CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200903281

A Triazacyclononane-Based Bifunctional Phosphinate Ligand for the Preparation of Multimeric ^{68}Ga Tracers for Positron Emission Tomography

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Abstract: For application in positron emission tomography (PET), **PrP9**, a *N*,*N*′,*N*″-trisubstituted triazacyclononane with methyl(2-carboxyethyl)phosphinic acid pendant arms, was developed as $^{68}Ga^{3+}$ complexing agent. The synthesis is short and inexpensive. Ga^{III} and Fe^{III} complexes of **PrP9** were characterized by single-crystal X-ray diffraction. Stepwise protonation constants and thermodynamic stabilities of metal complexes were determined by potentiometry. The Ga^{III} complex possesses a high thermodynamic stability ($\log K_{[GaL]} = 26.24$) and a high degree of kinetic inertness. ^{68}Ga labeling of **PrP9** is possible at ambient temperature and in a wide pH range, also at pH values as low as 1. This means that for the first time, the neat eluate of a TiO_2-based ^{68}Ge/^{68}Ga generator (typically consisting of 0.1 M HCl) can be directly used for labeling purposes. The rate of ^{68}Ga activity incorporation at pH 3.3 and 20 °C is higher than for the established chelators DOTA and NOTA. Tris-amides of **PrP9** with amino acid esters were synthesized to act as models for multimeric peptide conjugates. These conjugates exhibit radiolabeling properties similar to those of unsubstituted **PrP9**.

Keywords: gallium · macrocyclic ligands · potentiometry · radiopharmaceuticals · stability constants

Introduction

Positron emission tomography (PET) is a powerful tool in non-invasive and quantitative medical imaging, augmenting other imaging methods such as computer tomography (CT) or magnetic resonance imaging (MRI). For this technique, emission of positrons by β^+-emitters or, more precisely, the γ-radiation originating from their annihilation with electrons, is detected. At present, PET facilities are still dependent on an on-site cyclotron, since the predominantly used radioisotopes (^{18}F, ^{11}C, ^{13}N, and ^{15}O) are cyclotron-produced and have very short half-lives. This renders PET an expensive imaging technique. Recently, the positron emitter ^{68}Ga has gained more attention as it is now available from commercially distributed radionuclide generators. In such devices, ^{68}Ga is produced by decay of the parent nuclide ^{68}Ge ($T_{1/2} = 271$ d), which is absorbed on a TiO_2 or SnO_2 matrix. ^{68}Ga-based radiopharmaceuticals are thus available independently of an on-site cyclotron and enable medical PET scans at a fraction of the usual cost. It possesses a half-life of $\approx$68 min and is thus compatible with most bio-targeting applications. The topic of utilization of ^{68}Ga in PET has recently been reviewed.[1] In addition, there are two more cyclotron-produced gallium isotopes suitable for nuclear imaging: 1) ^{67}Ga is a γ-emitter ($T_{1/2} = 78.3$ h) used in single-photon emission computed tomography (SPECT) and therapy; 2) β^+-emitting ^{66}Ga with an intermediate half-life of

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.200903281.

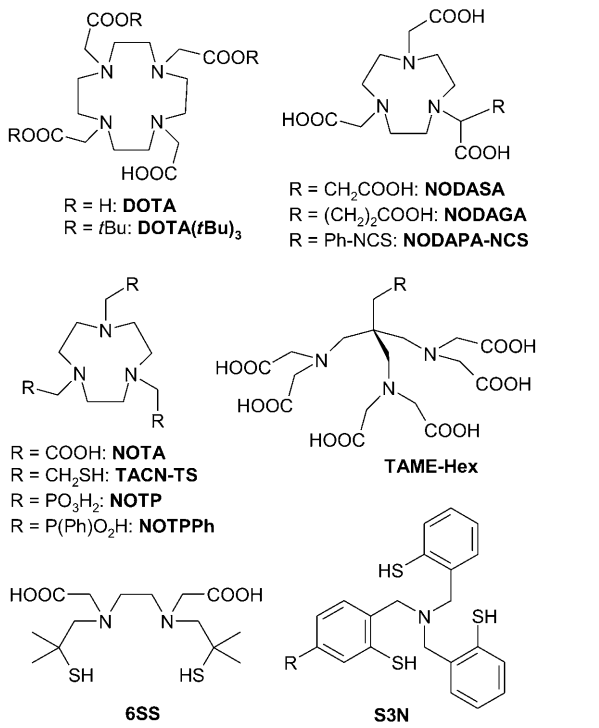
9.4 h did not receive much attention in the past,[2] but the interest in this radioisotope has revived recently.[3]

In aqueous solutions, gallium is stable only as a trivalent cation. It cannot be incorporated into the structure of targeting vectors by covalent bonding, but must be complexed by a ligand that is conjugated to the biological vector. The Ga^{3+} ion possesses a d^{10} electron configuration and accepts different coordination numbers (usually 4–6), while not displaying preference for any particular coordination polyhedron. At $pH > 4$, formation of colloidal hydroxide $[Ga(OH)_3]_n$ commences. Although this does not generally inhibit complex formation, radiolabeling is nevertheless substantially hampered due to formation of insoluble colloids (particularly at high activities) and their adhesion to the surface of the reaction vessel. At pH values above 8, a water-soluble hydroxo complex, $[Ga(OH)_4]^-$, is formed. As ligand exchange with the tetrahydroxo complex is a much slower process than complexation of free Ga^{3+}, complexation is achieved best at $pH < 4$.

Ligands for ^{68}Ga-based PET radiopharmaceuticals should ideally combine the following set of properties.

1) Stability: Ga^{III} complexes should be as stable as possible; a kinetic inertness of the complex is more important than high thermodynamic stability.
2) Quick complexation under radiochemical conditions: Formation of Ga^{III} complexes should be fast at low temperatures, low concentration, and minimal excess of the ligand. A desirable ligand will chelate Ga^{3+} in solutions of nanomolar concentration at room temperature within minutes.
3) Selectivity: The ligand should ideally be selective for Ga^{3+} ion. Particularly, complexation of serum metals like Ca^{2+}, Mg^{2+}, and Zn^{2+} ions (the last being produced by decay of ^{68}Ga) should be disfavored in order to avoid transmetallation in vivo or diminishing of radiochemical yield.
4) Conjugation ability: The chelating unit has to possess a functional group which allows covalent binding to the targeting vector (biomolecule) without a significant derogation of complexation performance.
5) Long shelf life: In medical applications, excellent chemical stability is necessary.
6) Accessibility: Preparation of the compound in practical amounts should be quick, facile, and inexpensive.

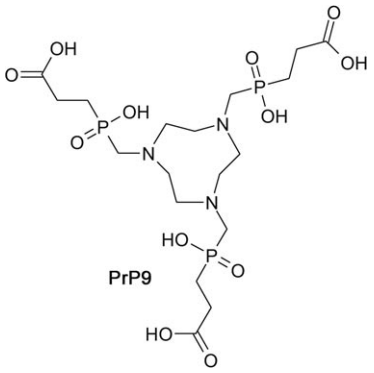
A choice of ligands (some of them representing a family of compounds) which have been proposed or have already been used for application in ^{68}Ga radiopharmaceuticals is depicted here (see references [8,12,18,19,21,23,27,58–64]). All of them have advantages and drawbacks. Aza-macrocycle based ligands generally form complexes with increased thermodynamic and, more importantly, kinetic stability compared to open-chain or tripod ligands.[4,5] However, in case of the first group, a considerable barrier has to be overcome in order to place the metal ion into the ligand cavity, thus causing slow complexation kinetics. The open-chain ligands

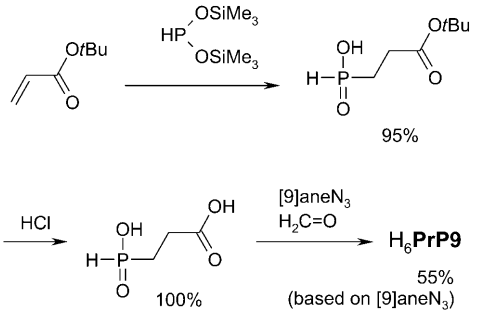


are less rigid and do not have a cavity of a particular size, which is why they are less selective for particular ions, but show faster complex formation.[6,7] Despite of the amazing thermodynamic stabilities of the resulting complexes,[8] ligands containing thiol groups are disfavored because of their tendency to degrade, albeit slowly, by oxidation. Also, the comparably low acidity of sulfhydryl groups hampers complex formation in acidic media, and derivatives suitable for bioconjugation are difficult to synthesize.[9]

DOTA derivatives are currently the working horses of bioconjugational chemistry related to medical imaging, including ^{68}Ga applications.[10,11] This is mainly due to the commercial availability of ready-to-use mono-unprotected precursors like DOTA(tBu)$_3$,[12] which, in turn, is rooted in the fact that lanthanide(III) complexes of DOTA derivatives and its conjugates have been extensively used as MRI contrast agents[13–16] and radiotherapeutics.[17] However, NOTA-like ligands show a much better selectivity towards Ga^{3+} ion and their complexes are more stable, as the size of the NOTA cavity is almost ideal for this ion.[18–20] Conjugable NOTA derivatives like NODASA,[21] NODAGA,[22] NODAPA-NCS[23] as well as other thiocyanato-equipped NOTAs[24,25] are thus much better suited for the synthesis of ^{68}Ga radiopharmaceuticals.

