Synthesis and Characterization of 1,4,7-Triazacyclononane Derivatives with Methylphosphinate and Acetate Side Chains for Monitoring Free Mg^{II} by ^{31}P and ^{1}H NMR Spectroscopy

Jurriaan Huskens[†] and A. Dean Sherry*[,†,‡]

Contribution from the Department of Chemistry, University of Texas at Dallas, P.O. Box 830688, Richardson, Texas 75083-0688, and Department of Radiology, The Rogers Magnetic Resonance Center, University of Texas Southwestern Medical Center, 5801 Forest Park Road, Dallas, Texas 75235-9085

Received November 9, 1995[⊗]

Abstract: A series of 1,4,7-triazacyclononane-based ligands containing one, two, or three methylphosphinate (MP) side chains has been prepared. The macrocycle with three methylphosphinates, NOTMP, appeared to be a very promising ligand for monitoring Mg^{II} in biological samples by ^{31}P NMR spectroscopy. The ^{31}P resonances of the free ligand and the Mg^{II} complex were well resolved, in slow exchange, and well downfield of typical tissue phosphate and phosphate ester metabolite resonances. The K_d value of the Mg(NOTMP) complex was 0.35 mM at physiological pH and temperature, a value which is optimal for accurate assessment of free Mg^{II} in cells and blood plasma. The latter was confirmed experimentally. Furthermore, the selectivity of NOTMP for Mg^{II} over Ca^{II} exceeded 2 orders of magnitude. The K_d values of the Mg^{II} complexes were sensitive to pH and temperature. The pH effects could be easily predicted from potentiometric pH titration data. Examination of the Mg^{II} ligand equilibria by ^{31}P NMR over a wide temperature range provided complexation enthalpies and entropies. It was shown that the smaller K_d values at higher temperatures were largely due to a temperature effect on the highest ligand protonation constant. Only a small endothermic complexation enthalpy contributed to this phenomenon.

Introduction

Magnetic resonance spectroscopy (MRS) is an attractive tool for monitoring physiological events *in vivo*. The continuing growth of MRS in clinical diagnosis raises interest in the development of auxiliary ligands for determining levels of biologically important ions in intact, functioning tissue. Among the divalent alkaline earth ions, most interest has focused on Ca^{II}, largely because of its well-established role as an intracellular second messenger.[1] Magnesium(II) has received less attention in this regard because its concentration is typically much higher in cells and, consequently, it is generally thought of as a relatively nonspecific, hard acid cation that binds with and helps stabilize phosphorus-containing metabolites like ATP. However, there has been evidence mounting in recent years that Mg^{II} may play a pivotal role in regulation of a much wider variety of intracellular events.[2]

The most common method for monitoring levels of free Mg^{II} ($[Mg]_f$) uses the chemical shift separation of the ^{31}P α- and γ-resonances of ATP.[3] Accurate assessment of $[Mg]_f$ is complicated by other factors contributing to the chemical shift of the observed resonances, including pH and coordination of monovalent cations.[4,5] The dissociation constant of Mg(ATP) (30−80 μM)[6−8] also is not favorable for accurate assessment of $[Mg]_f$, since ATP is more than 90% bound under typical intracellular conditions, thus making the determination of the ratio between free and bound ATP extremely sensitive to experimental error. More recently, chelators containing a fluorine reporter functionality have been used to measure $[Mg]_f$ in cells and perfused organs by ^{19}F NMR.[9,10] Due to line broadening of the ^{19}F resonances after loading the ligands into cells, the observed signal-to-noise ratios were not as high as expected.

Since ^{31}P NMR spectroscopy is quite valuable for monitoring intracellular pH, phosphorylation potentials, [ATP]/[ADP] ratios, and a wide variety of other energy status signals in cells, perfused organs, and intact animals, our objective was to design ligands containing one or, preferably, more NMR equivalent ^{31}P nuclei whose chemical shifts do not overlap with those typical phosphorus-containing metabolites, such as (poly)-phosphates and phosphate esters, but are sensitive to complexation with Mg^{II}. In previous studies,[11−13] we demonstrated the usefulness of the triazacyclononane ring, for both its synthetic versatility and its selectivity for binding Mg^{II} over Ca^{II}. The trisubstituted phosphonate monoester derivative NOTPME (see Figure 1) demonstrated the potential of these ligands for monitoring $[Mg]_f$ in isolated cells;[11] the free ligand and the Mg^{II} complex were in slow exchange, thus allowing the observation of both resonances separately and providing the ratio of their peak areas by signal integration. The conditional stability

* To whom correspondence should be addressed at either address. Phone: (214) 883-2907 or (214) 648-5877. FAX: (214) 883-2925 or (214) 648-5881. E-mail: sherry@utdallas.edu.

† University of Texas at Dallas.

‡ University of Texas Southwestern Medical Center.

⊗ Abstract published in *Advance ACS Abstracts,* April 15, 1996.

(1) Rasmussen, H.; Barrett, P. Q. *Physiol. Rev.* **1984**, *64*, 938−984.
(2) Grubbs, R. D.; Maguire, M. E. *Magnesium* **1987**, *6*, 113−127.
(3) Gupta, R. K.; Moore, R. D. *J. Biol. Chem.* **1980**, *255*, 3987−3993.
(4) Halvorson, H. R.; Vande Linde, A. M. Q.; Helpern, J. A.; Welch, K. M. A. *NMR Biomed.* **1992**, *5*, 53−58.
(5) Williams, G. D.; Mosher, T. J.; Smith, M. B. *Anal. Biochem.* **1993**, *214*, 458−467.
(6) Burt, C. T.; Cheng, H.-M.; Gabel, S.; London, R. E. *J. Biochem.* **1990**, *108*, 441−448.
(7) Williams, G. D.; Smith, M. B. *Magn. Reson. Med.* **1995**, *33*, 853−857.
(8) Golding, E. M.; Golding, R. M. *Magn. Reson. Med.* **1995**, *33*, 467−474.
(9) Levy, L. A.; Murphy, E.; Raju, B.; London, R. E. *Biochemistry* **1988**, *27*, 4041−4048.
(10) Murphy, E.; Steenbergen, C.; Levy, L. A.; Raju, B.; London, R. E. *J. Biol. Chem.* **1989**, *264*, 5622−5627.
(11) Ramasamy, R.; Lazar, I.; Brucher, E.; Sherry, A. D.; Malloy, C. R. *FEBS Lett.* **1991**, *280*, 121−124.
(12) Lazar, I.; Ramasamy, R.; Brucher, E.; Geraldes, C. F. G. C.; Sherry, A. D. *Inorg. Chim. Acta* **1992**, *195*, 89−93.
(13) Van Haveren, J.; DeLeon, L.; Ramasamy, R.; Van Westrenen, J.; Sherry, A. D. *NMR Biomed.* **1995**, *8*, 197−205.

S0002-7863(95)03771-1 CCC: $12.00

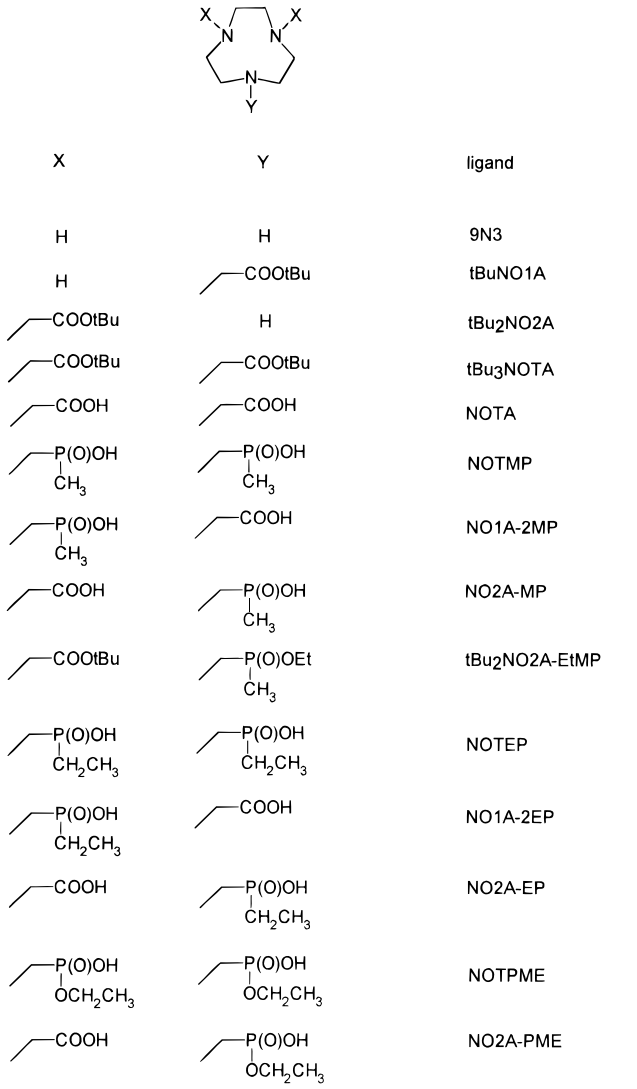


Figure 1. Ligands discussed in this study.

constant, $K_d = [Mg]_f[L]_f/[MgL]$, was too high, however, for accurate determination of the $[MgL]/[L]_f$ ratio. Ideally, this ratio should be about 1, which means that, for typical intracellular $[Mg]_f$ levels of about 0.5 mM, the K_d for the ideal ligand should also be about 0.5 mM. In a more recent study,[13] we demonstrated that changing the ligating side chain groups attached to the triazacyclononane ring allowed fine-tuning of the K_d. In that study, we concluded that a 1,4,7-triazacyclononane derivative containing two acetate and one ethylphosphinate side chains, NO2A-EP, was most promising for *in vivo* application.

