47.72; H, 9.82.

6-D-Glucopyranosyl Hexadecyl 5′-Thymidinyl Phosphate 3 (TGC). Procedure (a). Dowex 50W cation-exchange resin ($(n\text{-}C_4H_9)_4N^+$ form (1 g) was added to a solution of **8** (200 mg, 0.41 mmol) in water, and the mixture was stirred for 1 h at room temperature. The resin was removed by filtration, and the filtrate was lyophilized to a white solid (300 mg, 0.41 mmol). The tetrabutylammonium salt of phosphodiester **8** was dissolved in anhydrous acetonitrile (30 mL), and 1-bromohexadecane (2.5 mL, 8.2 mmol) was added. The reaction was heated for 24 h at 80 °C and cooled to room temperature to give two layers. The lower layer (1-bromohexadecane in excess) was discarded, and the solution was evaporated in vacuo. The residue was fractionated on a column of silica gel eluting with dichloromethane–methanol and then chromatographed on a column of Sephadex G10 with water as eluent: yield 130 mg (45%); r_f 0.35 (4:1 dichloromethane–methanol); $[\alpha]_{589}^{20}$ + 18° (*c* 1, water); UV (H_2O) 7400 (254 nm); MS (FAB^+) 731 (MNa^+); MS (Cl, isobutane) 709 (MH^+). Anal. Calcd for $C_{32}H_{57}N_2O_{13}P\cdot H_2O$: C, 52.89; H, 8.12; N, 3.85; P, 4.26. Found: C, 53.05, H, 8.35; N, 3.82; P, 4.02.

Procedure (b). The phosphodiester **9** (280 mg, 0.57 mmol) was converted into the tetrabutylammonium salt as described previously (416 mg). A solution of tetrabutylammonium salt of **9** (200 mg, 0.28 mmol) and 5′-iodothymidine[25] (200 mg, 0.57 mmol) in a mixture of 3:2 acetonitrile–tetrahydrofurane (50 mL) was heated for 24 h at 80 °C. The solution was evaporated in vacuo, and the yellow solid was chromatographed on silica gel (dichloromethane–methanol) and Sephadex G10 (water) to yield 40 mg of **3** (21%).

Hexadecyl 3′-*O*-Benzoyl-5′-thymidinyl Phosphate 7. β-Cyanoethyl 3′-*O*-benzoyl-5′-thymidinyl phosphate was obtained as previously described for phosphodiesters **5** and **6** from 3′-*O*-benzoylthymidine (1.45 mmol) and cyanoethyl phosphate with trichloroacetonitrile: yield 79%; r_f 0.34 (4:1 dichloromethane–methanol). To 300 mg (0.626 mmol) of this ester and 610 mg (2.52 mmol) of hexadecanol in 5 mL of anhydrous pyridine was added 476 mg of TPSNT.[17] After 2 h at room temperature, the solution was evaporated to dryness, and the residue was first chromatographed on silica gel column with dichloromethane–methanol and then on Sephadex LH20 with tetrahydrofurane–methanol (95:5). Although the reaction was complete according to TLC, only 13% of pure phosphodiester **7** was isolated: r_f 0.55 (9:1 dichloromethane–methanol).

Hexadecyl 5′-Thymidinyl Phosphate 10 (TC). **7** (120 mg, 0.17 mmol) was debenzoylated with 10 mL of 1% sodium methoxide for 15 min at room temperature. The same treatment previously described gave, after Sephadex LH20 chromatography, 39 mg (42%) of phosphodiester **10**: r_f 0.67 (7:3 dichloromethane–methanol); MS (EI), 547 (MH^+).

Vesicle Preparation. Phosphatidylcholine was extracted from egg yolk according to the method of Singleton et al.[26] Phosphatidic acid was prepared from the former as described by Allgyer and Wells.[27] Phospholipid purity was checked by recording ^1H NMR spectra in $CDCl_3$/MeOD solution. Large unilamellar vesicles (LUV) of defined size were prepared by reverse phase evaporation[28] using a mixture of egg phosphatidylcholine and phosphatidic acid (mole ratio 9:1). 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 (Nucleopore) in order to obtain a uniform size distribution.[29] The final phospholipid concentration was about 30 mM. For each experiment, LUV integrity was analyzed by recording ^{13}C (see text) and ^{31}P NMR spectra of a blank sample of vesicles.

NMR Experiments. For the NMR structural analysis, the phosphotriester molecule and its three phosphodiester precursors were dissolved in Me_2SO at a concentration of about 5 mM. A small amount of D_2O was added to the solutions in order to cancel the signals of exchangeable protons.

For the NMR transport study, small amounts of 20 mM stock solution of each compound were injected into two NMR tubes containing a buffered aqueous solution with or without LUV.

The final concentration of the phosphotriester compound was 1.8 mM. Since four isomers were simultaneously synthesized (see Results and Discussion), the lipid to phosphotriester ratio was about 67 (m/m) for each molecule. The concentration of the phosphodiester precursors was adjusted to respect this ratio, taking into account that those containing a glucose moiety (GC and GT) gave two isomers.