With all these considerations in mind, we devised a ligand structure that combines the advantages of the different structural motifs. The novel chelator **PrP9** (shown here) is structurally related to some known [9]aneN$_3$ derivatives.[26–29] Unlike the above-mentioned ligands, it contains phosphinates instead of carboxylates as primary coordination sites. Due to the lower pK_a value of phosphinic acids (mostly <1) compared to aliphatic carboxylic acids (typically ≈ 4),[30]





Scheme 1. Synthesis of the hexaprotonated (zwitterionic) form of the ligand **PrP9**.

metal-ion complexation should be possible at much lower pH values than in case of NOTA and DOTA, thereby expanding the pH range of application. Furthermore, the distant carboxylates are supposed to act as "pre-coordination" sites: at very low concentrations typical for radiochemistry, fast, open-chain-like interactions with metal ions are supposed to help to increase the effective metal concentration close to the ligand cavity and therefore increase complexation rate. The structure is thus expected to combine advantages of both macrocycle-based chelators (stability and selectivity) and open-chain ligands (fast complex formation). In addition, phosphinic acid and carboxylic groups exhibit different reactivity, which can be utilized to functionalize the carboxylates without having to protect the phosphinate moieties. The availability of three carboxylic acid moieties for conjugation furthermore paves the way towards multimeric tracers.

Results and Discussion

Throughout this paper the abbreviation **PrP9** (systematic name of **PrP9** is 1,4,7-triazacyclononane-1,4,7-tris[methyl(2-carboxyethyl)phosphinic acid]) is used regardless of protonation state, except in cases in which the distinction is necessary for comprehension.

Ligand synthesis: The preparation protocol for **PrP9** is extremely short; there are just three steps starting from stock chemicals and only the last reaction involves the azamacrocycle. All steps have satisfying to excellent yields. The complete synthesis is outlined in Scheme 1 and can be carried out in less than three days. All required materials are commercially available and, apart from the amine [9]aneN$_3$, they are also inexpensive. In addition, the synthesis requires very little workup effort. One extraction after the first step and just a simple ion exchange chromatography after the third step, followed by recrystallization, are necessary in order to obtain a very pure product. The procedure can be scaled up without problems, as has been proven by preparing 15 g of **PrP9** in a single batch. For these reasons, we hold the view that **PrP9** has good prospects in providing a basis for the synthesis of bioconjugated chelators.

Complex synthesis and solid-state structures: Although **PrP9** forms complexes with a variety of metal ions, precipitates suitable for X-ray diffraction and analysis could only be obtained for [Ga(H$_3$**PrP9**)] and [Fe(H$_3$**PrP9**)]. Two phases were obtained for each, differing only in the amount of water of crystallization. From acetone/water mixtures, the complexes crystallized as dihydrate and monohydrate, respectively, whereas a simple evaporation of an aqueous solution afforded isostructural hexahydrates. In both compounds the chelate cages in the two phases are quite similar and just one example is depicted (Figure 1; for the Fe^{III} complex see

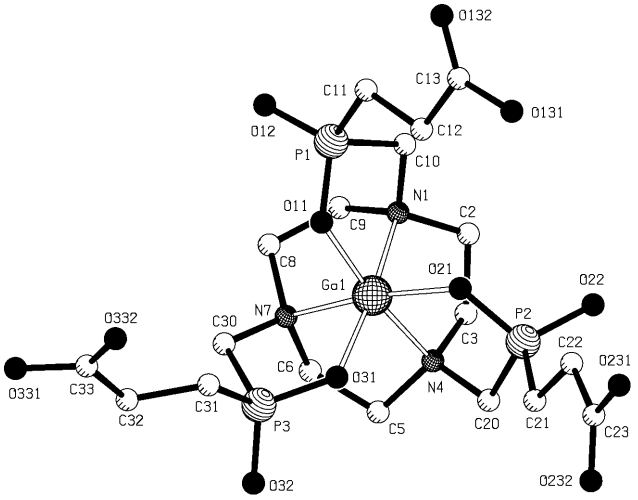


Figure 1. Molecular structure of the [Ga(H$_3$**PrP9**)] complex in [Ga(H$_3$**PrP9**)]·6H$_2$O (hydrogen atoms are omitted for clarity).

Supporting Information, Figure S1). Coordination bond lengths and angles of the two Ga^{III} structures are almost identical, whereas the two Fe^{III} phases exhibit small structural differences (see Table 1). The parameters of the [Ga(H$_3$**PrP9**)] structures are similar to other hexacoordinated Ga^{III} complexes.[31] The lattices contain enantiomeric Λδδδ-*RRR*/Δλλλ-*SSS* [Ga(H$_3$**PrP9**)] complex units with the metal in a trigonal-antiprismatic coordination sphere and the propionic acid moieties being located above the complex cage. An identical structural arrangement has been found in the

Table 1. Selected parameters of the complex moieties in [Fe(H$_3$**PrP9**)]·nH$_2$O and [Ga(H$_3$**PrP9**)]·nH$_2$O in the solid state [distances given in Å, angles in °].

	[Fe(H$_3$**PrP9**)]·nH$_2$O		[Ga(H$_3$**PrP9**)]·nH$_2$O	
	$n=1$	$n=6$	$n=2$	$n=6$
M–N1	2.210(2)	2.178(2)	2.120(2)	2.108(1)
M–N4	2.232(2)	2.198(2)	2.124(2)	2.128(1)
M–N7	2.189(2)	2.178(2)	2.125(2)	2.106(1)
M–O11	1.927(1)	1.948(1)	1.926(1)	1.937(1)
M–O21	1.932(1)	1.947(1)	1.924(1)	1.931(1)
M–O31	1.934(1)	1.946(1)	1.929(1)	1.932(1)
N1-M-O31	162.27(5)	164.54(6)	169.64(6)	170.07(5)
N4-M-O11	162.19(5)	164.73(6)	169.63(6)	169.88(5)
N7-M-O21	164.31(5)	165.41(6)	169.95(6)	170.21(4)
N1-NQ-OQ-O11[a,b]	48.41	49.91	52.08	51.82
N4-NQ-OQ-O21[a,b]	47.79	49.44	51.90	51.55
N7-NQ-OQ-O31[a,b]	51.01	51.37	52.78	53.16
NQ–OQ[a]	2.3929	2.3966	2.3722	2.3788
M–NQ[a]	1.487(2)	1.4540(2)	1.3594(2)	1.3506(2)
M–OQ[a]	0.9063(2)	0.9428(2)	1.0128(2)	1.0284(2)
N$_3$/O$_3$ planes ∡	3.57(8)	1.63(6)	0.51(3)	1.22(5)

[a] NQ and OQ are the barycenters of the N$_3$ and O$_3$ planes, respectively. [b] Torsion angle.

[Ga(NOTPPh)] complex.[32] A similar combination of chelate-ring conformation and pendant-arm helicity has been found in the structurally related compounds [Ga(NOTA)][19] and [Ga(NODASA)][21] (Table 2), as well as in other Ga^{III} complexes with six-membered pendant arm chelate rings.[33,34] Interestingly, a different situation is encountered for Fe^{III} complexes: for phosphorus-containing ligands, pendant arm helicity is opposite to macrocycle chelate rings (Λδδδ/Δλλλ, similarly as found in the Ga structures); for ligands with carboxylate pendant arms it is the same (Λλλλ/Δδδδ; for more information see the Supporting Information).

Table 2. Comparison of some structural parameters of [Ga(H$_3$**PrP9**)] with analogous complexes [distances given in Å, angles in °].

	N-Ga-O[a]	Torsion ∡[b]	OQ-NQ[c]	Ga-OQ[c]	Ga-NQ[c]
[Ga(H$_3$**PrP9**)][d]	169.9	52.2	2.376	1.355	1.021
[Ga(NOTPPh)][32]	168.3	52.1	2.370	1.391	0.979
[Ga(NOTA)][20]	167.4	47.6	2.329	1.318	1.012
[Ga(H-NODASA)][21]	165.5	44.4	2.363	1.333	1.030

[a] Average of values for opposing N and O atoms. [b] For definition of torsion angle see Table 1. [c] NQ and OQ are the barycenters of the N$_3$ and O$_3$ planes, respectively. [d] Average values of two structures.

Comparison of average "diagonal" N-Ga-O and torsion angles of structurally related Ga^{III} complexes (Table 2) reveals that the values of those derived from phosphinate ligands, and particularly of [Ga(H$_3$**PrP9**)], are more close to those of an ideal octahedron (180° and 60°, respectively). Also, the N$_3$ and O$_3$ planes are slightly more rotated. In all structures regarded, gallium is located closer to the N$_3$ than to the O$_3$ plane; the difference is most pronounced in the structure of the phenylphosphinate complex [Ga(NOTPPh)]. Comparing all the parameters characterizing the ligand cavity, it can be stated that, among these ligands, **PrP9** fits best for Ga^{III}.

The Fe–O and Ga–O distances in [Ga(H$_3$**PrP9)]** and (Fe(H$_3$**PrP9**)] (see Table 1) are almost identical, but Fe–N bonds are significantly longer than Ga–N linkages. Also, the coordination environment of Fe^{III} is more distorted, the average N-M-O and the torsion angles of the Ga^{III} complexes being closer to the ideal values. This means that the cavity size of **PrP9** is slightly less suitable for Fe^{III} (ionic radius 0.69 Å) than for Ga^{III} (ionic radius 0.76 Å).