In the present study, we investigated the possibility of changing the ethyl group of NO2A-EP to a methyl group to provide an easily monitored ^{1}H resonance as well. Surprisingly, this relatively minor structural alteration yielded ligands with significantly higher affinities for Mg^{II}. We report here the synthesis and characterization of the complete methylphosphinate family of ligands containing mixed acetate and methylphosphinate side chains. We show that the fully substituted methylphosphinate ligand NOTMP has an appropriate K_d for monitoring $[Mg]_f$, and demonstrate its utility for measuring $[Mg]_f$ in blood plasma.

Experimental Section

Synthesis. Chemicals. Diethoxymethylphosphine was obtained from Alfa and *tert*-butyl alcohol from Baker. Standard solutions for potentiometry (0.1000 M HCl and 1.000 M KOH) and the buffers (pH 4, 7, and 10) used for electrode calibration were obtained from Ricca. All other chemicals were obtained from Aldrich. *tert*-Butyl alcohol was dried over zeolite NaA. All other chemicals were used as received.

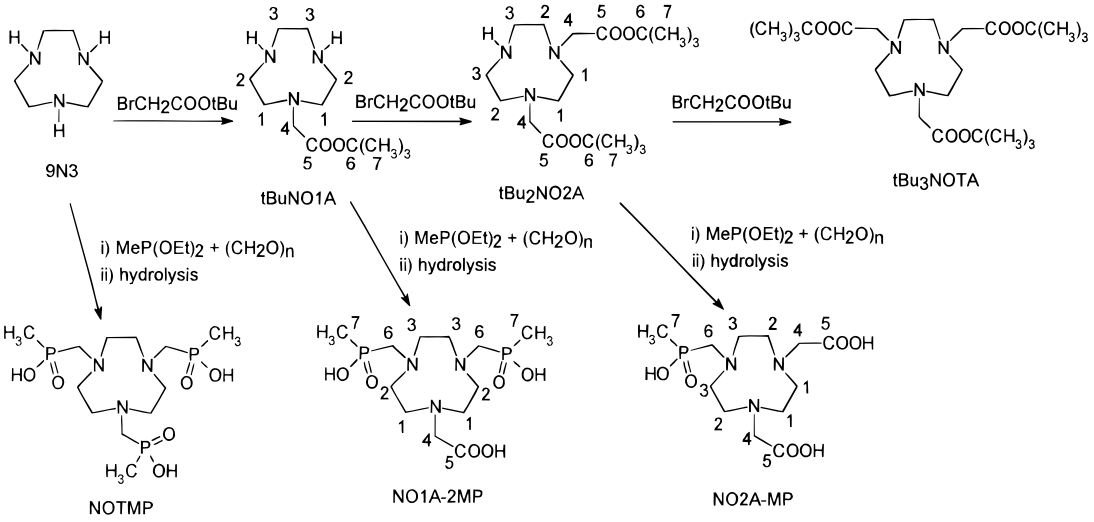
1,4,7-Triazacyclononane-*N*-(methylenemethylphosphinic acid)-*N′*,*N″*-bis(acetic acid) Hydrochloride (NO2A-MP). 1,4,7-Triazacyclononane (0.482 g, 3.74 mmol) was dissolved in 10 mL of tBuOH. Trifluoromethanesulfonic acid (TfOH; 0.588 g, 3.92 mmol) was added, and the solution was heated to 50 °C. Bromoacetic acid *tert*-butyl ester (1.457 g, 7.47 mmol in 6 mL of tBuOH) and KOtBu (0.838 g, 7.47 mmol in 6 mL of tBuOH) were added simultaneously in 20 equal aliquots over a period of 30 h. The reaction was stirred overnight at 50 °C. After cooling to room temperature, a ^{13}C NMR spectrum revealed that the mixture contained tBuNO1A, tBu_2NO2A, and tBu_3NOTA in a 1:5:1 ratio. Water was added, the pH was lowered to 5, and the mixture was washed with diethyl ether. A ^{13}C NMR spectrum of the ether extract showed the presence of tBu_3NOTA. The water layer was adjusted to pH 8 and extracted twice with diethyl ether. After drying over Na_2SO_4, evaporation of the ether gave tBu_2NO2A. TfOH. This was redissolved in water, and the pH was raised to 13 with aqueous NaOH. Extraction with diethyl ether gave the free base, tBu_2NO2A (0.914 g, 68%), which was judged 95% pure by ^{13}C NMR. Traces of tBu_3NOTA that were present were removed by repeating the extraction procedure, finally yielding 0.662 g (1.85 mmol, 50%) of the white solid, tBu_2NO2A. (Extraction of the original water layer at pH 13 yielded 77 mg of tBuNO1A (0.32 mmol, 9%) which was used for the NMR titration experiment; see below.) ^{13}C NMR ($CDCl_3$) (tBu_2NO2A, ppm): δ 171.4 (C5), 80.5 (C6), 57.8 (C4), 52.8 and 52.6 (C1 and C2), 46.7 (C3), 28.0 (C7) (numbering according to Scheme 1).

A mixture of tBu_2NO2A (0.399 g, 1.12 mmol) and diethoxymethylphosphine, $MeP(OEt)_2$ (0.263 g, 1.93 mmol), was prepared using the phosphine as the solvent. Nitrogen was bubbled into the flask for 5 min, after which the mixture was heated to 80 °C. Paraformaldehyde, $(CH_2O)_n$ (0.060 g, 2.0 mmol), was added, and this dissolved within 1 min. The mixture was stirred for 1 h. After cooling to room temperature, water was added and the pH lowered to 5. The solution was washed twice with diethyl ether. The pH of the water layer was raised to 13, and the product separated from the water layer. Diethyl ether was added, and the water layer was extracted twice with diethyl ether. The combined ether layers were dried over Na_2SO_4. Evaporation gave tBu_2NO2A-EtMP (0.450 g, 0.94 mmol, 84%) as a yellow oil. It appeared pure by NMR. ^{1}H NMR ($CDCl_3$) (tBu_2NO2A-EtMP, ppm): δ 3.96 (2H, $POCH_2$, "quintet", $^3J_{PH} = {}^3J_{HH} = 7$ Hz), 3.15 (4H, H4, s), 2.70−2.80 (10H, H2, H3, and PCH_2N, m), 2.70 (4H, H1, s), 1.40 (6H, PCH_3, d, $^2J_{PH} = 12$ Hz), 1.31 (18H, H7, s), 1.17 (3H, $POCH_2CH_3$, t, $^3J_{HH} = 7$ Hz). ^{13}C NMR: δ 171.2 (C5), 80.5 (C6), 59.9 ($POCH_2$, d, $^2J_{PC} = 8$ Hz), 59.6 (C4), 57.2 (C3, d, $^2J_{PC} = 8$ Hz), 57.1 (PCH_2N, d, $^1J_{PC} = 115$ Hz), 55.5 (C1 and C2), 28.0 (C7), 16.4 ($POCH_2CH_3$, d, $^3J_{PC} = 6$ Hz), 12.9 (PCH_3, d, $^1J_{PC} = 90$ Hz) (numbering (H1−H4, H7, C1−C7) according to tBu_2NO2A, Scheme 1).

