and dried under vacuum at 35 °C. Typical yields were better than 95%.

NMR Experiments. Proton-decoupled, ^{15}N NMR spectra were recorded at 9.4 T (40.53 MHz for ^{15}N and 400.134 MHz for ^{1}H) on a Bruker Instruments spectrometer, Model WM-400, in the pulse Fourier transform mode, with quadrature phase detection.

^{15}N measurements were made by using 15-mm sample tubes that contained 50–100 mM solutions of the aminosaccharides in 85:15 (v/v) H_2O:D_2O. The operating parameters included a spectral width of 20 kHz, a pulse width of 40 μs (75° flip angle), an acquisition time of 0.4 s, and a pulse repetition time of 3.0 s. Two-level, broad-band proton decoupling performed at high power (5 W) during acquisition of data and at low power (1 W) during the relaxation delay provided decoupled spectra *with* the NOE and avoided excessive heating, so that the samples remained at 26 ± 2 °C. In a typical experiment, 16K of memory was allocated for data acquisition and was then increased to 32K (16K real data) by zero filling. Before Fourier transformation of the data, 2 Hz of exponential line broadening was applied. Transformed spectra had a digital resolution of 1.2 Hz (or 0.03 ppm) per data point. The ^{15}N chemical shifts are estimated to be accurate to ±0.1 ppm. Many samples gave no useful ^{15}N NMR spectra until paramagnetic impurities had been removed. This was accomplished by passing the NMR solution through a small column of Chelex-100 ion-exchange resin[36] into a sample tube that had been pretreated with a solution of ethylenediaminetetraacetic acid, disodium salt. The pH of solutions for NMR was adjusted with ~1 N and ~4 N NaOH or HCl.

The pK_a values, ^{15}N protonation shifts ($\Delta\delta_N$), and ^{15}N chemical shifts (δ_N) of the protonated amino groups of neomycin B (see Table IV) were computed[23] by fitting the ^{15}N chemical shift titration data (Figure 1) to the function $\delta_N = \Delta\delta_N 10^{-pK}10^{pH}/(1 + 10^{-pK}10^{pH}) + \delta_{NH_3^+}$ by use of the on-line, mathematical modeling laboratory component (MLAB) of the NIH/EPA Chemical Information System. Experimental ^{15}N chemical shift titration curves were plotted by using MLAB and compared with the appropriate theoretical curves calculated from the parameters obtained from the best fit. These parameters were confirmed by separate computations in which the curve fitting and plotting were programmed on a Univac 1108 system.

(36) A chelating, cationic exchange resin sold by Biorad Laboratories.

^{15}N spin–lattice relaxation times (T_1) were measured by using the fast inversion–recovery (FIRFT) sequence[37] modified to include two-level, broad-band proton decoupling (vide supra). A fixed delay of 2.0 s was used between pulse sequences and ten variable delays (τ values) ranging from 0.03 to 15.0 s. Sample temperatures were maintained at 25 ± <1 °C throughout an entire T_1 experiment by using low decoupling power during variable and fixed delay times. The free induction decay for each τ value was acquired in 4K of computer memory by using a spectral width of 4 kHz and was then expanded to 16K (8K real data) by zero filling; finally, exponential line broadening (1 Hz) was applied [digital resolution = 0.5 Hz (or 0.01 ppm) per data point]. The 90° pulse (44.5 μs) was determined for 80% formamide in D_2O. Degassed solutions of neomycin B free base were treated with Chelex-100 resin prior to T_1 measurements. Paramagnetic solutions were prepared by the direct addition of solid (1,10-diaza-4,7,13,16,21-pentaoxabicyclo[8.8.5]tricosane)gadolinium(III) chloride, $[Gd(2.2.1)]Cl_3$, which was synthesized according to the procedure of Gansow et al.[38]

Pulse sequence recycling times used in nuclear Overhauser experiments were $>8T_1$. Spectrometer parameters identical with those for ^{15}N relaxation measurements were employed.