Solution structure of [Ga(PrP9)]: In both ^{31}P and ^{71}Ga NMR spectra, single resonances were found (42.4 and ≈135 ppm, respectively). The ^{31}P NMR thus proves that solid-state and solution structures are identical, that is, only one non-fluxional pair of enantiomers Λδδδ-*RRR*/Δλλλ-*SSS* is present. The half-width of the Ga resonance ($\nu_{1/2}$ ≈400 Hz) is larger than in case of [Ga(NOTA)] (210 Hz)[19] and more close to the values found for complexes of related phosphorus containing ligands, the methylenephosphonate analogue [Ga(NOTP)]$^{3-}$ (430 Hz)[35] or methylene(phenyl)-phosphinate analogue [Ga(NOTPPh)] (560 Hz).[32]

In the ^{1}H NMR spectra, only two sets of overlapping triplets at 2.16 and 2.76 ppm, originating from protons of the propionate side arms, can be unambiguously assigned. The resonances of the remaining protons form a set of overlapping multiplets, indicating a rigid structure of the whole complex, which appears to be conformationally stable as ^{1}H NMR spectra measured at temperatures of 20–80 °C indicate a high rigidity of the ligand skeleton. Up to 60 °C, the splitting pattern of the ring protons and the nitrogen-bound side-arm protons (N-CH$_2$-P) remains unchanged; some signal coalescence starts at 80 °C. This is in accordance with the respective observations made for [Ga(NOTA)], [Ga(NOTPPh)], and [Ga(NOTP)]$^{3-}$ complexes.[19,32,35]

In the ^{13}C NMR spectrum, the signals of the ring carbons (appearing as a single resonance in the solution of the free ligand) are split into two separate signals, one of them showing the C,P coupling (11 Hz, Figure S4). This further corroborates the conclusions drawn from ^{31}P NMR spectra. No coalescence of these signals was observed at temperatures up to 90 °C. Hence, the lower limit for the interconversion Gibbs free energy between the two enantiomers can be approximately assessed to $\Delta G^{int} > 100$ kJ mol^{-1}.

Thermodynamic and mechanistic studies: Potentiometry and NMR techniques were employed for determination of protonation constants and complex stabilities as well as for investigation of the mechanism of complex formation.

The stepwise protonation constants of the ligand (Figure 2) are $\log K_a = 11.48$, 5.44, 4.84, 4.23, 3.45, and 1.66. The protonation sequence can be estimated by a comparison

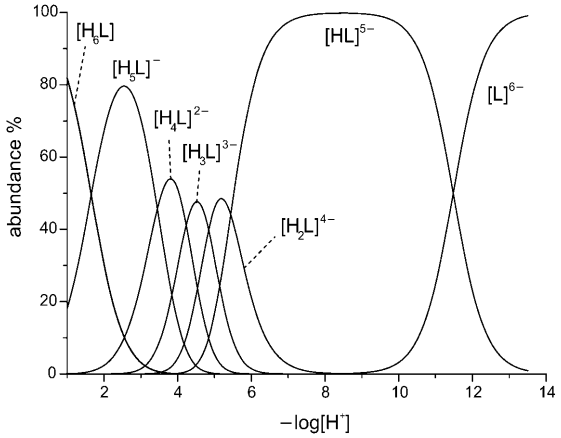


Figure 2. Distribution diagram for the protonation of **PrP9**.

of these data with those for similar ligands (see also Supporting Information, Table S2).[27,28,36] The first two protonations should occur on the ring nitrogen atoms, followed by three protonations on the side-arm carboxylates. The other protons are most likely bound on the remaining ring nitrogen atom and/or phosphinate groups. Thus, not all phosphinate groups are protonated, even in solutions with a very low pH (<1) and therefore are still able to complex Ga^{3+} ion. Basicity of the ring nitrogen atoms is somewhat lower than in case of NOTA,[37] but higher than for triazacyclononane-derived methylphosphinic acid ligands,[27,28] presumably due to higher overall negative charges of the **PrP9** anions. Basicity of the distant carboxylate groups is in the usual range for carboxylic acids.[38,39]

The stability constants were determined for complexes of **PrP9** with several biologically important metal ions as well as some lanthanides. For Y^{3+} and Lu^{3+} ions, the measurements were not possible, as insoluble precipitates were obtained, most likely due to formation of complex polymers containing protonated ligand molecules. The data given in Table 3 clearly show the preference of **PrP9** for the Ga^{3+} ion. Complexes with Cu^{2+} and Zn^{2+} are about ten, and those with Mg^{2+} and Ca^{2+} approximately 20 orders of magnitude less stable. Furthermore, stability constant values for the related metal-ion pairs Mg^{2+}/Ca^{2+} and La^{3+}/Gd^{3+} illustrate the ligand's selectivity for small ions, as for both couples the stability constant of the larger ion is about two orders of magnitude lower. Similar selectivities have been observed for NOTA[37,40] and its phosphinate analogues.[27,28] (For a comparison of stability constants and more distribution diagrams see Supporting Information; Table S4, Figures S2 and S3). Also, **PrP9** fits into the correlation of overall basicity of these ligands with the stability constants of their Cu^{II} and Zn^{II} complexes.[30]

The distribution diagram (Figure 3) shows that the 1:1 complex is triply negatively charged under physiological pH. In solutions containing two equivalents of Ga^{3+}, the primary complex appears to be able to bind an additional Ga^{3+} ion, most likely by weak interaction with the side-arm carboxylates and/or the phosphoryl oxygen atoms (that is, in the form of an "out-of-cage" complex). This shows the ability of the carboxylates to direct Ga^{3+} ions towards the ligand cavity, which we believe is the main cause for the unusually fast complex formation.

From Figure 3 it is furthermore apparent that the Ga^{III} complex is fully formed, even at the beginning of titration, in the form of a fourfold protonated species, $[Ga(H_4\mathbf{PrP9})]^+$, the corresponding fourth protonation constant being $\log K_a \approx 0.7$ (Table 3). Hence, the $K_{[GaL]}$ value obtained from potentiometry is determined by the competitive ligand exchange reaction with an hydroxide anion, which, in order to represent the complexes' actual thermodynamic stability, must be in thermodynamic equilibrium. This reaction, however, turned out to proceed surprisingly slowly and this had to be taken into account in order to obtain correct results. During the normal "in-cell" titration, we observed no precipitate of $Ga(OH)_3$ and pH readout quickly stabilized over the whole pH range of measurement (1.5–12). From the data thus collected, a stability constant of $\log K_{[GaL]} = 35.65$ was calculated. However, this value appeared unrealistically high compared to the one of the [Ga(NOTA)] complex ($\log K_{[GaL]} = 31.0$),[18] since **PrP9** exhibits a lower overall basicity than NOTA. When the reaction was given a timespan of about four weeks in order to reach equilibrium ("out-of-cell" titration), we obtained a more realistic value of $\log K_{[GaL]} = 26.24$, which is, as expected, lower than that of [Ga(NOTA)]. Even at pH 11, the reaction half-life was ≈ 60 h (see Supporting Information, Figure S7); finally, at pH 13 the reaction was completed within minutes. A possible explanation is the high negative charge of the $[Ga(\mathbf{PrP9})]^{3-}$ ion, which could be hampering the approach of OH^- to the central

Table 3. Stability constants [$\log K$] and stepwise protonation constants [$\log K_A$] of complexes of **PrP9** with selected metal ions.

	Ga^{3+}	Cu^{2+}	Zn^{2+}	Mg^{2+}	Ca^{2+}	La^{3+}	Gd^{3+}
$L+M \rightleftharpoons LM$	**26.24**	**16.85**	**16.88**	**7.84**	**6.04**	**11.26**	**13.46**
$LM+H \rightleftharpoons HLM$	5.2	5.14	5.17	6.49	7.94	6.22	4.80
$HLM+H \rightleftharpoons H_2LM$	4.5	4.66	4.68	5.00	4.98	5.00	4.80
$H_2LM+H \rightleftharpoons H_3LM$	3.8	3.95	3.96	4.74	4.50	3.95	3.78
$H_3LM+H \rightleftharpoons H_4LM$	0.7	1.33		4.29		3.32	3.38
$LM(OH)+H \rightleftharpoons LM(H_2O)$	9.9	12.24	12.63		13.04	11.32	10.42
$LM(OH)_2+H \rightleftharpoons LM(OH)(H_2O)$						12.30	11.23
$LM+M \rightleftharpoons LM_2$	7.3	3.32	2.43	2.95	2.80		
$LM_2+H \rightleftharpoons HLM_2$		4.79	4.71	5.9			
$LM_2(OH)+H \rightleftharpoons LM_2(H_2O)$	2.5			8.5	12.8		