The ester product tBu_2NO2A-EtMP (0.406 g, 0.85 mmol) was dissolved in 1 M aqueous HCl and heated at 80 °C for 15 h. This removed the tBu groups completely, but about 50% of the Et groups were still intact. Therefore, the pH was raised to 12, and the mixture was heated at 50 °C for 15 h. A small fraction (10%) showed cleavage of the methylphosphinate group to NO2A. The product was purified on a cation exchange column (DOWEX-H^+) eluted by a gradient of 0.1−2.0 M HCl. Evaporation gave NO2A-MP (0.264 g, 65%) as a white solid, also containing HCl and H_2O, which was judged pure by ^{1}H and ^{31}P NMR. Potentiometry gave the molecular weight (477) and the approximate formula NO2A-MP·2HCl·3.6H_2O. ^{1}H NMR (D_2O) (NO2A-MP, ppm): δ 4.01 (4H, H4, s), 3.53 (4H, H1, s), 3.37−3.43 (8H, H2 and H3, br d), 3.33 (2H, H6, d, $^2J_{PH} = 6$ Hz), 1.44 (3H, H7, $^2J_{PH} = 14$ Hz). ^{13}C NMR: δ 173.1 (C5), 59.6 (C4), 57.8 (C6, d, $^1J_{PC} = 97$ Hz), 54.1 and 53.6 (2:1) (C1, C2, and C3), 17.5 (C7, d, $^1J_{PC} = 91$ Hz) (numbering according to Scheme 1).

1,4,7-Triazacyclononane-*N*,*N′*-bis(methylenemethylphosphinic acid)-*N″*-(acetic acid) Hydrochloride (NO1A-2MP). tBuNO1A was prepared in a similar procedure using 1,4,7-triazacyclononane (0.215 g, 1.67 mmol), 5 mL of tBuOH, TfOH (0.266 g, 1.77 mmol), bromoacetic acid *tert*-butyl ester (0.492 g, 2.52 mmol in 3 mL of tBuOH), and KOtBu (0.281 g, 2.50 mmol in 3 mL of tBuOH). The reaction was stirred overnight at 50 °C. Subsequent extractions of the

Scheme 1



water layer at pH 7 and 13 with diethyl ether gave an oily mixture of tBuNO1A and tBu_2NO2A (7:3, 0.113 g, 0.29 mmol of tBuNO1A, 17%), which was used without further purification. ^{13}C NMR ($CDCl_3$) (tBuNO1A, ppm): δ 171.6 (C5), 81.0 (C6), 57.8 (C4), 52.8 (C1), 46.8 and 46.2 (C2 and C3), 28.2 (C7) (numbering according to Scheme 1).

Phosphorylation and subsequent hydrolysis were performed in a procedure analogous to the one described for NO2A-MP, using the mixture of tBuNO1A and tBu_2NO2A (0.113 g), $MeP(OEt)_2$ (0.082 g, 0.60 mmol), and $(CH_2O)_n$ (0.018 g, 0.60 mmol). NO1A-2MP was obtained (51 mg, 33%), also containing HCl and H_2O, which was judged pure by ^{1}H and ^{31}P NMR. Potentiometry gave the molecular weight (535), and the approximate formula NO1A-2MP·3HCl·3H_2O was derived. ^{1}H NMR (D_2O) (NO1A-2MP, ppm): δ 3.99 (2H, H4, s), 3.39 (8H, br s) and 3.29 (8H, br s) (H1, H2, H3, and H6), 1.38 (6H, H7, $^2J_{PH}$ = 15 Hz). ^{13}C NMR: δ 172.2 (C5), 59.3 (C4), 57.4 (C6, d, $^1J_{PC}$ = 96 Hz), 54.3, 53.9, and 53.5 (C1, C2, and C3), 17.6 (C7, d, $^1J_{PC}$ = 92 Hz) (numbering according to Scheme 1).

1,4,7-Triazacyclononane-*N*,*N*′,*N*″-tris(methylenemethylphosphinic acid) Hydrochloride (NOTMP). The phosphorylation of 1,4,7-triazacyclononane (0.634 g, 4.91 mmol) was performed in a procedure analogous to the one for NO2A-MP, using $MeP(OEt)_2$ (2.429 g, 17.86 mmol) and $(CH_2O)_n$ (0.536 g, 17.87 mmol). The ester product Et_3NOTMP was purified on a silica column eluted with MeOH/CH_2Cl_2 (1:1 v/v). The pure ester was obtained as a yellow oil (1.705 g, 3.49 mmol, 71%). ^{1}H NMR ($CDCl_3$) (Et_3NOTMP, ppm): δ 4.00 (6H, $POCH_2$, "quintet", $^3J_{PH} = {}^3J_{HH}$ = 7 Hz), 2.81−2.92 (18H, ring CH_2 and PCH_2N, m), 1.46 (9H, PCH_3, d, $^2J_{PH}$ = 14 Hz), 1.24 (9H, $POCH_2CH_3$, t, $^3J_{HH}$ = 7 Hz). ^{13}C NMR: δ 60.2 ($POCH_2$, d, $^2J_{PC}$ = 8 Hz), 57.9 (PCH_2N, d, $^1J_{PC}$ = 113 Hz), 57.6 (ring CH_2, d, $^2J_{PC}$ = 7 Hz), 16.6 ($POCH_2CH_3$, d, $^3J_{PC}$ = 6 Hz), 13.3 (PCH_3, d, $^1J_{PC}$ = 91 Hz).

The hydrolysis to and purification of NOTMP was performed similarly as described above to yield NOTMP (1.58 g, 87%), also containing HCl and H_2O, which was judged pure by ^{1}H and ^{31}P NMR. Potentiometry gave the molecular weight (557), and the approximate formula NOTMP·3HCl·2H_2O was derived. ^{1}H NMR (D_2O) (NO1A-2MP, ppm): δ 3.37 (6H, PCH_2N, d, $^2J_{PH}$ = 8 Hz) and 3.34 (12H, ring CH_2, s), 1.38 (9H, PCH_3, $^2J_{PH}$ = 14 Hz). ^{13}C NMR: δ 56.1 (PCH_2N, d, $^1J_{PC}$ = 94 Hz), 52.8 (ring CH_2), 16.5 (PCH_3, d, $^1J_{PC}$ = 92 Hz).

Potentiometry. Potentiometric titrations were conducted at 298 and 310 K using an Accumet 925 pH meter (Fisher), an Orion 8103 Ross combination electrode, and a Metrohm 665 Dosimat automatic buret (Brinkman Instruments). The ionic strength was adjusted to 0.1 M using KCl in all titrations, and all measurements were performed under a N_2 atmosphere. Hydrogen ion activities were obtained from millivolt readings, and the electrode was calibrated using Ricca high-precision buffers, which are valid from 278 to 318 K. Hydrogen ion activities were translated into concentrations using pK_w = 13.79 (298 K) and 13.40 (310 K) and an activity coefficient of 0.82, which were determined from separate titrations of 0.001 and 0.01 M KOH with 0.1 M HCl.

All solutions were prepared from distilled, demineralized, and degassed water, and were saturated with and stored under N_2. Ligand solutions were typically 2−3 mM, and excess KOH was added. To determine the ligand protonation constants, these solutions were titrated with standardized 0.1000 M HCl. For the metal ligand stability determinations, a concentrated metal chloride solution (0.2 M) was added to the cup solution to reach a metal ligand ratio of about 1, after which the solution was also titrated with 0.1 M HCl. Exact ligand concentrations (and molecular weights of the isolated salts) were evaluated after multiple titrations of ligand and metal ligand solutions. All titrations were performed at least twice.

The calculation of the constants was performed using a general spreadsheet program with an approach described earlier.[14,15] Only the three highest protonation constants could be evaluated in the pH range of 2−12 for all ligands. Formation of polynuclear complexes M_nL was not observed in any system.

NMR Spectroscopy. All analytical ^{1}H (200 MHz) and ^{13}C (50 MHz) NMR spectra, as well as the protonation titrations of tBuNO1A, tBu_2NO2A, and NO2A-MP, were recorded on a JEOL FX200 NMR spectrometer at 295 K. The protonation titration of NO1A-2MP was monitored by ^{1}H NMR spectroscopy (500 MHz), using a 5 mm ^{13}C/^{1}H probe. ^{1}H NMR spectra of the hydrolyzed ligands were recorded in D_2O using HDO (4.80 ppm) as the internal standard, while their ^{13}C NMR spectra were recorded in 10% aqueous D_2O with tBuOH (31.2 ppm) as the internal standard. More experimental details about the protonation titrations are provided in the supporting information together with all ^{1}H and ^{13}C resonance data and assignments.