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Registry No. 1a, 4146-30-9; **1b**, 119-04-0; **1c**, 84107-25-5; **1a**, 62906-25-6; **2a**, 3947-65-7; **2b**, 15446-43-2; **2c**, 15373-06-5; **3a**, 52433-41-7; **3b**, 68405-91-4; **3c**, 84051-62-7; **4a**, 2037-48-1; **4b**, 14429-30-2; **4c**, 84107-26-6; **4d**, 14187-81-6; α-**5a**, 84056-78-0; β-**5a**, 84107-24-4; α-**5b**, 59433-00-0; β-**5b**, 84064-34-6; α-**6a**, 14131-62-5; β-**6a**, 14131-63-6; α-**6b**, 10036-64-3; β-**6b**, 14131-68-1; α-**7a**, 84064-35-7; β-**7a**, 84064-36-8; α-**7b**, 84064-37-9; β-**7b**, 55298-35-6.

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Affinities of Phosphoric Acids, Esters, and Amides for Solvent Water

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Abstract: The equilibrium constant for transfer of trimethyl phosphate to the vapor phase from dilute aqueous solution was found to be 3×10^{-7}, which is lower than that of methyl acetate by a factor of approximately 10^4. As each alkyl group was removed from trialkyl phosphates, affinity for solvent water was enhanced by an additional factor of more than 10^4, as indicated by distribution coefficients between chloroform and water. Differences in free energy of solvation between reactants and products were found to be more than sufficient to account for the favorable free energy of hydrolysis of phosphate esters in water, so that these reactions would be endergonic in the vapor phase. Distribution properties of phosphoric amides, methyl ethylene phosphate, and triethylphosphine oxide were also investigated.

Derivatives of phosphoric acid are widespread constituents of lipids, nucleic acids, proteins, and carbohydrates and serve as reactive intermediates in the action of several enzymes. These compounds are distinguished from other classes of biological molecules by their extreme polarity. In considering possible influences of solvation effects on equilibria of their chemical transformations in water and on their binding affinities for biological receptors, it would be helpful to have quantitative information about the relative affinities of various phosphorus derivatives for watery surroundings. Substituted phosphonic acids, for example, have sometimes been prepared as analogues of unstable phosphoric acid derivatives in the hope that they might serve as stable enzyme inhibitors: are they more or less hydrophilic than the parent compounds? Vapor pressure measurements have shown that the large negative free energy of carboxylic ester aminolysis, a central reaction in protein biosynthesis, is more than completely matched by the difference in free energy of solvation between reactants and products.[1] Can the negative free energy of hydrolysis of phosphoric acid esters[2-4] be analyzed in similar terms?

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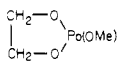
Table I. Partition Coefficients for Transfer of Phosphorus Derivatives from Water to Nonpolar Environments at 20 °C, Ionic Strength 0.30

	$CHCl_3$	$(ClCH_2)_2$	C_6H_6	CCl_4	cyclohexane	hexane	heptane	vapor
triesters								
$(PrO)_3PO$	4.7×10^3		3.0×10^2	64 (72[b])	15	6.1 (7.5[b])	10.8	2.8×10^{-5}
$(EtO)_3PO$	1.9×10^2	21	1.13	0.63	0.73	0.18	0.15	1.5×10^{-6}
$(MeO)_3PO$	5.8	0.78	0.20	8.5×10^{-2}	6.0×10^{-3}	7.8×10^{-3}	5.2×10^{-3}	3.0×10^{-7}
diesters								
$(BuO)_2PO(OH)$	1.8[a]		0.38[a]					
$(PrO)_2PO(OH)$	9×10^{-2}	3.1×10^{-2}	2.2×10^{-2}	1.3×10^{-2}	1.2×10^{-3}	1.12×10^{-3}	9.8×10^{-4}	
$(EtO)_2PO(OH)$	2.7×10^{-3}						2.7×10^{-5}	
$(MeO)_2PO(OH)$	1.5×10^{-4}							
monoester								
$PrOPO(OH)_2$	2.5×10^{-6}							
amides								
$(BuO)_2PON(CH_3)_2$	6.5×10^3							
$(BuO)_2PONHCH_3$	1.2×10^3							
$(BuO)_2PONH_2$	50							
others								
	0.88							
	0.54		5.0×10^{-3}	1.9×10^{-3}		8.1×10^{-4}		

[a] Reference 5. [b] Reference 23.