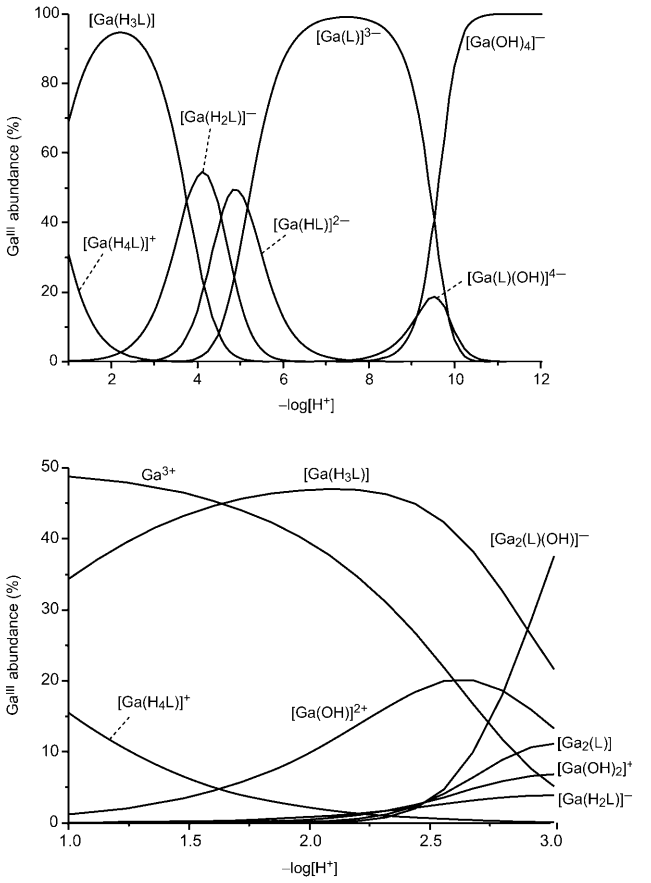


Figure 3. Distribution diagrams for the Ga^{III}/**PrP9** systems (ligand-to-metal-ratio: upper: L/M = 1:1, lower: L/M = 1:2; c_L = 4 mM).

ion.[18] This shows that neglecting the kinetics of the ligand–hydroxide competition for the gallium(III) ion can easily lead to erroneous values for thermodynamic stabilities; such kinetic inertness may give a false impression of a higher stability constant. For polydentate/rigid ligands, equilibration time generally should be checked carefully, even in the alkaline pH region at which, until now, equilibriums have been supposed to establish quickly. The carboxylate protonation constants for the Ga^{III} complex obtained by both "non-equilibrium" ($\log K_a$ = 5.14, 4.54, and 3.65) and "out-of-cell" titrations were identical (Table 3). This confirms a fast and quantitative complexation of Ga^{3+} ions even at pH ≈ 1.5, which was also observed during NMR measurements and under radiochemical conditions (see below).

In addition, we like to draw attention to another aspect of the observed kinetics. Although at physiological pH hydroxide anions will compete with the ligand in the [Ga(**PrP9**)] complex, this process is very slow compared to the ^{68}Ga half-life of 68 min. In practice, decay limits the lifetime of any ^{68}Ga-labeled compound to a couple of hours. For applications in nuclear medicine, the high "non-equilibrium" stability constant value of $\log K_{[GaL]} \approx 35$ that has been measured in a comparable timeframe is therefore more decisive than the true long-term thermodynamic stability, since equilibration requires weeks which renders it irrelevant in terms of ^{68}Ga radiochemistry.

The complexation mechanism was also investigated by ^{31}P and ^{71}Ga NMR spectroscopy. Between pH 1.5 and 8, quantitative complexation occurs immediately after mixing equimolar amounts of Ga^{3+} ions and **PrP9** without any intermediates being detectable. However, at initial pH values of 1.3, 1.0, and 0.8, quantitative complexation requires 8, 65, and 90 min, respectively (Figure 4 and Figures S6 and S7 in

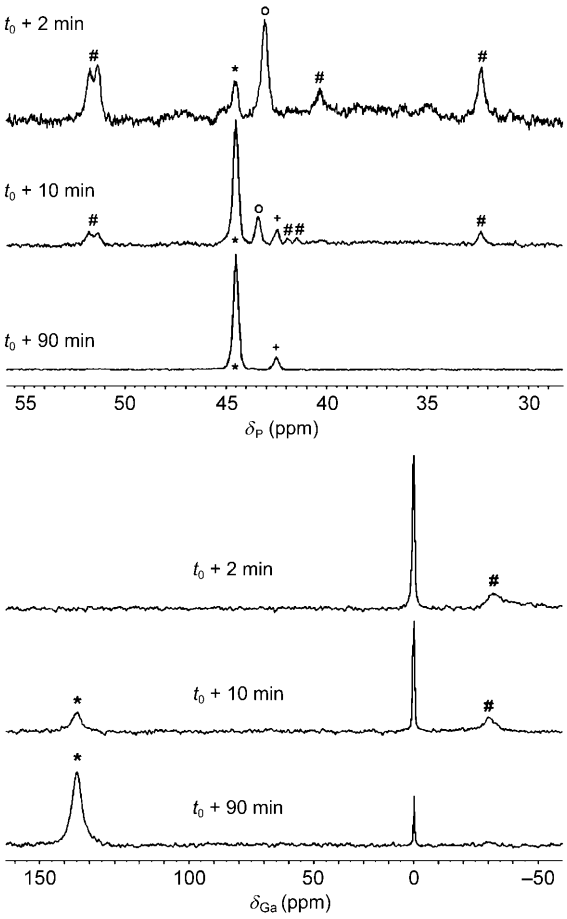


Figure 4. Complexation of Ga^{3+} with **PrP9** as followed by ^{31}P (top) and ^{71}Ga NMR (bottom) (M/L = 1, pH 0.8, 25 °C). Peak labeling: *: final ("in cage") complex, #: intermediates, ○: free ligand. In the ^{31}P NMR spectra, the small peak at ≈42.5 ppm (labeled +) probably corresponds to a diastereomeric complex.

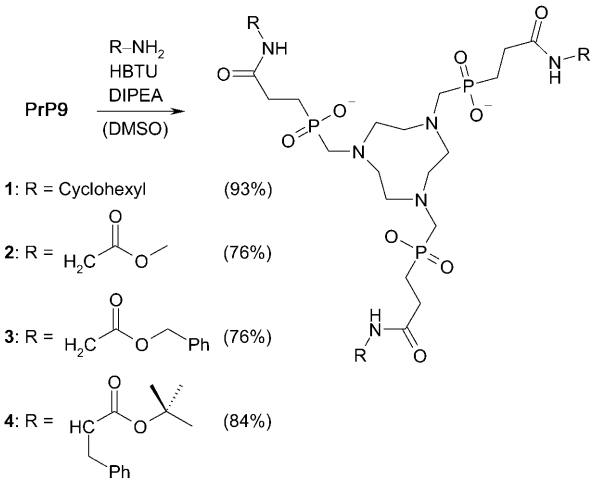
the Supporting Information). Thus, additional NMR signals could be observed, presumably corresponding to "out-of-cage" complexes. The chemical shift of the ^{71}Ga NMR signal at ≈ −30 ppm belongs to some species possessing a rather symmetric O_6 coordination environment that is very probably formed from phosphinate and carboxylate oxygen atoms (Figure 4). The transient ^{31}P and ^{71}Ga NMR peaks should correspond to the "out-of-cage" complexes as interconversion of isomeric "in-cage" octahedral complexes is energetically demanding and thus does not occur at room temperature. The ^{31}P NMR peak at ≈42.5 ppm was observed in all

final reaction mixtures and can be assigned to a minor diastereomer formed during the process of complex formation.

Moreover, complexation could be observed even under extremely acidic conditions, that is, in 1, 2, or 5 M hydrochloric acid. In 1 M HCl, complete extinction of the $[Ga(H_2O)_6]^{3+}$ signal in the ^{71}Ga NMR spectrum requires about 12 d, whereas in 2 M HCl solution an equilibrium was reached within 30 d (in which approximately 30% of "free" gallium is present). Finally, in 5 M HCl the ^{71}Ga resonance of the "in-cage" complex could not be detected after a period of 30 d. However, a ^{31}P NMR resonance of $\delta \approx 40$ ppm and some other signals appeared, indicating that some Ga^{3+}–ligand interactions (probably formation of the "out-of-cage" complex) occurs even in extremely acidic media.

When a sample of [Ga(**PrP9**)] was dissolved in 6 M $HClO_4$ at 25 °C, no decomplexation was observed over a period of seven months. A comparable degree of stability has been observed for [Ga(NOTA)] in aqueous HNO_3 (pH −0.7).[19] Hence we conclude that even under harsh conditions, protons are not able to effectively compete with Ga^{3+} ion for the ligand's basic sites (the ring nitrogen atoms), which renders the complex extremely inert. As observed by potentiometry, the "in-cage" complex, once formed, is protonated on the phosphinate phosphoryl oxygen atom(s) instead, resulting in $[Ga(H_4L)]^+$ (Figure 3) or even multiply protonated species.

Conjugation: Clearly, the carboxylate moieties of **PrP9** are predestined to act as conjugation sites for biomolecules. However, the scope of this study is mainly to deliver proof of principle, rather than the development of actual PET tracers. At this stage, we only intended to assess the general feasibility of such derivatization as well as the radiolabeling properties of such conjugates. Therefore, triamides of **PrP9** with cyclohexylamine and amino acid esters were prepared as model compounds for conjugates with for example, (oligo)peptides (Scheme 2). From some common peptide coupling agents investigated, uronium reagents like HBTU (2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and TBTU (2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) gave the best results. Five equivalents of amine and about eight equivalents of coupling agent were found to be sufficient for complete derivatization. Subsequently the triamides **1–4** could be purified very conveniently by diafiltration through a membrane with 0.5 kDa cutoff. The compounds, obtained as the Na^+ salts, were easily soluble in water.



Scheme 2. Functionalization of **PrP9** with amines, leading to conjugates **1–4**.

Concerning derivatization, the most significant advantage of **PrP9** is that protection of the phosphinate moieties during the coupling reaction was found to be unnecessary. Hence, no final deprotection step following conjugation has to be taken into account, which simplifies conjugation protocols. On the one hand, this could render further protection of certain targeting vectors unnecessary; on the other hand, utilization of a vector that does not require any protection at all offers the possibility of a single-step synthesis of a desired bioconjugate. Moreover, due to the availability of three equal conjugation sites, the access to multimeric tracers is provided in a very convenient way.