All ^{31}P NMR spectra (202 MHz), as well as the ^{1}H (500 MHz) monitored K_d determinations of NO2A-MP and NOTMP, were recorded on a General Electric GN500 NMR spectrometer using a 10 mm broadband probe. Spectra were recorded at room temperature (295 K) or at another temperature using the standard GN variable temperature control unit. For the ^{31}P NMR spectra, aqueous 85% phosphoric acid (0 ppm) was used as the external standard, and ^{1}H decoupling was applied unless stated otherwise. For the K_d determinations, performed at 295 and 310 K, 3 mL samples were prepared of 2−3 mM ligand in an aqueous solution containing 10% D_2O, 115 mM of KCl, 20 mM of NaCl, 10 mM of glucose, and 10 mM of HEPES. The pH was adjusted to 7.40 (at room temperature) using aqueous NaOH. Aliquots of 0.2 M $MgCl_2$ were added, and the ^{31}P spectrum was recorded after each addition. The pH was checked and adjusted if necessary. Resonance areas were determined by peak integration using standard GE software. Evaluation of the K_d values was performed by treating the ligand concentration as a variable, as described in the text. Calculated ligand concentrations always appeared to match the molecular weights obtained by potenti-

(14) Van Westrenen, J.; Khizhnyak, P. L.; Choppin, G. R. *Comput. Chem.* **1991**, *15*, 121−129.

(15) Huskens, J.; Van Bekkum, H.; Peters, J. A. *Comput. Chem.* **1995**, *19*, 409−416.

ometry. The ^{31}P NMR shifts of the ligands were measured as a function of pH, using 3−5 mM samples.

For the studies of the behavior of K_{eq} (for the equilibrium $MgL + H^+ \rightleftharpoons Mg^{II} + HL$, see below) *versus* temperature, similar samples were used containing 2.00 mM ligand, and 2.00 mM (for NOTMP) or 1.62 mM (for NO1A-2MP and NO2A-MP) $MgCl_2$. Spectra were measured from 283 to 353 K in steps of 10 K. For NOTMP, K_{eq} values were calculated from the peak areas, and the enthalpy, ΔH, and entropy, ΔS, were determined from a plot of $-R \ln K_{eq}$ *versus* $1/T$. For NO1A-2MP and NO2A-MP, the $[Mg]_{tot}/[L]_{tot}$ ratio was treated as a variable in the calculation of ΔH, while ΔS was fixed at −132 J/(mol K) (see text). In both cases, the calculated $[Mg]_{tot}/[L]_{tot}$ ratio appeared to be equal to the experimental one (0.81). No corrections for a possible change of pH *versus* temperature were made.

The spin−lattice relaxation times (T_1) of NOTMP and its Mg^{II} complex were determined using the inversion−recovery pulse sequence with a three-parameter curve-fitting procedure.[16] The T_1 of the free ligand was also determined in a sample without $MgCl_2$. Selective saturation of either the free ligand or the Mg^{II} complex resonance, using the former sample, was performed by applying a low-power long pulse (1 s). In both cases, no effect on the peak area of the nonirradiated resonance was observed, thus excluding spin transfer between the resonances.

Plasma from one of the authors (A.D.S.) was used for a determination of the K_d of Mg(NOTMP) monitored by ^{31}P NMR, as described above. An accurately known amount of ligand (3 mM) was used, and 10% D_2O was added for locking. The pH (7.80) was measured and appeared to be stable during the titration.

Results and Discussion

Synthesis. Previous work[13] has shown that the ethylphosphinate derivative NO2A-EP (Figure 1) had an appropriate K_d (0.43 mM) for *in vivo* purposes, and the ^{31}P NMR spectrum showed two well-separated ($\Delta\delta = 2.4$ ppm) signals for the free ligand and the Mg^{II} complex. Since our goal was to design a ligand that might have a ^{1}H resonance also sensitive to Mg^{II} binding, our first target compound was NO2A-MP. This ligand was synthesized via a new strategy, in which no protecting groups are required. Starting from 1,4,7-triazacyclononane (9N3), two acetate groups (tBu esters) were attached by reaction with 2 equiv of *tert*-butyl bromoacetate in tBuOH, giving tBuNO1A and tBu_2NO2A (Scheme 1). The HBr produced in the reaction was neutralized by the simultaneous addition of KOtBu. The formation of the trisubstituted product tBu_3NOTA was inhibited by protonating a single macrocyclic nitrogen using 1 equiv of trifluoromethanesulfonic acid (TfOH). This method using H^+ as the "protecting group" relies on the fact that there is a large gap between the stability constants (in water) of the first and the second protonation steps of 9N3 and its derivatives. In this way, a selectivity of 70% toward tBu_2NO2A was reached. Earlier attempts without TfOH showed a selectivity of only 30%.

The use of TfOH as a protection agent in the synthesis of tBu_2NO2A was further investigated by titrating tBuNO1A and tBu_2NO2A with TfOH, while monitoring their ^{1}H and ^{13}C NMR spectra. In general, protonation of an amine induces a large downfield shift on α-hydrogen nuclei and a large upfield one on β-carbons. Therefore, H4 and C5 were expected to be good indicators for the protonation sites. Both nuclei exhibited marginal shifts for both protonations of tBuNO1A and for the first protonation step of tBu_2NO2A, while large effects were observed for the second protonation step of the latter compound. From these results it was concluded that protonation takes place preferentially at a secondary nitrogen atom. A more elaborate description, together with all assignments of the ^{1}H and ^{13}C resonances, is given in the supporting information. The strong preference for protonation at secondary nitrogen atoms necessitated a careful tuning of the $[H^+]/[9N3]$ ratio in the synthesis of tBu_2NO2A. To achieve the maximal selectivity, this ratio has to be 1 during the course of the reaction. A larger ratio will lead to protection of tBuNO1A, and therefore a competitive reaction rate between tBuNO1A and tBu_2NO2A, while a smaller ratio fails to protect tBu_2NO2A from the consecutive reaction toward tBu_3NOTA. This selectivity was reached by using 1 equiv of TfOH, and by adding *tert*-butyl bromoacetate and KOtBu simultaneously in small equal quantities.

The usual hydrolysis procedure of refluxing in 6 M HCl appeared unsuitable for this ligand, since more than 20% of the methylphosphinate side chain was cleaved off after 15 h at 120 °C. Even lower acid concentrations and lower temperatures yielded small amounts of NO2A. The product NO2A-MP was further purified on a cation exchange column eluted with aqueous HCl, finally yielding the free acid. The ligand as well as its Mg^{II} complex appeared to be quite stable in neutral aqueous solution. No degradation was observed for several weeks; the same was true for the other ligands discussed here.

Two other compounds, NO1A-2MP and NOTMP, were synthesized using similar methods. NO1A-2MP was prepared from tBuNO1A isolated from a reaction of 9N3 with 1.5 equiv of *tert*-butyl bromoacetate, applying 1 equiv of TfOH. We also attempted to use the protective strategy described above for tBu_2-NO2A in the selective synthesis of tBuNO1A, by using 2 equiv of TfOH. However, the reactivity of the diprotonated 9N3 appeared to be too low to protect the diprotonated tBuNO1A from consecutive reaction toward tBu_2NO2A.

The intermediate tBuNO1A was reacted with excess $MeP(OEt)_2$ and $(CH_2O)_n$, followed by hydrolysis and purification with cation exchange column chromatography. NOTMP was prepared in one step as the triethyl ester, starting from 9N3 and excess $MeP(OEt)_2$ and $(CH_2O)_n$, similar to a known procedure.[17] Hydrolysis in aqueous NaOH, followed by purification on a cation exchange column, gave the free acid. These latter two ligands were prepared to measure the K_d of their Mg^{II} complexes, because the K_d of Mg(NO2A-MP) was, surprisingly, too low (see below) for our intended application.

NMR Characterization of the Ligands and their Mg^{II} Complexes. All three ligands NOTMP, NO1A-2MP, and NO2A-MP displayed a single ^{31}P NMR resonance in the 35−45 ppm range, well downfield of resonances from biologically important phosphates. For all ligands, the resonances of the free ligand and the Mg^{II} complex were well resolved and in slow exchange at 202 MHz. There was no line broadening of either resonance up to 80 °C. We also used selective saturation of either the free ligand or the Mg^{II} complex resonance of NOTMP to investigate the time scale of the interchange between them. In both experiments, saturation of either resonance had no effect on the peak area of the nonsaturated resonance. Thus, exchange between ligand and complex was slow relative to their spin−lattice relaxation rates ($1/T_1 = 0.63$ s^{-1} for these ligands).