Distribution properties of monomeric phosphoric acid derivatives have not been widely investigated, perhaps because simple phosphodiesters tend to dimerize even at low concentrations in organic solvents.[5,6] In order to secure further information and compare the solvation of undissociated phosphoric and carboxylic acid derivatives, we have therefore investigated the partition coefficients of phosphoric and phosphonic acid derivatives between water and various nonpolar environments using ^{32}P-labeled compounds of high specific activity.

Materials and Methods

Dibutylphosphoric amides were prepared by the general method of Nikonorov et al.[7] by reacting the amine with dibutylphosphoryl chloride prepared by the method of de Roos and Toet.[8] The amide, *N*-methyl amide, and *N*,*N*-dimethyl amide showed boiling points and infrared spectra consistent with those reported by Nikonorov et al.[7] Triethyl phosphite was obtained from Aldrich Chemical Co. and used without further purification. Methyl ethylene phosphate was prepared by the method of Covitz and Westheimer.[9]

Trialkyl phosphates were synthesized by the method of Clermont,[10] modified for rapid use with ^{32}P. $H_3{}^{32}PO_4$ was first converted to the trisodium salt and then precipitated as Ag_3PO_4 by addition of $AgNO_3$. The dried silver salt (1 mmol) was refluxed with alkyl iodide (50 mmol) in dimethylformamide (2 mL) overnight. The precipitated AgI was removed by filtration and washed with $CHCl_3$ to remove last traces of trialkyl phosphate. The combined washings were extracted with potassium phosphate buffer (0.1 M, pH 6.8) to remove DMF and trace quantities of radioactive contaminants and then concentrated to a small volume by evaporation. TLC (see below) revealed no detectable radiochemical impurities.

Dialkyl [^{32}P]phosphates were obtained by quantitative hydrolysis of trialkyl phosphates in the presence of aqueous KOH (0.3 M) for 10 days at room temperature.[11] The alkaline solution was then extracted several times with $CHCl_3$ to remove last traces of trialkyl phosphate and neutralized with HCl. The resulting solution of dialkyl phosphate contained no detectable radioactive contaminants.

Monoalkyl [^{32}P]phosphates were prepared enzymatically by a modification of the procedure of McVicar.[12] Sodium [^{32}P]phosphate adjusted to pH 9 (0.4 mmol) and alcohol (40 mmol) were treated with dialyzed alkaline phosphatase from *Escherichia coli* (10 units, obtained from Sigma Chemical Co.) in a total volume of 6 mL of water. After 15 h of incubation at 37 °C, approximately 15% conversion to alkyl phosphate had occurred, as shown by TLC (see below). The product was concentrated and purified by descending chromatography on Whatman 3MM paper, with butanol/acetic acid/water (4:1:1). The band corresponding to monoalkyl phosphate was eluted, and TLC showed no traces of radiochemical impurities.

On Eastman Chromogram cellulose TLC, observed R_f values in butanol/acetic acid/water (4:1:1) were inorganic phosphate 0.02, Me_3PO_4 0.97, Me_2HPO_4 0.42, MeH_2PO_4 0.10, EtH_2PO_4 0.65, and PrH_2PO_4 0.60. R_f values in wet butanol containing 2% acetic acid were as follows: Et_3PO_4 0.98, Et_2PO_4 0.8, Pr_3PO_4 0.98, and Pr_2HPO_4 0.82.

Solvent–solvent distribution experiments at ionic strength 0.3 M were carried out by shaking two phases, each previously saturated with countersolvent, in separatory funnels maintained at 20 °C by immersion in a water bath. The aqueous phase contained 0.3 M KCl (for triesters) or 0.3 M HCl (for other compounds). Even in the case of methyl ethylene phosphate, the only unstable solute examined in the present series, less than 5% hydrolysis occurred in the time required for distribution experiments. For this solute 0.05 M potassium phosphate buffer (pH 7.0) was added to the aqueous phase, which was made up to ionic strength 0.3 with KCl; under these conditions the observed half-time for hydrolysis was in the neighborhood of 25 min at 20 °C. In experiments involving direct determination of the solute by NMR, deuterated solvents were used as indicated under Results. Quantitative analysis was then performed by integrating the major solute peak in both phases by using added pyrazine as an integration standard. In experiments involving ^{32}P-labelled solutes, standard quenching corrections were applied as necessary to samples containing organic solvents. Radioactive solute was normally determined by direct scintillation counting of aliquots from both phases. In extreme cases, where distribution was extremely one-sided, relative large volumes of the less-favored solvent were used, and the apparent distribution was calculated from the concentration of solute in the more favored solvent before and after distribution. Alternatively, solute was recovered from large volumes of the less favored solvent by back-extraction with a small volume of the more favored solvent, identical with the volume used in the first extraction, and the distribution coefficient was calculated by comparing A and B in eq 1 where $K_{M \to L}$ =