^{68}Ga labeling: Labeling of the unsubstituted ligand **PrP9** with ^{68}Ga was performed by employing various temperatures and pH values, according to an established protocol reported earlier.[41,42] It allows for a very precise adjustment of pH and temperature and is therefore well suited for the investigation of labeling properties. Figure 5 shows that at pH 3, nearly complete (>95%) incorporation of activity is achieved almost instantaneously at 60 °C and above. Even at 40 °C and room temperature, decrease in labeling performance is just marginal, requiring three instead of one minute to reach a >95% plateau. Variation of pH at a constant temperature of 60 °C revealed that instantaneous labeling occurs for pH values from 3–5, whereas for complete radioactivity incorporation at lower pH, slightly longer reaction times are required. Moreover, for the derivatives **1–4** an almost complete incorporation of activity occurs nearly as rapidly, as in the case of the unsubstituted compound (see Supporting Information, Figure S8). Hence we conclude that functionalization of the side arms does not substantially affect complex formation; this result is somewhat surprising but nevertheless quite satisfying.

The most striking feature of **PrP9** and its derivatives is their ability to incorporate $^{68}Ga^{3+}$ at pH values as low as 1, which has two major advantages. Firstly, at a pH below 3 there is no perturbing formation of the colloidal hydroxide and all activity is available for radiolabeling in form of free $[^{68}Ga(H_2O)_6]^{3+}$. The second aspect is more a practical one: The very popular commercially available TiO_2-based $^{68}Ge/^{68}Ga$ generator systems are commonly eluted with 0.1 M HCl, the eluate thus having pH of ≈1. None of the common chelators used for ^{68}Ga radiopharmaceuticals can be sufficiently labeled directly with this eluate. Hence, processing of the eluate[41] or addition of buffers has been mandatory, whereby the latter do not play an innocent role in terms of

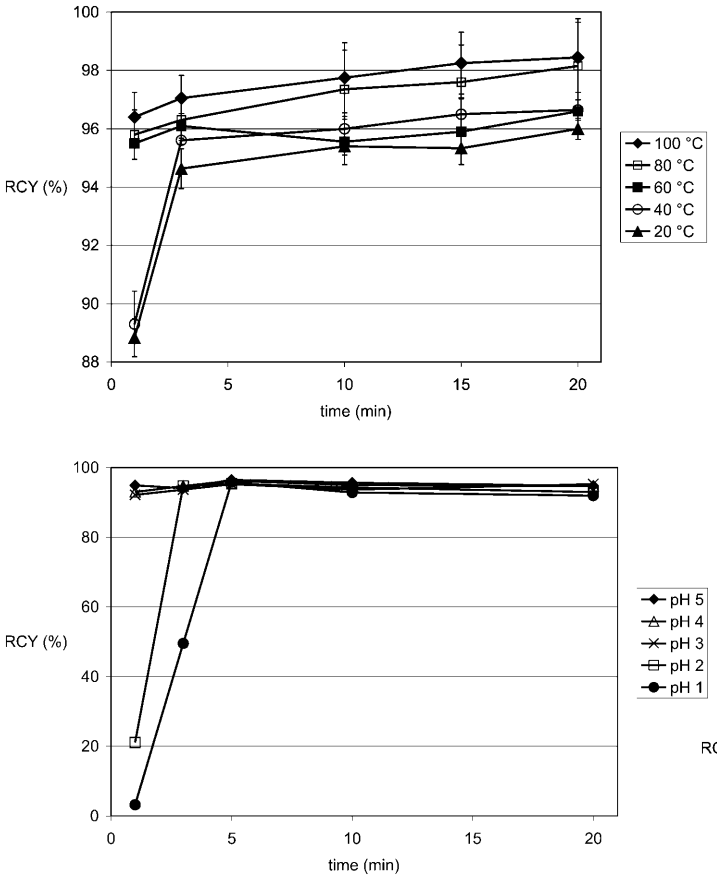


Figure 5. Radiochemical yield (RCY; ^{68}Ga incorporation) for labeling of **PrP9** (14 nmol in 5 mL buffered water, 120–160 MBq) upper: pH 3, 20–100 °C; lower: pH 1–5, 60 °C.

chemical interactions during the labeling process, as well as with respect to legal and regulatory issues in radiopharmaceutical production. We therefore exemplarily performed labeling of the benzylglycine conjugate **3** using the neat eluate from a TiO_2-based ^{68}Ga generator (Cyclotron Co., Obninsk, Russia). Figure 6 illustrates that although radioactivity incorporation occurs significantly slower than at pH 3, a radiochemical incorporation of >95% after 10 min is achieved by using 14 nmol of the ligand, an amount which is in the usual range for clinical production of ^{68}Ga tracers.[42,43] This shows the feasibility of such labeling under conditions common in clinical practice.

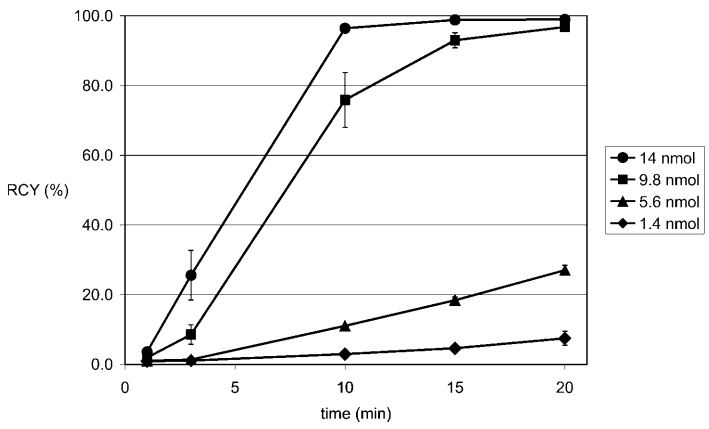


Figure 6. Radiochemical yield (^{68}Ga incorporation) for labeling of **3** (60 MBq in 400 μL, 60 °C, pH 1, different molar amounts of **3**).

We also performed a direct comparison of **PrP9** with NOTA and DOTA, the most common chelating units in ^{68}Ga chemistry. Here, we employed another commonly used labeling method.[10] HEPES buffer was used to adjust the pH of the eluate from a SnO_2-based generator (from iThemba LABS, South Africa, eluted with 1 M HCl) to a value of 3.3. We chose this pH in order to ensure a fair and realistic comparison of the ligands, since it has been found to be optimal for both DOTA-[43] and NOTA-conjugated peptides.[44] The results of the labeling at ambient temperature (≈20 °C), depicted in Figure 7, show that **PrP9** exhibits superior performance than DOTA and is also ahead of NOTA, although the velocity of radioactivity incorporation of the latter is in a comparable range. It is evident that at lower pH, the advance of **PrP9** will increase because both competitors exhibit drastically reduced labeling yields in more acidic media.[43,44]

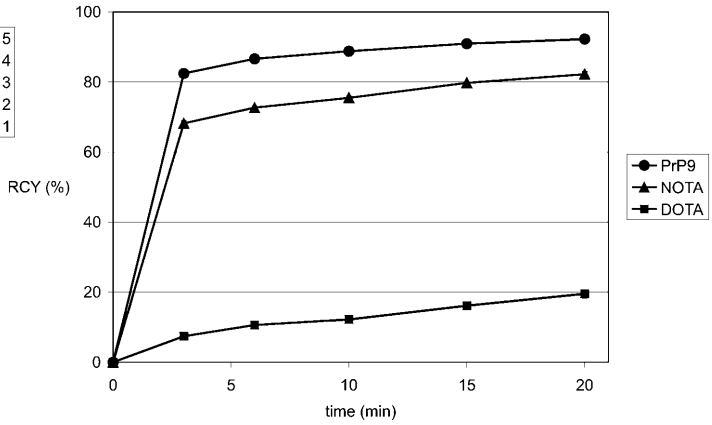


Figure 7. Radiochemical yield (^{68}Ga incorporation) for labeling of **PrP9**, NOTA, and DOTA (10 nmol of ligand, 60 MBq in 110 μL HEPES-buffered solution, pH 3.3, 20 °C). Errors were too small to be displayed at this scale.

Conclusion

Desirable properties of Ga^{III} chelators for application in ^{68}Ga-based nuclear imaging include high complex stability, fast and selective complex formation, ability to be conjugated, long shelf life, and accessibility. Our data show that the novel ligand system **PrP9** introduced herein does fulfill all of these requirements.

Complex formation kinetics under common chemical as well as under radiochemical conditions is exceptionally fast, being superior to the established ligand systems DOTA and

NOTA. This is owed to the assumed complexation mechanism: initially, the Ga^{3+} ion is coordinated very quickly by the pendant carboxylates (open-chain-like interaction, leading to "out-of-cage" complexes). The ion is thereby brought into direct vicinity of the main chelation site, thus accelerating transfer to the cage by increasing the effective concentration close to the macrocyclic cavity. Moreover, decomplexation under very acidic as well as under neutral and moderately alkaline conditions is extremely slow. This is of utmost importance as problems caused by dissociation/transmetallation in biosystems can therefore be ruled out, and in ^{68}Ga radioimaging, the risk of uncontrolled distribution of $^{68}Ga^{3+}$ activity is eliminated. The fast complex formation allows for milder labeling conditions (e.g., at ambient temperature), thereby expanding the scope of ^{68}Ga radiolabeling towards targeting vectors with low thermal stability (as for example, antibodies or their fragments).