Accurate assessment of [ligand]/[complex] integral ratios is vital for measuring free Mg^{II} levels using the strategy employed here. The ^{1}H coupled and decoupled ^{31}P line widths, nuclear Overhauser effects (NOE), and longitudinal relaxation times (T_1) were measured for NOTMP and its Mg^{II} complex. Since the T_1 values were essentially equal for the free ligand (1.6 ± 0.1 s) and the Mg^{II} complex (1.7 ± 0.1 s), short delays without complete relaxation may be applied without loss of integral accuracy. Similarly, equal values of the NOEs (1.3 and 1.2,

(16) Canet, D.; Levy, G. C.; Peat, I. R. *J. Magn. Reson.* **1975**, *18*, 199.

(17) Broan, C. J.; Cole, E.; Jankowski, K. J.; Parker, D.; Pulukkody, K.; Boyce, B. A.; Beeley, N. R. A.; Millar, K.; Millican, A. T. *Synthesis* **1992**, 63−68.

Table 1. ^{31}P (202 MHz) and ^{1}H (500 MHz) Chemical Shifts[a] of NOTMP, NO1A-2MP, and NO2A-MP and Their Mg^{II} Complexes at pH 7.4 and 295 K

	^{31}P NMR			^{1}H NMR		
L	δ_L	δ_{MgL}	$\Delta\delta$	δ_L	δ_{MgL}	$\Delta\delta$
NOTMP	36.8	42.3	5.5	1.33	1.40	0.07
NO1A-2MP	39.6	42.1	2.5	1.30	1.41	0.11
NO2A-MP	40.1	41.8	1.7	1.30	1.41	0.11
NOTEP[b]	40.5	45.5	5.0			
NO1A-2EP[b]	42.9	45.3	2.4			
NO2A-EP[b]	42.7	45.1	2.4			
NOTPME[c]	17.5	23.5	6.0			
NO2A-PME[b]	21.3	23.4	2.1			

[a] In parts per million; measured with 85% phosphoric acid (0 ppm) as the external and HDO (4.80 ppm) as the internal standard for the ^{31}P and ^{1}H NMR spectra, respectively. [b] Reference 13. [c] Reference 11.

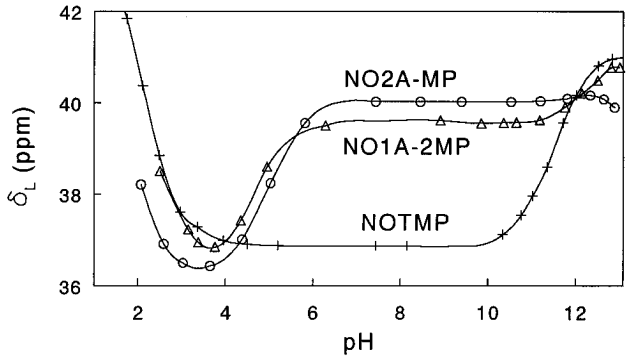


Figure 2. ^{31}P chemical shift of the free ligand resonances, δ_L, of NOTMP, NO1A-2MP, and NO2A-MP as a function of pH, as determined at 202 MHz at 295 K.

respectively) allow proton decoupling without loss of integral accuracy. The line width of the free NOTMP resonance decreased from 30 Hz (multiplet) to 4 Hz (singlet) upon proton decoupling, while the line width of the Mg(NOTMP) resonance decreased from 37 to 12 Hz. So, in both cases the resonances of the complex were somewhat broader than for the free ligand, probably due to the formation of diastereomeric complexes due to the methylphosphinate groups becoming chiral upon coordination to Mg^{II}. Assuming that exchange between the diastereomeric MgL species is slow or intermediate on the NMR time scale, the chemical shift difference between such species must be small.

The ^{31}P NMR chemical shifts of NOTMP, NO1A-2MP, and NO2A-MP and of their Mg^{II} complexes are listed in Table 1, together with some values of previously described ligands.[11,13] The most striking phenomenon is the large shift difference, $\Delta\delta$ ($=\delta_{MgL} - \delta_L$), for NOTMP, NOTEP,[13] and NOTPME.[11] Since the chemical shifts of the Mg^{II} complexes, δ_{MgL}, were nearly equal for all members of the same family, the large $\Delta\delta$ values for the triphosphinate and tris(phosphonate ester) ligands must be due to differences in the values of δ_L. Therefore, we investigated the behavior of δ_L *versus* pH for NOTMP, NO1A-2MP, and NO2A-MP (Figure 2). It appeared that the values of δ_L were about the same for all three ligands at high pH, where the ligands are nonprotonated. Upon addition of 1 equiv of acid, the NOTMP resonance shifted to lower frequency (4.1 ppm), while the effects on the shift of NO1A-2MP (1.2 ppm) and NO2A-MP (0 ppm) were much smaller. This indicates that the first protonation of NO1A-2MP and NO2A-MP occurred largely at a nitrogen atom with an acetate side chain, while in NOTMP protonation must occur at a site near a phosphinate side chain. This results in shielding of the ^{31}P nuclei that are in a β-position relative to the protonated N atom.

This microscopic protonation sequence was confirmed by ^{1}H NMR shifts (see the supporting information). The first two protonation steps occurred at ring nitrogens, and were selective for protonation at a nitrogen atom near an acetate. This selectivity can probably be ascribed to a stronger hydrogen bond formed between a protonated nitrogen and an adjacent acetate *versus* a methylphosphinate group. The third protonation produces very little change in chemical shift of the ring protons. This indicates that this protonation does not take place at a nitrogen, but probably at the carboxylate group of an acetate side chain. This may lead to a rearrangement of a proton from the N atom adjacent to the protonated acetate to the N atom adjacent to the still deprotonated methylphosphinate group. Also, the large increase of the ^{31}P NMR chemical shift of all three ligands during the third protonation (Figure 2) suggests that a conformational change occurs, which may be ascribed to a rotation of the acetate group. The protonation sequence for NO2A-MP is schematically illustrated by Scheme 2. The microscopic protonation sequence, observed here for the methylphosphinate family, also explains the ^{31}P NMR chemical shift differences observed for the ethylphosphinate and the phosphonate ethyl ester derivatives (see Table 1).

The methyl proton resonances of NOTMP, NO1A-2MP, and NO2A-MP appear as a sharp doublet ($^{2}J_{PH} = 13$ Hz) while their Mg^{II} complexes show slightly broadened resonances. However, $\Delta\delta$ ($=\delta_{MgL} - \delta_L$) is only 0.07 (NOTMP) or 0.11 ppm (NO1A-2MP and NO2A-MP; see Table 1). Although these resonances are well resolved at high field, this small shift difference may be too small to be useful *in vivo*. The observation that the shift difference is smaller for NOTMP compared to NO1A-2MP and NO2A-MP can be explained by the fact that protonation of a nitrogen atom near a methylphosphinate side chain causes a (small) downfield shift of the δ_L of the methyl protons, in contrast to the upfield effect on the δ_L of the ^{31}P resonance. If the free and the Mg^{II}-bound ligand resonances could be resolved *in vivo*, then spin-echo heteronuclear editing or multiple quantum NMR techniques should allow monitoring of the methyl ^{1}H resonance of the methylphosphinates without interference from other ^{1}H signals.

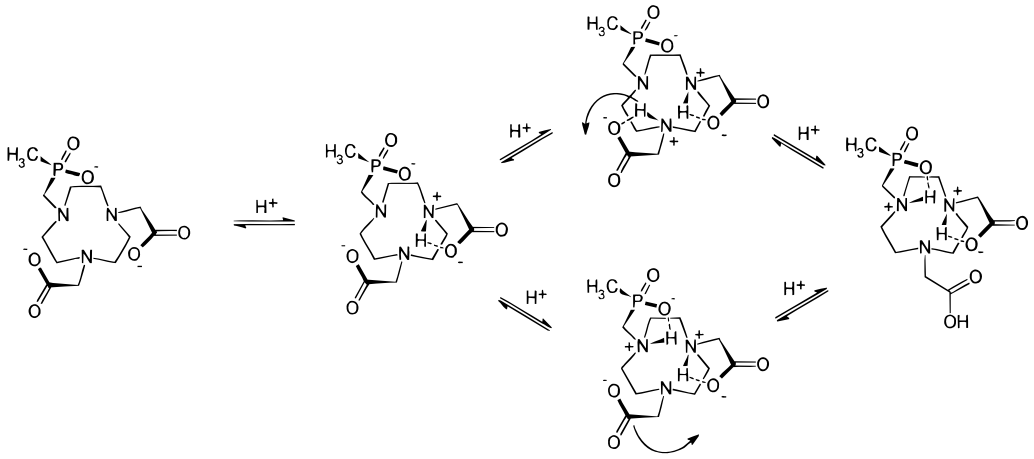
Thermodynamics. Potentiometry was used to measure the protonation constants of the ligands NOTMP, NO1A-2MP, and NO2A-MP and their metal ligand stability constants with Mg^{II} and Ca^{II}. All constants were measured in 0.1 M KCl to mimic intracellular conditions, and all were determined at 25 and 37 °C. Typical titration curves for NOTMP (at 25 °C) are presented in Figure 3. All results are summarized in Table 2, together with previous results for NOTA and the ethylphosphinate derivatives NOTEP, NO1A-2EP, and NO2A-EP.