$$K_{M \to L} = \frac{BM}{LA - LB} \quad (1)$$

equilibrium constant for transfer to the less favored solvent, M = volume of more favored solvent (usually 2 mL), used in both extractions, B = solute concentration in back-extract, L = volume of less favored solvent (usually 1000 mL), and A = initial solute concentration in more favored solvent, before distribution. The identity of material appearing in the less favored solvent was established by chromatographic mobility on TLC or by NMR spectroscopy, and comparison with the authentic solute.

Water-to-vapor distribution coefficients of ^{32}P-labeled phosphotriesters were determined by using a dynamic method described previously[13] in

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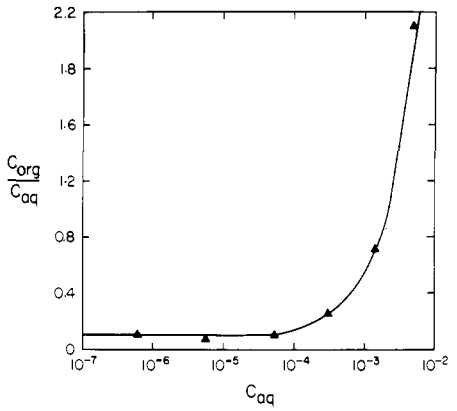

Figure 1. Apparent distribution coefficient of dipropyl phosphate at 20 °C, plotted as a function of solute concentration in the aqueous phase. The organic phase was wet chloroform, and the aqueous phase was 0.3 M HCl, saturated with chloroform. The solid line is a theoretical curve calculated for a true distribution coefficient of 0.10 and an association constant of 2.3×10^4 M^{-1} for dimerization of solute in the organic phase (comparable with values reported for diethyl and dipropyl esters in ref 6).

which water-saturated nitrogen was passed through a series of gas washing bottles containing radioactive solute dissolved in 0.3 M aqueous KCl at 25 °C. Material accumulating in the water trap was identified as intact phosphotriester by its chromatographic mobility upon TLC and by comparing its distribution coefficient from water to chloroform with that of the authentic compound.

Results

As noted by Dyrssen and his associates,[5,6] dialkylphosphates exhibit a strong tendency to dimerize in nonaqueous solvents, resulting in an alteration in apparent distribution from water toward the nonpolar solvent at high solute concentrations. Typical behavior is shown for dipropylphosphate in Figure 1. Table I shows partition coefficients from water to chloroform, determined for the neutral compounds at 20 °C and ionic strength 0.3, in concentration ranges where the value of the observed partition coefficient did not change with changing solute concentration. The tendency to form dimers was more pronounced with phosphodiesters than with other solutes listed in Table I, none of which showed any appreciable deviation in apparent distribution coefficient at concentrations below 0.1 M. For several solutes, nonpolar solvents other than chloroform were also used as indicated. In the exceptional case of the phosphotriesters, it proved possible, by using ^{32}P-labeled material of very high specific activity, to measure equilibria of transfer from water to the vapor phase.

The influence of temperature on water-to-chloroform distribution was also examined for dimethyl and trimethyl phosphate. In the range of temperatures from 0 to 50 °C, the water-to-chloroform distribution coefficient of trimethyl phosphate decreased from 7.1 to 5.13, corresponding to a van't Hoff enthalpy loss of 3.19 kJ for this transfer. In the range of temperatures from 0 to 45 °C, the water-to-chloroform distribution coefficient of dimethyl phosphate increased from 4.6×10^{-5} to 6.9×10^{-4}, corresponding to a van't Hoff enthalpy gain of 41.6 kJ for this transfer.