Another useful effect of the complexation mechanism is that formation of colloidal hydroxide is prevented; no formation of precipitates was observed during complexation reactions over the entire pH range. Hence, the ligand can be considered suitable for ^{68}Ga radiolabeling even at pH$\approx$5 and above. This expansion of applicable labeling pH range, together with straightforward labeling at room temperature, enables its use in radiotracers featuring more sensitive targeting vectors (e.g., antibodies or their fragments). Furthermore, the Ga^{III} complex is quantitatively formed even below pH 1. This entails the most significant improvement concerning ^{68}Ga radiochemistry, as the pH range for optimal labeling is expanded towards quite acidic solutions. For the first time, ^{68}Ga labeling can be performed using the neat eluate from TiO_2-based ^{68}Ge/^{68}Ga generators (typically 0.1 M HCl, pH 1). Due to possible degradation of sensitive biomolecules at such pH, this might finally turn out to be of lesser practical relevance. However, it means that for **PrP9** there is no strict necessity to keep the pH in a very narrow range from 2.5–4 during the labeling procedures (as required, for example, for DOTA peptides). This might render the fully automated syntheses of ^{68}Ga radiotracers more robust, in which adjustment of labeling pH is often a crucial issue.

In addition, the synthesis of **PrP9** itself is fast, simple, and scalable; particularly in comparison with other ligand systems bearing additional carboxylic groups suitable for conjugation like DOTA(tBu)$_3$ or NODAGA(tBu)$_3$. Another novel and important characteristic is that conjugation by amide formation needs no protection (and therefore no final deprotection) as metal coordination sites and functional groups for conjugation are different in nature. However, the most striking advantage of **PrP9** is to provide easy access to multimers. We are not aware of any other system of such simple structure that could be multiply conjugated to amine substrates in a single synthetic step. We therefore hold the view that although monofunctionalization can be easily achieved as well by performing the coupling reaction by using a large excess of the ligand, the true designation of the compound is the preparation of multimeric tracers.

All things considered, we believe that **PrP9** has truly the potential to boost the development of ^{68}Ga-based nuclear medicine and molecular imaging by providing facile and cost-efficient access to ^{68}Ga radiopharmaceuticals with superior properties. Further investigations concerning the behavior in biosystems as well as the preparation of conjugates with targeting vectors are currently under way.

Experimental Section

Materials and methods: All reagents used were of analytical grade. Dry solvents were used only where indicated and dried according to established procedures. The preparation of [9]aneN_3 followed essentially a published procedure,[45] with little alterations as described earlier.[46] NMR spectra were recorded using a UNITY Inova (400 MHz) or a VNMRS (300 MHz) spectrometer from VARIAN. ^{1}H and ^{13}C NMR shifts are referenced to TMS. ^{31}P NMR shifts are given relative to 85% aq. H_3PO_4. ^{71}Ga NMR shifts are referenced to a 1.0 M aqueous solution of $Ga(NO_3)_3$. Elemental analysis was performed using a HERAEUS Vario EL III system. Ultrafiltration/diafiltration was performed using a Millipore setup (consisting of a 50 mL stirred cell model 8050, CDS10 selector valve, and RC800 mini-reservoir), in combination with Ultracel cellulose acetate membranes, filter code YC05, NWML 500Da. Diluted HCl for elution of ^{68}Ge/^{68}Ga generators was prepared from HCl (suprapure) and water (ultrapure; both from Merck).

Syntheses: The full synthetic route to of H_6**PrP9** includes two steps to obtain the phosphorus precursor; its synthesis has been developed in our laboratory and published by some of us before.[47] However, as efficacy and yields were significantly improved, we report the complete protocol starting from commercially available chemicals, including the mentioned steps.

Synthesis of H_6PrP9: Dry ammonium hypophosphite (0.3 mol, 25 g) and hexamethyldisilazane (0.48 mol, 77 g, 100 mL) were heated to 105 °C under argon atmosphere with stirring, whereupon gaseous ammonia was evolved. *Caution! The intermediate HP(OSiMe$_3$)$_2$ is pyrophoric!* After 4 h the mixture was cooled to room temperature, dry dichloromethane (200 mL) was added and the solution was cooled in an ice bath. Then *tert*-butyl acrylate (213 mmol, 27.3 g, 31 mL) was added through a syringe and the mixture was stirred for additional 12 h at room temperature. The mixture was hydrolyzed by transferring it into ethanol (500 mL) by means of a capillary and evaporated to dryness. The crude product was dissolved in chloroform (300 mL) and extracted with two portions (60 mL each) of 3% aq. HCl. The aqueous phases were combined and re-extracted with chloroform (3×50 mL). The combined organic extracts were dried over anhydrous sodium sulfate and the solvent removed to afford [2-(tert-butyloxycarbonyl)ethyl]phosphinic acid as a colorless, viscous oil (39.5 g, ca. 203 mmol, 95%), with a purity of 96% according to ^{31}P NMR. The remaining 4% impurity is the P-disubstituted product, which is inert in the following reactions and thus does not need to be removed. The crude phosphinic acid was dissolved in a mixture of ethanol (100 mL) and conc. aq. HCl (100 mL) and heated under reflux for 12 h. Then all volatiles were removed in vacuo to yield (2-carboxyethyl)phosphinic acid (34.2 g, ca. 203 mmol, 100%) as a colorless oil which solidifies upon standing. This compound and 1,4,7-triazacyclononane (45 mmol, 5.8 g) were dissolved in 6 M aq. HCl (120 mL) and heated to 70 °C. Paraformaldehyde (0.6 mol, 18 g) was added in small portions with stirring during a period of 24 h, while progress of the reaction was monitored with ^{31}P NMR spectroscopy. Then the solvents were distilled off in vacuo and the remaining HCl was removed by repeatedly adding small portions of water and evaporating to dryness. The crude product was purified by chromatography on ion exchange resin (DOWEX 50×8, H^+-form, column size 25×6 cm, eluent: water). Impurities were removed with the first 700 mL of eluate. The next fraction of approx. 1.8 L containing the pure product was concentrated in vacuo to a volume of 40 mL and methanol (300 mL) was added, whereupon the product slowly crystallized.

After 1 h, isopropanol (150 mL) was added and the suspension was cooled for several hours in order to ensure a complete precipitation. The product was filtered off, washed with isopropanol and diethyl ether, and dried in vacuo to yield H_6**PrP9**·2 H_2O (15.4 g, 55% based on triazacyclononane) as a colorless, fine-crystalline powder. M.p. 218–219°C; ^{1}H NMR (300 MHz, D_2O): δ = 2.07 (dt, J_3^{HH} = 7.8 Hz, J_3^{PH} = 13.7 Hz, 6H; C(O)-CH_2), 2.68 (dt, J_3^{HH} = 7.7 Hz, J_2^{PH} = 13.2 Hz, 6H; P-CH_2), 3.46 (d, J_2^{PH} = 5.7 Hz, 6H; N-CH_2-P), 3.53 ppm (s, 12H; ring-CH_2); ^{13}C{^{1}H} NMR (75.4 MHz, D_2O): δ = 25.1 (d, J_1^{PC} = 94 Hz, P-*C*-C), 26.8 (C(O)-*C*), 51.5 (ring-*C*), 54.6 (d, J_1^{PC} = 89 Hz, N-*C*-P), 177.3 ppm (d, J_3^{PC} = 13 Hz, *C*=O); ^{31}P{^{1}H} NMR (121.4 MHz, D_2O): δ = 40.0 ppm; MS (ESI positive): *m*/*z* (%): 602 (21) [*M*+Na]$^+$, 626 (100) [*M*+2Na−H]$^+$, 648 (22) [*M*+3Na−2H]$^+$; MS (FAB positive): *m*/*z*: 580 [*M*+H]$^+$; elemental analysis calcd (%) for $C_{18}H_{36}N_3O_{12}P_3$·2 H_2O (615.44): C 35.13, H 6.55, N 6.83; found C 35.04, H 6.60, N 6.67.

Synthesis of [Ga(H_3PrP9)]·*n*H_2O: H_6**PrP9**·2 H_2O (0.65 mmol, 400 mg) and $GaCl_3$ (0.65 mmol, 115 mg) were dissolved in water (1 mL). After brief heating, the pH value of the solution was found to be −0.4. An aqueous solution of 5% NH_3 was added until a pH of 2.3 was reached and the complex was allowed to precipitate for 24 h. The solid was filtered off and recrystallized from water (1 mL). The crystals were filtered off and dried in vacuo, whereupon the material apparently loses a fraction of the co-crystallized water, as the initially large transparent crystals disintegrate to give 320 mg of a colorless powder. The single crystals of [Ga(H_3**PrP9**)]·6 H_2O suitable for X-ray diffraction were obtained from water and those of [Ga(H_3**PrP9**)]·2 H_2O by diffusion of acetone vapor into an aqueous solution of the complex. ^{1}H NMR (300 MHz, D_2O): δ = 2.13–2.21 (m, 6H; C(O)-CH_2), 2.69–2.79 (m, 6H; P-CH_2), 3.10–3.28 (m, 6H; N-CH_2-P), 3.35–3.48 ppm (m, 12H; ring-CH_2); ^{13}C{^{1}H} NMR (101 MHz, D_2O): δ = 21.8 (d, J_1^{PC} = 100 Hz, P-*C*-C), 24.2 (C(O)-*C*), 49.7 (ring-*C*), 53.3 (d, J_3^{PC} = 11 Hz, ring-*C*), 55.4 (d, J_1^{PC} = 85 Hz, N-*C*-P), 174.0 ppm (d, J_3^{PC} = 13 Hz, *C*=O); ^{31}P{^{1}H} NMR (121.4 MHz, D_2O): δ = 42.4 ppm; ^{71}Ga NMR (122.0 MHz, D_2O): δ = 135.2 ppm; MS (ESI negative): *m*/*z*: 644/646 [*M*−H]$^-$; MS (ESI positive): *m*/*z*: 668/670 [*M*+Na]$^+$, 690/692 [*M*+2Na−H]$^+$, 712/714 [*M*+3Na−2H]$^+$; elemental analysis calcd (%) for $C_{18}H_{33}N_3O_{12}P_3Ga$·1.3 H_2O (669.53): C 32.29, H 5.36, N 6.28; found C 32.27, H 5.40, N 6.12.