The NOTMP protonation constants and stability constant with Mg^{II} differed considerably from values reported previously by others,[18] especially the second protonation constant which they report to be near pH 7. Figure 3, however, clearly shows the absence of a pK_a in this pH region, suggesting that the previous literature values are in error.

The first protonation constants of the ligands described herein are all somewhat higher than the values for the corresponding ethylphosphinate derivatives.[13] Also, the complexes with Mg^{II} and Ca^{II} are more stable for the methylphosphinates than for the corresponding ethylphosphinates. The selectivity for Mg^{II} over Ca^{II} ($\log K_{MgL} - \log K_{CaL}$) decreased with an increase of the number of acetate side chains, as reported previously.[13] At 37 °C, the first protonation constants decreased as expected (see below), while the Mg^{II}−ligand stability constants were some-

(18) Broan, C. J.; Jankowski, K. J.; Kataky, R.; Parker, D. *J. Chem. Soc., Chem. Commun.* **1990**, 1738−1739.

Scheme 2



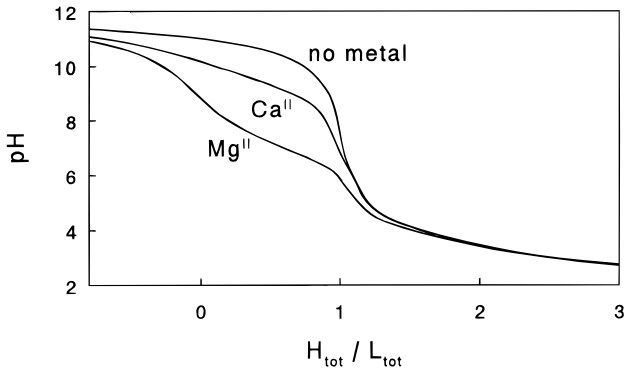


Figure 3. Potentiometric titration curves for NOTMP (2 mM) in the absence and presence of 1 equiv of Mg^{II} or Ca^{II}, as determined at 298 K.

what higher. The species MgHL was observed in most of the experiments, but was a minor species throughout the titrations, while CaHL was not observed in any of the titrations.

For biological applications, the more critical binding constant is that given by the conditional dissociation constant, $K_d = [Mg]_f[L]_f/[MgL]$, near physiological pH values. Here, $[Mg]_f$ is the free Mg^{II} concentration, and $[L]_f$ is the total concentration of uncomplexed (free) ligand, *i.e.*, the sum of the concentrations of (nonprotonated) L and its protonated forms. Since the first protonation constants for all these ligands are much higher than physiological pH values while the second protonation constants are much lower, $[L]_f$ can conveniently be written as [HL] at this pH.

K_d values can be assessed at physiological pH from ^{31}P NMR titrations performed under buffered conditions. The experiments described below were performed in HEPES-buffered solutions (pH 7.40), containing KCl, NaCl, and glucose to mimic intracellular conditions. In general, 2−3 mM ligand solutions were titrated with small aliquots of a concentrated $MgCl_2$ solution. The ^{31}P NMR spectrum was recorded after each addition, and the peak areas corresponding to L and MgL were measured to give the integral ratio $y = [MgL]/[L]_f$. Since the exact ionic form and, hence, molecular weight of each ligand were unknown when these titrations were begun (the potentiometric titrations were performed later), the total ligand concentration, $[L]_{tot}$, was treated as a variable in each NMR titration. In order to present the results graphically, the definition of K_d was rewritten as eq 1, using the mass balances for L ($[L]_{tot} = [L]_f + [MgL]$) and Mg^{II} ($[Mg]_{tot}$ (the total Mg^{II} concentration) $= [Mg]_f + [MgL]$). Plots of $[Mg]_{tot}/y$ *versus* $1/(1 + y)$ gave straight lines with $[L]_{tot}$ as the slope and K_d as the intercept.

$$[Mg]_{tot}/y = [L]_{tot}/(1 + y) + K_d \qquad (1)$$

The results of the ^{31}P NMR titrations at 25 °C for NOTMP, NO1A-2MP, and NO2A-MP are shown in Figure 4. The calculated $[L]_{tot}$ values obtained from these plots agreed well with those calculated using the molecular weights of the ligands, as determined later by potentiometry. The intercepts of Figure 4 indicate that the K_d values for the three ligands are in the order NO2A-MP < NO1A-2MP < NOTMP. The K_d of Mg(NO2A-MP) estimated from this curve was 0.07 mM, a value that is much smaller than the actual $[L]_{tot}$ (3 mM) used in the experiment. These experimental conditions gave rise to a large relative error in the K_d for this complex. However, the concentration of NO2A-MP could not be lowered in this experiment because this ligand has only one ^{31}P nucleus per molecule and, consequently, the signal-to-noise ratio became limiting. The ligand concentrations of NO1A-2MP and NOTMP (2 mM) were of the same order of magnitude as the K_d values of their Mg^{II} complexes estimated from these plots.

Similar experiments were performed using ^1H NMR spectroscopy. Table 2 lists the K_d values of the methylphosphinate derivatives measured experimentally by ^{31}P and ^1H NMR and the corresponding values estimated from the potentiometric data ($\log K_d = \log K_{HL} - pH - \log K_{MgL}$). All K_d values appeared to be equal to their potentiometric estimates within experimental error. Somewhat to our surprise, the K_d value for Mg(NO2A-MP) (0.07 mM) was about 6-fold lower than that for Mg(NO2A-EP) (0.43 mM), too low for measurement of typical intracellular free Mg^{II} levels. However, NO1A-2MP and even NOTMP proved to have K_d values in a useful range for monitoring $[Mg]_f$. An additional advantage of these ligands is their higher ^{31}P NMR sensitivity, because they have more equivalent ^{31}P nuclei per molecule. NOTMP is especially suited, since it combines the highest ^{31}P (and ^1H) NMR sensitivity with a relative ease of preparation and the largest ^{31}P NMR shift difference between L and MgL (see above).

A remarkable effect was the temperature dependence of K_d. Both NMR and potentiometry indicated that the K_d of Mg(NOTMP) decreased by about a factor of 3 between 25 and 37 °C (see Table 2). In order to investigate this effect further, ^{31}P NMR spectra of samples containing mixtures of L and MgL were measured as a function of temperature. For a sample containing 2 mM NOTMP and 2 mM $MgCl_2$, the temperature effect on the integral ratio was dramatic: at 10 °C there was more free ligand than Mg^{II} complex ($y = 0.8$), while at 80 °C the free ligand peak was barely visible ($y > 20$). From measurements over this temperature range, it became possible to calculate thermodynamic parameters (ΔH and ΔS) for the

Table 2. Stepwise Protonation and Metal−Ligand Stability Constants of NOTMP, NO1A-2MP, and NO2A-MP with Mg^{II} and Ca^{II} As Determined by Potentiometry at 298 and 310 K (I = 0.1 M KCl), and Conditional Mg^{II}−Ligand Dissociation Constants, K_d, Measured from ^{31}P (202 MHz) or ^{1}H (500 MHz) NMR Spectra at pH 7.40

		log K_i[a]			log K_{ML}[a]		log β_{MHL}[b]	K_d of MgL (mM)		
L	T (K)	HL	H_2L	H_3L	MgL	CaL	MgHL	pH-metry[c]	^{31}P NMR	^{1}H NMR
NOTMP	298	10.92(2)	3.97(3)	2.09(2)	6.66(5)	4.45(4)	12.76(15)	0.8	1.1(1)[d]	1.2(1)[d]
	310	10.68(4)	3.98(2)	2.05(3)	6.67(5)	4.40(4)	*e*	0.4	0.35(3)	
NO1A-2MP	298	11.7(1)	4.24(5)	2.10(2)	8.0(1)	6.3(1)	14.5(3)	0.2	0.40(3)[d]	
	310	11.4(2)	4.1(1)	2.0(2)	8.1(1)	6.6(1)	14.0(2)	0.08		
NO2A-MP	298	12.0(1)	4.99(3)	2.73(3)	8.9(2)	7.5(1)	14.8(2)	0.05	0.07(3)[d]	0.06(3)[d]
	310	11.7(1)	5.04(3)	2.90(3)	9.0(2)	7.7(1)	14.4(2)	0.02		
NOTEP[f]	298	10.27(5)	4.72(3)	3.26(3)	5.54(2)	3.79(2)				
NO1A-2EP[f]	298	10.45(5)	4.42(1)	2.76(2)	5.57(3)	3.56(4)				
NO2A-EP[f]	298	11.15(4)	4.89(4)	2.82(4)	7.26(3)	6.43(1)				
NOTA[g]	298	11.41	5.6	2.9	9.69	8.92				