Discussion

Free energies of transfer from water to the vapor phase, providing an absolute measure of hydrophilic character, could be measured only for the least polar compounds included in the present study. Vapor pressures of other solutes were below the limits of detection, even when using concentrated carrier-free ^{32}P. Comparison of partition coefficients in Table I shows that the *relative* water-leaving tendencies of the various solutes were roughly similar, regardless of whether chloroform or a hydrocarbon was used as the nonpolar phase. Thus the distribution of solutes between water and chloroform may provide a reasonable indication

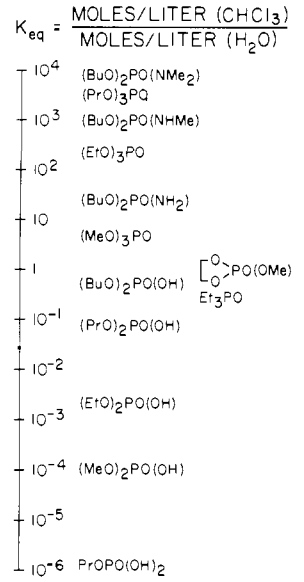

Figure 2. Equilibrium constants for transfer from chloroform-saturated water to wet chloroform at 20 °C, ionic strength 0.30.

of the relative water-leaving character that would be observed by using any other reference phases.

Comparing the behavior of phosphoric and carboxylic acid derivatives, one finds that the >P=O function appears to be considerably more hydrophilic than the <C=O function. Thus, despite the neutral or slightly hydrophobic character of alkoxy groups,[14] the equilibrium constant for transfer of trimethyl phosphate from the vapor phase to dilute aqueous solution (Table I) exceeds that of methyl acetate[15] by a factor of 12 400, and the water-to-chloroform distribution of dipropylphosphoric acid (Table I) is similar to that of unsubstituted propionic acid.[16] The apparently greater hydrogen-bonding tendencies of phosphorus oxyacids are expressed in a somewhat different way in dimerization equilibria. Dyrssen et al.[6] found that the association constant of dialkylphosphoric acids in benzene were approximately 7×10^5 M^{-1} regardless of alkyl chain length, whereas Pohl et al.[17] reported an equilibrium constant of 4×10^2 M^{-1} for dimerization for acetic acid in the same solvent.

Amides of dibutylphosphoric acid are found to be *less* hydrophilic than the parent acid, and their preference for chloroform increases montonically with increasing N-methylation. In contrast, acetamide and its N-methylated derivatives were found to be much more hydrophilic than acetic acid itself, with water affinity reaching a maximum in *N*-methylacetamide.[13]

Water-to-chloroform distribution coefficients are displayed in Figure 2. Successive alkylation of phosphoric acid, in the series of propyl derivatives, leads to major losses in relative affinity for solvent water, amounting to more than 10^4 per alkyl group in distribution coefficient. In contrast, affinities of propanol and water for aqueous surroundings are relatively narrowly separated, differing by a factor of about 30.[18] Changing solvation accordingly provides a substantial contribution to the observed free energy of hydrolysis of phosphate esters in water. The observed equilibrium constant for hydrolysis of methyl phosphate in neutral aqueous solution is in the neighborhood of unity, expressed in terms of the molarity of water.[19] In an anhydrous environment phosphoric acids might thus be expected to be considerably less stable than their esters. This may help to explain the observation that at the active site of the enzyme alkaline phosphatase, phosphate esters are formed spontaneously from inorganic

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phosphate and the hydroxyl group of a serine residue (for a review, see ref 20).

The free energy of hydrolysis of a phosphotriesters in water does not appear to have been determined experimentally. A large contribution to the negative free energy of hydrolysis, amounting to approximately −30 kJ/mol at pH 7, must arise from the ionization of the product phosphodiesters, a strong acid with a pK_a typically in the neighborhood of 1.4.[21] The contribution of product ionization is presumably enought to raise the triester to a group-transfer potential comparable with or exceeding that of ATP. This unusual phosphorylating capacity, often exploited in synthetic chemistry, has evidently not proven useful in the evolution of living organisms. Steric hindrance may limit the usefulness of triesters as biosynthetic intermediates, and they would presumably be difficult to synthesize.