^1H NMR 500 MHz spectra were recorded on a Bruker WM-500 spectrometer and referenced relative to internal tetramethylsilane (TMS) in Me_2SO or trimethylsilyl propionic acid (TMP) in H_2O/D_2O. Two-dimensional COSY, relayed coherence transfer (RCT) COSY, and NOESY spectra were performed by using the standard pulse sequences: ($90°\text{-}t_1\text{-}90°\text{-}t_2$),[30] ($90°\text{-}t_1\text{-}90°\text{-}T\text{-}180°\text{-}T\text{-}90°\text{-}t_2$),[20] and ($90°\text{-}t_1\text{-}90°\text{-}T_m\text{-}90°\text{-}t_2$)[31] with a mixing time T_m of 0.3 s. Proton spectra in aqueous solution (20% D_2O, 80% H_2O) were recorded at 300 MHz on a Bruker MSL 300 by using a two pulse sequence ($45°\text{-}T\text{-}45°$).[32,33]

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^{31}P NMR (121.5 Hz) and ^{13}C NMR (75.5 MHz) spectra were recorded on a Bruker MSL 300 spectrometer and referenced relative to external trimethyl phosphate and dioxane, respectively. Two levels of broad band proton decoupling were applied for all the experiments. In the case of ^{31}P spectra, a Hahn spin echo sequence (90°–T–180°–T) was used with a T value of 1 ms in order to cancel the signal of membrane phospholipids whose transverse relaxation times (T_2) is less than 0.5 ms.

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Calculations of Charge-Transfer Transition Energies and Spectroscopic Properties of a Molecular Crystal: Methylbacteriopheophorbide *a*

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Abstract: Theoretical expressions are developed for calculating the spectroscopic properties of molecular crystals. The theory is applied to crystalline methylbacteriopheophorbide *a* as a test case for a similar analysis of the properties of photosynthetic reaction centers. The treatment starts by writing π molecular orbitals for the individual molecules as linear combinations of atomic orbitals. Intermolecular interactions are included in the form of configuration interactions. Exciton-type interactions of intramolecular transitions are evaluated in terms of transition monopoles, and intermolecular charge-transfer (CT) transitions are included explicitly with the aid of semiempirical atomic resonance integrals and electron–electron repulsion integrals. The diagonal transition energies for the intramolecular transitions are obtained from the transition energies for a monomeric molecule in solution. CT transition energies are calculated by using experimentally measured reduction potentials in conjunction with calculations of electrostatic interactions in the crystal and in solution. This is done by taking into account the microscopic dielectric effect associated with the polarizabilities of the crystal atoms and by evaluating the solvation free energies of the oxidized and reduced molecules in solution. The calculated CT transition energies are close to the energies that are required to bring the calculated spectroscopic properties into alignment with the measured absorption spectrum and linear dichroism of the methylbacteriopheophorbide crystal. This is particularly encouraging, because no adjustable parameters are included in the construction of the crystal wave functions from those of the isolated molecules. The satisfactory agreement between theory and experiment lends support to attempts to calculate the spectroscopic properties and electron-transfer dynamics of photosynthetic reaction centers.

In previous papers,[1-4] we developed a quantum mechanical treatment of the spectroscopic properties and electron-transfer kinetics of reaction centers of photosynthetic bacteria. To evaluate the intermolecular interactions of the four molecules of bacteriochlorophyll and two molecules of bacteriopheophytin in the reaction center, the theory used semiempirical atomic resonance integrals and electron–electron repulsion integrals that had been parametrized in earlier studies of pyrene dimers and other excimers. Because the data set employed for this parametrization is limited, it is important to test the theory by applying it to oligomers of other molecules closely related to bacteriochlorophyll. Such a test will be most meaningful if the geometry of the oligomer is known accurately, because the resonance integrals are particularly strong functions of the molecular positions and orientations. One system that meets this criterion is crystalline methylbacteriopheophorbide *a* (MeBPh, Figures 1 and 2). Barkigia et al.[5] have solved the crystal structure by X-ray diffraction, and Hanson and Hofrichter[6] have measured the crystal's optical absorption spectrum and linear dichroism. The distances between neighboring MeBPh molecules in the crystal are similar to some of the intermolecular distances between bacteriochlorophylls in the reaction center, and some of the crystal's spectroscopic properties resemble those of the reaction center. In both cases, there is a strong absorption band at long wavelengths, shifted far to the red of the corresponding band in the spectrum of monomeric MeBPh or bacteriochlorophyll. An analysis of intermolecular charge-transfer (CT) transitions is more complicated for the crystal than for the reaction center, because each MeBPh molecule in the crystal has strong orbital overlap with a larger number of neighboring molecules. Exciton interactions among the pigments also extend over many more molecules in the crystal than in the reaction center. Nevertheless, a crystal provides a critical test case for calculations of the energetics of CT transitions in a well-defined environment.

The theory of optical absorption by molecular crystals was treated by Davydov for the case of a molecule with a single excited state or a set of noninteracting excited states.[7] In a more general situation the molecule will have a manifold of excited states that are mixed by intermolecular interactions, in addition to CT transitions that can mix with the intramolecular transitions.[8,9] We

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