[a] Standard deviations derived from two or three separate titrations: $K_i = [H_iL]/[H_{i-1}L][H]$, $K_{ML} = [ML]/[M][L]$. [b] $\beta_{MHL} = [MHL]/[M][H][L]$. [c] Estimated values from potentiometry: $\log K_d = \log K_{HL} - pH - \log K_{ML}$ at pH 7.40. [d] T = 295 K. [e] Not observed. [f] Reference 13. [g] Reference 19.

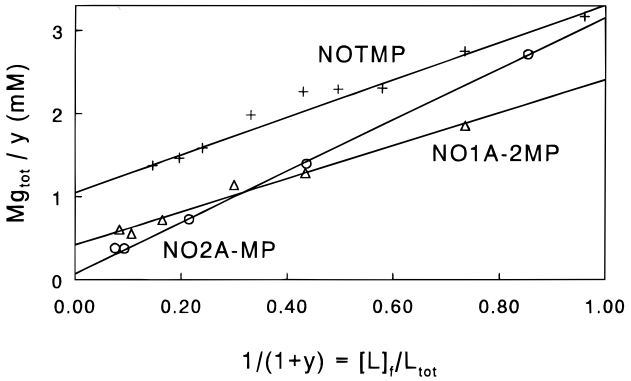


Figure 4. $[Mg]_{tot}/y$ ($y = [MgL]/[L]_f$) *versus* the free ligand fraction, $[L]_f/[L]_{tot}$ ($=1/(1+y)$), for NOTMP, NO1A-2MP, and NO2A-MP, as determined by ^{31}P NMR (202 MHz) at 295 K and pH 7.4. The slopes represent $[L]_{tot}$ values, whereas the intercepts give K_d values.

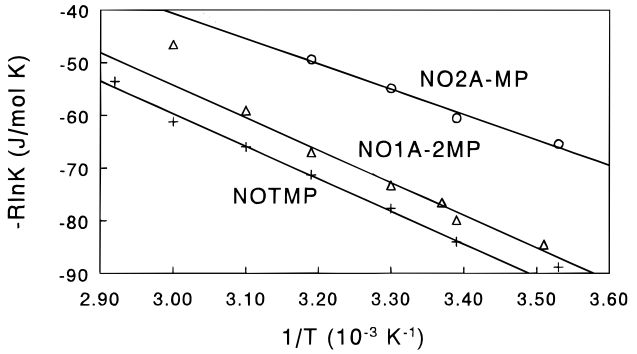


Figure 5. $-R \ln K_{eq}$ (K_{eq} is defined for the equilibrium $MgL + H^+ \rightleftharpoons Mg^{II} + HL$) *versus* $1/T$ for NOTMP ($[L]_{tot}$ = 2.00 mM, $[Mg]_{tot}$ = 2.00 mM), and NO1A-2MP and NO2A-MP ($[L]_{tot}$ = 2.00 mM, $[Mg]_{tot}$ = 1.62 mM), as determined by variable temperature ^{31}P NMR (202 MHz) at pH 7.4. The slopes represent ΔH values for this equilibrium.

equilibrium $MgL + H^+ \rightleftarrows HL + Mg^{II}$. The equilibrium constant, K_{eq} ($=[HL][Mg]_f/[MgL][H]$), could be obtained from the NMR data once the ligand and Mg^{II} concentrations were accurately known. At physiological pH, $[L]_f = [HL]$ as described above. Therefore, the relationship between K_{eq} and K_d is straightforward ($K_{eq} = K_d/[H]$). Since the equilibrium is the decomplexation of MgL to Mg^{II} and L followed by the protonation of L to HL, the free energy of the equilibrium, ΔG, can be written as the difference in free energy of the protonation, ΔG_{HL}, and the complexation, ΔG_{ML}, as given in eq 2. Plots of

$$\Delta G = -RT \ln K_{eq} = \Delta H - T\Delta S = \Delta G_{HL} - \Delta G_{ML} \quad (2)$$

$-R \ln K_{eq}$ *versus* $1/T$ gave straight lines with ΔH as the slope and $-\Delta S$ as the intercept, as shown in Figure 5. The enthalpy ($\Delta H = \Delta H_{HL} - \Delta H_{ML}$) and entropy ($\Delta S = \Delta S_{HL} - \Delta S_{ML}$) changes determined from such plots are obviously related to the protonation and complexation values.

In order to obtain the complexation enthalpy, ΔH_{ML}, and entropy, ΔS_{ML}, from the data shown in Figure 5, we fixed the protonation entropy, ΔS_{HL}, at a value of 52 J/(mol K) for all three ligands NOTMP, NO1A-2MP, and NO2A-MP. This approximation seems valid since the literature values[19] for 9N3 (54 J/(mol K), Table 3) and NOTA (50 J/(mol K)) are very close to each other, even though protonation in NOTA is probably assisted by hydrogen bonding. Furthermore, the $T\Delta S_{HL}$ term contributes only 25% to the ΔG_{HL} term at 298 K. ΔH_{HL} values for the methylphosphinate ligands were calculated from the protonation constants (at 298 K) as given in Table 2.

Table 3. Enthalpies, ΔH (in kJ/mol), and Entropies, ΔS (in J/(mol K)), of the Equilibrium $MgL + H^+ \rightleftharpoons Mg^{II} + HL$ for NOTMP, NO1A-2MP, and NO2A-MP As Measured by Temperature Dependent ^{31}P NMR, and Resulting Values for the Protonation and Mg^{II} Complexation As Derived in Combination with Potentiometric Data

L	ΔH	ΔS	ΔH_{HL}	ΔS_{HL}	ΔH_{MgL}	ΔS_{MgL}
NOTMP	−62(3)	−126(8)	−47	(52)[a]	15	178(8)
NO1A-2MP	−62(4)	(−132)[a]	−51	(52)[a]	11	(184)[a]
NO2A-MP	−57(3)	(−132)[a]	−53	(52)[a]	4	(184)[a]
NOTA[b]			−46	50	0	184
9N3[b]			−44	54		

[a] Value estimated from literature values for 9N3 and/or NOTA. [b] Reference 19.

For the NOTMP data shown in Figure 5, no further assumptions were necessary: ΔH_{ML} and ΔS_{ML} were calculated from ΔH and ΔS. The value of ΔS_{ML} of NOTMP (178 ± 8 J/(mol K)) was equal to the literature value for NOTA (184 J/(mol K)) within experimental error. This suggests that the Mg^{II} ion in both complexes is coordinated in a similar manner, very likely with three nitrogens and three side chains. The endothermic enthalpy of Mg(NOTMP) (ΔH_{ML} = 15 kJ/mol) reflects the difference in coordinating strength of the phosphinate group compared to the acetate group in NOTA (ΔH_{ML} = 0 kJ/mol). This contributes to lower Mg^{II}−ligand stability constants for the methylphosphinate family compared to NOTA (see Table 2) and to a (slightly) higher stability constant of Mg(NOTMP)

(19) Martell, A. E.; Smith, R. M.; Motekaitis, R. J. *NIST Critical Stability Constants of Metal Complexes Database, NIST Standard Reference Database 46*; NIST Standard Reference Data, Gaithersburg, MD 20899, 1993.

with an increase in temperature from 25 to 37 °C. However, the largest contributing factor to the decrease in K_{eq} (and K_d) with T comes from the exothermic protonation enthalpy (−47 kJ/mol). Thus, the greater tendency of HL to dissociate at higher temperatures contributes most to the greater overall stability of Mg(NOTMP) at physiological temperature than at room temperature.