The distribution behavior of methyl ethylene phosphate was found to be similar to that of trimethyl phosphate, suggesting that solvation makes no unusual contribution to the thermodynamic properties of cyclic esters of this kind.

When comparing trimethyl phosphate with triethylphosphine oxide, in which ester oxygens are replaced by methylene groups, only a small difference in apparent water affinity is observed (somewhat surprisingly triethylphosphine oxide is slightly *more* hydrophilic than trimethyl phosphate). Phosphonic acids should accordingly serve as reasonable analogues for phosphate esters in the design of enzyme antagonists, in terms of their solvation properties. A number of such inhibitors have been developed, of which the best known is probably the herbicide glyphosate, an inhibitor of the biosynthesis of anthranilic acid.[22]

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Registry No. $(PrO)_3PO$, 513-08-6; $(EtO)_3PO$, 78-40-0; $(MeO)_3PO$, 512-56-1; $(BuO)_2PO(OH)$, 107-66-4; $(PrO)_2PO(OH)$, 1804-93-9; $(EtO)_2PO(OH)$, 598-02-7; $(MeO)_2PO(OH)$, 813-78-5; $PrOPO(OH)_2$, 1623-06-9; $(BuO)_2PON(CH_3)_2$, 84108-32-7; $(BuO)_2PONHCH_3$, 2014-81-5; $(BuO)_2PONHz$, 870-52-0; $(C_2H_5)_3PO$, 597-50-2; methyl ethylene phosphate, 2196-04-5.

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Cyclopeptide Alkaloids. Conformational Analysis of the Dihydro-*p*-phencyclopeptine Nucleus

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Abstract: A combined NMR spectroscopic and X-ray crystallographic approach to the analysis of the conformations of two synthetic dihydro-*p*-phencyclopeptines, (5*S*)-5,6-trimethylene-8-deamino-1,2-dihydro-*p*-phencyclopeptine (**2d**) and its *N*-methyl derivative, (5*S*)-3-methyl-5,6-trimethylene-8-deamino-1,2-dihydro-*p*-phencyclopeptine (**2a**), is described. The one-dimensional ^1H NMR spectra of both cyclopeptides are assigned by means of two-dimensional homonuclear (^1H) *J*-spectral analysis, homonuclear decoupling experiments, computer-generated spectral simulations, and steady-state ^1H NOE measurements at 270 and 360 MHz. On the basis of Karplus relationships derived from these experimental results, together with ^{13}C NMR spectrscopic analysis, predictions for the solution conformations of both cyclopeptides are deduced. The solution conformation of the *N*-methyl cyclopeptide **2a** is compared with its solid-state conformation determined by X-ray crystallography, and the two are found to be in good agreement. Comparative NMR studies of the two *p*-phencyclopeptines reveal only subtle conformational differences between the two cyclopeptides. These differences occur in the region near the N3–C4 amide where the structural difference between the two cyclopeptides, the *N*-methyl group, is localized. Of the eight possible conformations of the *p*-phencyclopeptine nucleus, both cyclopeptides adopt the same overall geometry with both amides trans. The small conformational differences that do exist between **2a** and **2d** reflect the ability of NH cyclopeptide **2d** to form an intramolecular hydrogen bond similar to those observed in γ turns in proteins. Conformational implications with respect to ion binding of the *p*-phencyclopeptines nucleus are considered.

The physical properties and biological activity of proteins are primarily dependent on levels of structure other than just the primary amino acid sequence. The analysis of peptide backbone conformations therefore provides an important route to understanding the three-dimensional arrangement of amino acid residues in proteins. In recent years, a great deal of attention has been focused on conformational studies of small cyclic peptides both natural and synthetic.[2] These substances, in addition to being interesting because of their biological activity, have been used as models for larger proteins because of the simplified analysis that is possible.

Of the spectroscopic techniques that are applicable to conformational analysis, nuclear magnetic resonance (NMR) has found increasing use.[3-5] NMR also has been one of the important techniques used for the elucidation of the structures of the cy-

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