Synthesis of [Fe(H_3PrP9)]·*n*H_2O: H_6**PrP9**·2 H_2O (0.2 mmol, 123 mg) and [Fe(acac)$_3$] (0.2 mmol, 72 mg) were dissolved in water (0.5 mL) and isopropanol (0.1 mL). When heated to reflux, a red solution initially obtained turned into a light yellow one after several minutes. After cooling to ambient temperature and standing for several hours, yellow crystals had formed, which were filtered off and recrystallized from water (0.5 mL). The precipitate was filtered off and dried in vacuo. During drying, it apparently loses a fraction of the co-crystallized water, as the initially transparent crystals disintegrate to result in a yellow powder (88 mg). The single crystals suitable for X-ray diffraction were obtained directly from water as [Fe(H_3**PrP9**)]·6 H_2O and by diffusion of acetone vapor into the aqueous solution as [Fe(H_3**PrP9**)]·H_2O. MS (ESI negative): *m*/*z*: 631 [*M*−H]$^-$; MS (ESI positive): *m*/*z*: 655 [*M*+Na]$^+$, 677 [*M*+2Na−H]$^+$, 699 [*M*+3Na−2H]$^+$, 721 [*M*+4Na−3H]$^+$; elemental analysis calcd (%) for $C_{18}H_{37}N_3O_{14}P_3Fe$ (668.26): C 32.35, H 5.58, N 6.29; found: C 32.20, H 5.63, N 6.09.

Preparation of conjugates—general procedure: H_6**PrP9**·2 H_2O (0.2 mmol, 123 mg) was dissolved in dry DMSO (3 mL). Then diisopropylethylamine (DIPEA, 2 mmol, 260 mg, 0.35 mL) was added; in case the coupled amine was in the hydrochloride form, 3 mmol (390 mg, 0.5 mL) of DIPEA were used. The amine or amino acid ester hydrochloride (1 mmol) was added and the mixture was stirred for 5 min. Then TBTU (1.6 mmol, 0.5 g) was added in small portions within 10 min. The mixture was left to react for variable time (see individual compound data) and afterwards diluted with water (50 mL). For workup, solutions were concentrated to 15 mL by ultrafiltration through a membrane with 0.5 kDa MWCO. Diafiltration with aqueous NaCl (0.05 M, 300 mL) removed all impurities. The solutions were further concentrated to 10 mL and desalted by diafiltration with pure water (100 mL). Subsequent lyophilization of the retentate afforded the sodium salts of the products as off-white to pale yellow, voluminous solids.

Tris(cyclohexylamide)-PrP9 (1): Cyclohexylamine (1 mmol, 100 mg, 115 μL), reaction time: 10 min. Yield: 179 mg (93%); ^{1}H NMR (400 MHz, D_2O): δ = 1.08–1.27 (m, 15H), 1.52–1.55 (m, 3H), 1.64–1.67 (m, 6H), 1.72–1.80 (m, 12H), 2.30–2.37 (m, 6H; P-CH_2), 3.05 (d, J_2^{PH} = 6.0 Hz, 6H; N-CH_2-P), 3.19 (br s, 12H; ring-CH_2), 3.45–3.52 ppm (m, 3H); ^{13}C{^{1}H} NMR (75.4 MHz, D_2O): δ = 24.3, 24.9, 26.9 (d, J_1^{PC} = 92 Hz, P-*C*-C), 28.7, 31.9, 48.9, 50.6, 53.6 (d, J_1^{PC} = 92 Hz, N-*C*-P), 174.2 ppm (d, J_3^{PC} = 17 Hz, *C*=O); ^{31}P{^{1}H} NMR (161.9 MHz, D_2O): δ = 38.5 ppm; MS (ESI negative): *m*/*z*: 843 [*M*+Na−2H]$^+$; elemental analysis calcd (%) for Na(H_2**1**)·6 H_2O ($C_{36}H_{80}N_6O_{15}P_3Na$; 952.96): C 45.37, H 8.46, N 8.82; found: C 45.20, H 8.35, N 8.74.

Tris{(*O*-methyl)glycyl}PrP9 (2): Glycine methyl ester hydrochloride (1 mmol, 125 mg), reaction time: 45 min. Yield: 138 mg (76%); ^{1}H NMR (400 MHz, D_2O): δ = 1.70–1.81 (m, 6H; CH_2-C(O)N), 2.36–2.45 (m, 6H; P-CH_2), 3.08 (d, J_2^{PH} = 6.0 Hz, 6H; N-CH_2-P), 3.16 (br s, 12H; ring-CH_2), 3.68 (s, 9H; CH_3), 3.94 ppm (s, 6H; CH_2-C(O)O); ^{13}C{^{1}H} NMR (75.4 MHz, D_2O): δ = 24.7 (d, J_1^{PC} = 90 Hz, P-*C*-C), 31.0, 43.9, 53.1, 55.4, 56.0 (d, J_1^{PC} = 99 Hz, N-*C*-P), 174.8, 178.6 ppm (d, J_3^{PC} = 16 Hz, *C*=O); ^{31}P{^{1}H} NMR (161.9 MHz, D_2O): δ = 38.5 ppm; MS (ESI negative): *m*/*z*: 813 [*M*+Na−2H]$^-$; elemental analysis calcd (%) for Na_2(H_2**2**)·4.8 H_2O ($C_{27}H_{59.6}N_6O_{19.8}P_3Na$; 901.1): C 35.99, H 6.67, N 9.33; found C 35.98, H 6.35, N 9.19.

Tris{(*O*-benzyl)glycyl}PrP9 (3): Glycine benzyl ester hydrochloride (1 mmol, 202 mg), reaction time: 50 min. Yield: 173 mg (76%); ^{1}H NMR (400 MHz, D_2O): δ = 1.73–1.80 (m, 6H; CH_2-C(O)N), 2.38–2.43 (m, 6H; P-CH_2), 3.05 (d, J_2^{PH} = 5.6 Hz, 6H; N-CH_2-P), 3.14 (br s, 12H; ring-CH_2), 3.75 (s, 6H; CH_2-C(O)O), 4.85 (s, 6H; CH_2-Ph), 7.05–7.12 ppm (m, 15H; C_6H_5); ^{13}C{^{1}H} NMR (100.6 MHz, D_2O): δ = 24.1 (d, J_1^{PC} = 90 Hz, P-*C*-C), 25.7, 38.6, 47.6, 50.6 (d, J_1^{PC} = 93 Hz, N-*C*-P), 64.5, 125.5, 125.8, 126.0, 132.5, 168.4, 173.1 ppm (d, J_3^{PC} = 16 Hz, *C*=O); ^{31}P{^{1}H} NMR (161.9 MHz, D_2O): δ = 38.3 ppm; MS (ESI negative): *m*/*z*: 1042 [*M*+Na−2H]$^-$; elemental analysis calcd (%) for Na(H_2**3**)·5 H_2O ($C_{45}H_{72}N_6O_{20}P_3Na$; 1132.99): C 47.70, H 6.41, N 7.42; found C 47.52, H 6.37, N 7.38.

Tris{(*O*-*tert*-butyl)-L-phenylalanyl}PrP9 (4): L-Phenylalanine *tert*-butyl ester hydrochloride (1 mmol, 258 mg), reaction time: 50 min. Yield: 223 mg (84%); ^{1}H NMR (400 MHz, D_2O): δ = 1.10 (s, 27H; CH_3), 1.73 (br s, 6H; CH_2-C(O)N), 2.36 (br s, 6H; P-CH_2), 2.77–2.86 (br s, 6H; CH_2-Ph), 3.04 (br s, 6H; N-CH_2-P), 3.16 (br s, 12H; ring-CH_2), 4.33 (t, *J* = 4.8 Hz, 3H; C*H*), 6.97–7.07 ppm (m, 15H; C_6H_5); ^{13}C{^{1}H} NMR (100.6 MHz, D_2O): δ = 24.2 (d, J_1^{PC} = 103 Hz, P-*C*-C), 24.7, 25.7, 34.6, 47.6, 50.6 (d, J_1^{PC} = 92 Hz, N-*C*-P), 52.3, 79.7, 124.0, 125.7, 126.7, 133.9, 169.1, 172.1 ppm (d, J_3^{PC} = 16 Hz, *C*=O); ^{31}P{^{1}H} NMR (161.9 MHz, D_2O): δ = 38.2 ppm; MS (ESI negative): *m*/*z*: 1210 [*M*+Na−2H]$^-$; elemental analysis calcd (%) for Na(H_2**4**)·6 H_2O ($C_{57}H_{98}N_6O_{21}P_3Na$): C 51.89, H 7.49, N 6.37; found C 51.70, H 7.61, N 6.24.