For NO1A-2MP and NO2A-MP, the measurements of K_{eq} *versus* temperature were subject to the same problem as in the experiment in which the K_d of Mg(NO2A-MP) at 25 °C was determined (see above): the concentration of the ligand had to be (much) higher than the K_d itself, providing relatively large uncertainties in the $[Mg]_f$ values. This problem was overcome by using another approximation: since the ΔS_{ML} values were the same for Mg(NOTMP) and Mg(NOTA), we fixed the values for Mg(NO1A-2MP) and Mg(NO2A-MP) at 184 J/(mol K). This set the intercept ($-\Delta S$) in Figure 5 for these two ligands at 132 J/(mol K). The values of ΔH were then obtained from a fit of the data presented in Figure 5, while the ΔH_{HL} values were calculated from the protonation constants at 298 K and ΔS_{HL} (see above). Thus, endothermic ΔH_{ML} values of 11 and 4 kJ/mol for NO1A-2MP and NO2A-MP, respectively, were obtained. An overview of the ΔH_{ML} data (Table 3) for the methylphosphinate derivatives and NOTA shows that substituting a methylphosphinate side chain for an acetate group (from NOTA to NOTMP) leads to an endothermic contribution to ΔH_{ML} of about 5 kJ/mol per group.

Performance of NOTMP in Blood Plasma. To investigate the behavior of NOTMP in blood plasma, we performed an *in vitro* titration of (human) blood plasma with $MgCl_2$ at 37 °C while monitoring the sample by ^{31}P NMR. For locking, a small amount of D_2O was added, yielding a sample containing 81% plasma (pH 7.8). At any point of the titration, the Mg^{II} mass balance was described by eq 3. $[Mg]_{ini}$ represents the free Mg^{II}

$$[Mg]_{ini} + [Mg]_{add} = [Mg]_f + [MgL] \quad (3)$$

concentration initially present, while $[Mg]_{add}$ represents the amount of Mg^{II} added during the experiment. It was assumed that the initially bound Mg^{II}, by other components of the plasma, would not be released during the experiment. Therefore, the definition for K_d can be rewritten as eq 4. Plotting $[Mg]_{add} -$

$$[Mg]_{add} - [MgL] = yK_d - [Mg]_{ini} \quad (4)$$

$[MgL]$ *versus* y will give linear plots with K_d as the slope and $-[Mg]_{ini}$ as the intercept. The results for the titration of the blood plasma (pH 7.8) are presented graphically in Figure 6, together with the ^{31}P spectrum of the plasma sample before addition of Mg^{II}.

Figure 6 shows that the K_d (slope) of NOTMP was lower in the blood plasma (0.14 ± 0.01 mM, pH 7.8) than in the aqueous buffer experiments (0.35 ± 0.03 mM at pH 7.4, Table 2). The difference can be completely ascribed to the pH difference in the two experiments. The most remarkable feature of Figure 6, however, is the intercept (−0.66 mM). Since the plasma was diluted to 81% at the beginning of the experiment, this means that $[Mg]_{ini}$ in pure plasma is 0.82 ± 0.02 mM. The same value (0.82 mM) was obtained from the initial sample without added Mg^{II}, by measuring the ^{31}P peak integrals and using a K_d = 0.14 mM. The total Mg^{II} concentration in pure plasma was 1.08 ± 0.01 mM, as measured by atomic absorption spectroscopy. This indicates that the free Mg^{II} concentration in plasma is 76% of the total Mg^{II} concentration, identical to results reported recently using an ion specific electrode.[20] The

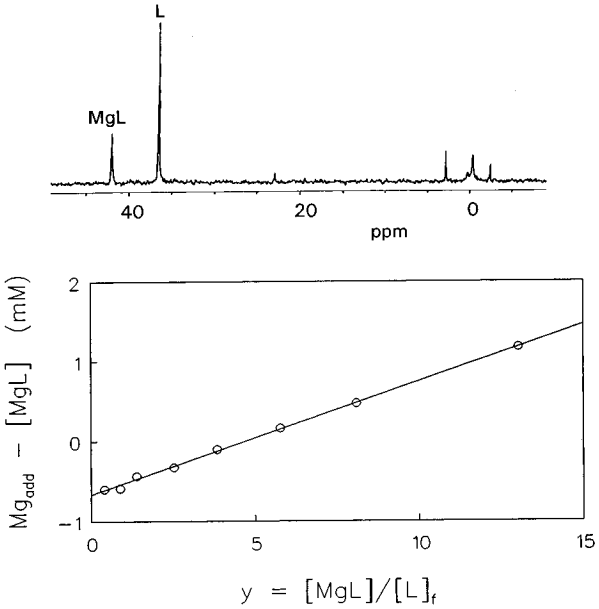


Figure 6. $[Mg]_{add} - [MgL]$ *versus* y (=$[MgL]/[L]_f$) for NOTMP (3 mM) as determined in human blood plasma (pH 7.8) by ^{31}P NMR (202 MHz) at 310 K. The slope represents the K_d value, whereas the intercept gives $-[Mg]_{ini}$. Spectrum: ^{31}P NMR spectrum of the plasma sample before addition of Mg^{II}.

remaining 24% (0.26 mM) must be bound to the proteins present in the plasma with a much higher affinity than that of NOTMP. Simulation of a 1:1 binding model for Mg^{II} plus protein showed that the K_d values of the protein binding sites must be less than 0.1 mM. The NMR titration results presented in Figure 6 are inconsistent with a large number of weaker Mg^{II}−protein sites.

Conclusions

The main goal of this research was to prepare a Mg^{II} chelator which contained easily monitored ^{31}P and ^{1}H resonances. Switching from ethyl- to methylphosphinate side chains proved to be a significant advantage in that the number of phosphorus-containing side chains could be increased without sacrificing the targeted K_d values of the ligands with Mg^{II}. In fact, the tris(methylphosphinate) derivative NOTMP appeared to have exactly the desired K_d (0.35 mM) at 37 °C and pH 7.4. Not only does this derivative have a 3-fold greater NMR sensitivity compared to NO2A-EP, but its synthesis is much easier and it is more selective for Mg^{II} over Ca^{II}. The latter appears to be a general phenomenon for triazacyclononane derivatives containing fewer acetate side chains. Furthermore, NOTMP shows a much larger chemical shift difference between the ^{31}P resonances of the free ligand and those of the Mg^{II} complex, owing to shielding of the ^{31}P nuclei that takes place upon protonation of a single amine. This is in contrast to the mixed side chain derivatives, in which protonation occurs preferentially at a N atom near an acetate group.

The relative concentrations of free NOTMP and its Mg^{II} complex can be assessed accurately from integration of ^{31}P resonance areas without rigorous experimental conditions, since their NOEs and longitudinal relaxation rates are equal. The effects of pH and temperature on the K_d can be evaluated using the thermodynamic data presented here, allowing an accurate calculation of free Mg^{II} levels under a variety of solution conditions. The pH effects can be easily evaluated using the

(20) Altura, B. T.; Shirey, T. L.; Young, C. C.; Hiti, J.; Dell'Orfano, K.; Handwerker, S. M.; Altura, B. M. *Methods Find. Exp. Clin. Pharmacol.* **1992**, *14*, 297−304.

equation $K_d(pH) = 10^{(7.4-pH)}K_d^{7.4}$, because of the large gap between the first and second protonation steps.

The methyl ^1H resonance of the methylphosphinate group is a sharp doublet *in vitro*, for both the free ligand and the Mg^{II} complex. The chemical shift difference, however, is small (0.07 ppm). For *in vivo* applications, NO1A-2MP might be more useful because its shift separation is somewhat larger (0.11 ppm). Still, ^{31}P decoupling and the use of multiple pulse sequences might be necessary to obtain reliable free Mg^{II} levels using ^1H *in vivo* NMR spectroscopy.

The results presented here suggest that the ligand NOTMP may prove useful for determining free Mg^{II} levels in plasma or for monitoring changes in $[Mg]_f$ in perfusates during ^{31}P NMR studies of perfused organs. For intracellular use, derivatization will be necessary.

Acknowledgment. This research was supported in part by grants from the Robert A. Welch Foundation (AT-584) and the NIH Biotechnology Research Program (P41-RR02584).

Supporting Information Available: Text describing the detailed synthesis of NO1A-2MP and NOTMP, figure showing the ^1H NMR spectrum of NOTMP and Mg(NOTMP), and tables giving the NMR shift data for tBuNO1A and tBu_2NO2A (^1H and ^{13}C), and for NO1A-2MP and NO2A-MP (^1H) (7 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

JA953771P