Crystal structure determination: The diffraction data were collected on a Nonius Kappa CCD diffractometer (Enraf–Nonius) at 150(1) K with $Mo_{K\alpha}$ radiation (λ = 0.71073 Å) and analyzed by using the HKL program package.[48,49] The structures were solved using direct methods and refined by full-matrix least-squares techniques (SIR92[50] and SHELXL97[51]). Scattering factors for neutral atoms were included in the SHELXL97 program. All non-hydrogen atoms were refined anisotropically. The hydrogen atoms were located in electron density map. Hydrogen atoms at carbon atoms were fixed in the theoretical positions. Those belonging to carboxylate and water oxygen atoms were fixed in original positions using the riding model with U_{eq}(H) = 1.2 U_{eq}. In the structure of [Ga(H_3**PrP9**)]·2 H_2O, one of the oxygen atoms of one carboxylate moiety was best refined disordered in two positions with mutual occupancy of 79 and 21%. CCDC-749080 ([Fe(H_3**PrP9**)]·H_2O), -749083 ([Fe(H_3**PrP9**)]·6 H_2O), -749081 ([Ga(H_3**PrP9**)]·2 H_2O), and -749082 ([Ga(H_3**PrP9**)]·6 H_2O) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif. For crystallographic parameters see also Supporting Information, Table S5.

Potentiometry: The stock/titration solutions used (aq. HCl, ≈0.03 M; NMe_4OH, ≈0.2 M) and metal chlorides or nitrates were the same as in previous studies;[52,53] the known amount of HCl was added to the stock

solution of $GaCl_3$ to prevent hydrolysis. Titration conditions: 25.0 ± 0.1 °C; $I = 0.1$ M (NMe_4Cl); $-\log[H^+]$ range of 1.7–11.9 or until precipitation of a metal hydroxide occurred; starting volume 5 mL; ligand concentration ≈0.004 M; presaturated wet argon as an inert gas. The titration system consisted of a PHM 240 pH meter, a 2 mL ABU 900 automatic piston burette and a GK 2401B combined electrode (all Radiometer, Denmark). At least three parallel titrations were carried out for each metal-to-ligand molar ratio (1:1 and 2:1); ≈40 points per each titration. In the case of Ga^{3+} ("equilibrium" titration) and Gd^{3+} ions, the complexation was too slow for a conventional titration. Thus the "out-of-cell" method was used: 30 (Ga^{3+}) or 25 (Gd^{3+}) points per titration; $-\log[H^+]$ range of 1.5–10.5 (Ga^{3+}/L = 1:1), 1.5–3.1 (Ga^{3+}/L = 2:1) or 1.8–6.0 (Gd^{3+}/L = 1:1); starting volume 1 mL; prepared under argon; equilibrium time at room temperature four weeks (Ga^{3+} systems) or 1 d (Gd^{3+} system). Solutions were kept in tightly closed ground glass tubes below $-\log[H^+] < 6$. For $-\log[H^+] > 6$, solutions were flame sealed into ampoules in order to protect the solutions against atmospheric CO_2 during standing, as alkaline solutions in the closed ground tubes gave irreproducible results. The solution $-\log[H^+]$ in each tube/ampoule for out-of-cell titration was measured separately with a freshly calibrated electrode (as given below). The constants determined by this technique showed higher standard deviations due to less precise measurements and a smaller number of experimental points. At least two parallel titrations for each metal-to-ligand ratio (1:1 and 2:1) were performed. The constants (with standard deviations) were calculated using the OPIUM program.[54–56] The program minimizes the criterion of the generalized least-squares method using the following calibration function:

$$E = E_0 + S\log[H^+] + j_1[H^+] + j_2[OH^-]$$

in which the additive term E_0 contains the standard potentials of the electrodes used and contributions of inert ions to the liquid-junction potential, S corresponds to the Nernstian slope (the actual value of which should be close to the theoretical value) and the $j_1[H^+]$ and $j_1[OH^-] = j_2K_w/[H^+]$ terms are the contributions of the H^+ and OH^- ions to the liquid-junction potential. It is clear that j_1 and j_2 cause deviation from a linear dependence of E on pH only in strongly acidic and strongly alkaline solutions. The calibration parameters were determined from titration of the standard HCl with the standard NMe_4OH before each ligand or ligand–metal titration to give a pair of calibration/titration, which was used for calculations of the constants. All constants determined are concentration constants. The water ion product pK_w (13.81) and stability constants of the $M^{2/3+}$–OH^- systems included into the calculations were taken from literature.[38,39,57]

NMR measurements: The Ga^{III}–**PrP9** complex formation was followed by ^{31}P and ^{71}Ga NMR spectroscopy at room temperature (25 °C). Typical conditions: $GaCl_3$ hydrate (15 mg) was dissolved in distilled water (5 mL) and the pH was adjusted to 1.3, 1.0 or 0.8 with 0.5 M aq. HCl. The ligand hydrate (10.3 mg) was pre-weighed into a 3 mL vial and quickly dissolved in this $GaCl_3$ solution (1 mL) to give a solution containing metal ions and ligand in equimolar amounts. This moment represented zero on the timeline ($t = 0$). The reaction mixture was transferred as quickly as possible to a 5 mm NMR tube and placed in the NMR spectrometer. Typically, the first spectra were obtained at $t = 2$ min. After the end of complexation reaction, the final pH of the solution was determined again. The ^{71}Ga NMR spectra were quantified against signal of $[Ga(OH)_4]^-$ (5 mM, pH 13) in the insert tube. Identity of the major isomer in the solution with that isolated in the solid state was confirmed by addition of the isolated complex into NMR sample after finishing the complexation reaction.

The dissociation of the Ga^{III}–**PrP9** complex in alkaline media was investigated by ^{71}Ga NMR spectroscopy ([GaL] = 5 mM, 25 °C). The complex was preformed by mixing of the equimolar amount of Ga^{3+} salt and the ligand dissolved in water. This solution was evaporated in vacuum and dissolved in water to give a stock solution of the complex. An increasing abundance of $[Ga(OH)_4]^-$ was quantified against 5 mM $[Ga(H_2O)_6]^{3+}$ solution in 0.1 M HNO_3 in an insert tube. The experiments were carried out at pH 11.0 (0.2 M CAPS buffer) and pH 13 (0.1 M NaOH). For decomplexation in acidic media, the complex was preformed in the same way and dissolved in 5 M $HClO_4$ The tube was stored at room temperature for seven months while ^{31}P and ^{71}Ga NMR spectra were recorded regularly.

^{68}Ga labeling—standard labeling procedure:[41] A 10 mL Mallinckrodt standard glass vial was charged with Millipore water (5 mL) containing the ligand (14 nmol). Elution of the TiO_2-based $^{68}Ge/^{68}Ga$ generator (from Cyclotron Co, Obninsk, Russia) was performed with 0.1 M HCl (7 mL). ^{68}Ga activity was concentrated on a small cation-exchange resin (AG 50-W × 8, 400 mesh, H^+-form) and purged with an acetone/HCl-mixture (1 mL). [^{68}Ga]$GaCl_3$ (120–160 MBq, ≅1.2–1.6 pmol) was eluted with a 2.44 % solution of 0.05 M HCl in acetone (400 μL) directly into the reaction vessel. The vial was heated to the specified temperature for 20 min. Samples were withdrawn from the reaction mixture at 1, 3, 10, 15 and 20 min and analyzed by radio-TLC (TLC sheets silica gel 60, mobile phase: 0.1 M sodium citrate in water).

The labeling of **3** with the neat generator eluate was performed by directly dissolving the specified amount of ligand **3** in the eluate (1.7 mL, typically containing ≈60 MBq of ^{68}Ga, ≅0.6 pmol) and subsequent heating of the solution to 60 °C. Probing and analysis were performed as described above. The stability of **3** under these conditions was checked by heating a solution of **3** (2 mg) dissolved in HCl (1 mL, 0.1 M) to 60 °C for 20 min. HPLC analyses (using a 20 × 4 mm C18 column, flow rate 1 mL min^{-1}, gradient: in 20 min from 30 to 70 % MeCN in H_2O, both eluents containing 0.1 % trifluoroacetic acid), were performed immediately after dissolving and after 20 min of heating. The chromatograms showed no significant differences and were practically identical to those of the compound dissolved in pure water.

For labeling in HEPES-buffered solution (comparison of **PrP9**, NOTA, and DOTA), a generator with an SnO_2 matrix (from iThemba LABS, South Africa, total eluted ^{68}Ga activity ca. 1500 MBq) was eluted with 1 M HCl. A fraction of 1.25 mL containing the highest activity was mixed with a solution of HEPES (600 mg) in water (Merck ultrapure, 500 μL). From this solution aliquots of 100 μL (containing about 60 MBq (≅0.6 pmol) each) were transferred into eppendorf cups. 10 μL of solutions of the ligand (1 μmol mL^{-1}) were added and mixed well. The pH of the mixture was 3.3. Samples were withdrawn after 4, 7, 10, 15, and 20 min and analyzed by TLC as described above.

Acknowledgements

J.N. gratefully acknowledges a postdoc grant from the German Academic Exchange Service (DAAD). This work was supported by the Grant Agency of the Czech Republic (grant no. 203/09/1056), the Czech Academy of Sciences (grant no. KAN201110651) and the Ministry of Education, Youth and Sport of the Czech Republic (grant no. MSM0021620857). This work was carried out in the framework of COST D38 and BM607 Actions and DiMI (grant no. LSHB-2005-512146) projects. We thank Dr. I. Císařová for collection of X-ray data.

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Received: November 30, 2009
Revised: February 4, 2010
Published online: May 12, 2010

