J. Med. Chem. **2002,** *45,* 3963−3971

Synthesis and Initial Evaluation for In Vivo Chelation of Pu(IV) of a Mixed Octadentate Spermine-Based Ligand Containing 4-Carbamoyl-3-hydroxy-1-methyl-2(1*H*)-pyridinone and 6-Carbamoyl-1-hydroxy-2(1*H*)-pyridinone

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Received December 11, 2001

An improved synthesis for a series of 1-hydroxy-2(1*H*)-pyridinone-based octadentate ligands is reported. The mixed chelate, octadentate ligand, 3,4,3-LI(1,2-Me-3,2-HOPO), was designed, synthesized, and tested for in vivo chelation of Pu in a mouse model. This ligand incorporates both 1,2-HOPO and Me-3,2-HOPO metal chelating units; the latter has higher affinity toward actinide ions than does 1,2-HOPO at physiological pH. Injected or administered orally to fasted or normally fed mice at the standard clinical dose 30 μmol/kg, both 3,4,3-LI(1,2-HOPO) and 3,4,3-LI(1,2-Me-3,2-HOPO) remove significantly more Pu than injected $CaNa_3DTPA$. Injected doses of 0.1 μmol/kg of these HOPO ligands are as effective as 30 μmol/kg of injected $CaNa_3$-DTPA. Ten daily injections of 30 μmol/kg of a HOPO ligand did not induce detectable acute toxicity in mice. The mixed HOPO ligand is somewhat more effective than 3,4,3-LI(1,2-HOPO) when given orally, and the enhanced reduction of liver Pu by the mixed ligand is statistically significant. Thus, both octadentate HOPO ligands meet the criterion of low toxicity at doses that are more effective than the standard dose of $CaNa_3DTPA$. Their improved effectiveness at low dose along with great oral activity (despite low gastrointestinal absorption) implies that new treatment regimens can be developed using the HOPO ligands alone or as adjuncts to $CaNa_3DTPA$ therapy, which will greatly exceed the amount of Pu excretion that is achievable with $CaNa_3DTPA$ alone.

Introduction

Plutonium is an industrial and environmental hazard. Nuclear weapons tests, military and power reactor accidents, and inadequate waste disposal practices have contaminated radiation workers[1,2] and the environment.[3,4] After intake via inhalation, ingestion, or deposition in a wound, Pu enters the blood stream where complexation by the nonfilterable iron transport protein transferrin (TF) severely inhibits its renal excretion.[5] The Pu−TF complex circulating in the blood dissociates, and the displaced Pu forms more stable long-lived complexes with bioligands in the skeleton and liver. Inhaled Pu is released very slowly from the respiratory tract. The accumulated radiation doses damage tissue and induce cancers in the three target organs: bone, liver, and lung.[6,7] Aggressive, and often protracted, treatment with chelating agents that form excretable low molecular weight Pu complexes is the only practical way to reduce the health consequences of internally deposited Pu.[2,8−10] Diethylenetriaminepentaacetic acid (DTPA), the only clinically accepted Pu removal agent, has clinical defects. In particular, $CaNa_3DTPA$ or less potent $ZnNa_3DTPA$ must be given to avoid depletion of essential divalent metal ions. Furthermore, clinically acceptable doses of DTPA do not remove Pu from bone mineral or liver ferritin. Finally, DTPA must be administered by injection, because it is not effective when given orally.[8,11−14]

The similar coordination properties of Fe(III) and Pu(IV)[5,9,11,15,16] suggested that synthetic actinide sequestering agents containing the iron-binding units of siderophores (microbial iron chelators) would be effective complexing agents for Pu(IV).[5,9,15,16] Our initial synthetic actinide sequestering agents contained catechol, the functional group of the potent siderophore enterobactin.[9,14,17] However, no catecholate ligand tested to date significantly improves in vivo Pu chelation with acceptably low toxicity at an effective dose when compared to $CaNa_3DTPA$.[10,18−21]

The heterocyclic hydroxypyridinone (HOPO) isomers are highly selective for “hard” metal ions with large charge/radius ratios, such as Fe(III) and Pu(IV). The HOPO monoanions have zwitterionic resonance forms that are isoelectronic with the catechol dianion. The HOPO metal binding units, which are monoprotic (deprotonated) at physiological pH, were expected to form stable Pu(IV) complexes at physiological pH.[22−25] Among the multidentate 1,2-HOPO ligands synthesized to date, spermine-based octadentate 3,4,3-LI(1,2-HOPO) (Figure 1, **1**) is the most effective Pu chelator. (The abbreviation of **1** stands for the 1,2-HOPO ligand with a linear tetramine backbone, abbreviated as LI, in which the four amine nitrogen atoms are separated by 3, 4, and 3 carbon atoms, respectively.) Given by injection or orally, its efficacies for in vivo chelation of Pu(IV), Am(III), and Th(IV) greatly exceed those of $CaNa_3DTPA$

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10.1021/jm010564t CCC: $22.00 © 2002 American Chemical Society
Published on Web 07/31/2002

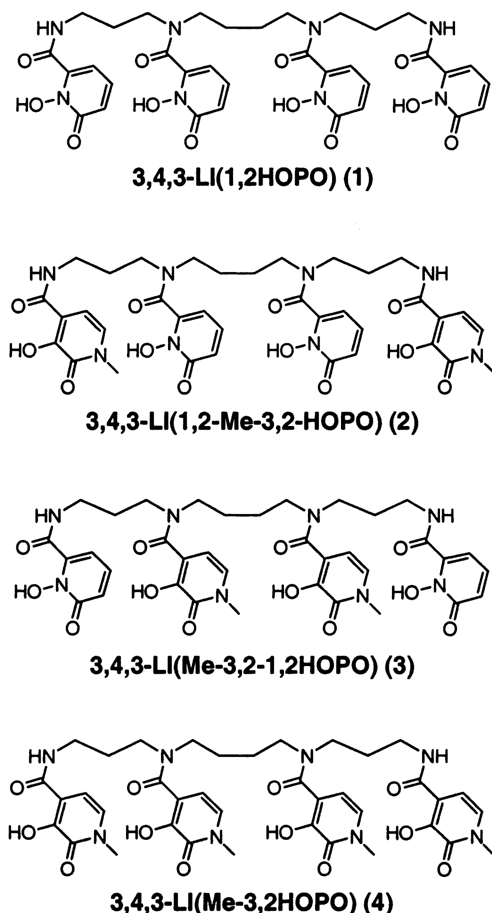


Figure 1. Possible octadentate 1,2-HOPO and Me-3,2-HOPO ligands based on spermine scaffolds.

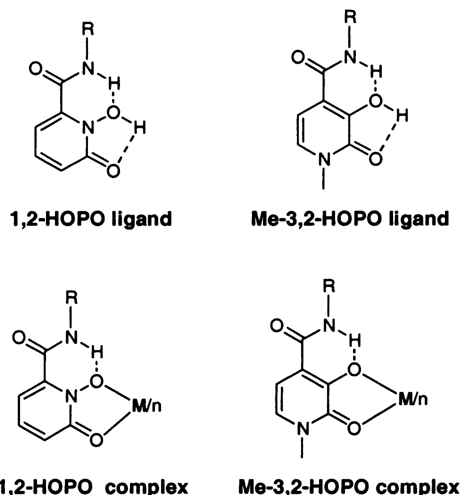


Figure 2. Functionalized HOPOs and the metal complexes of these predisposed ligands.

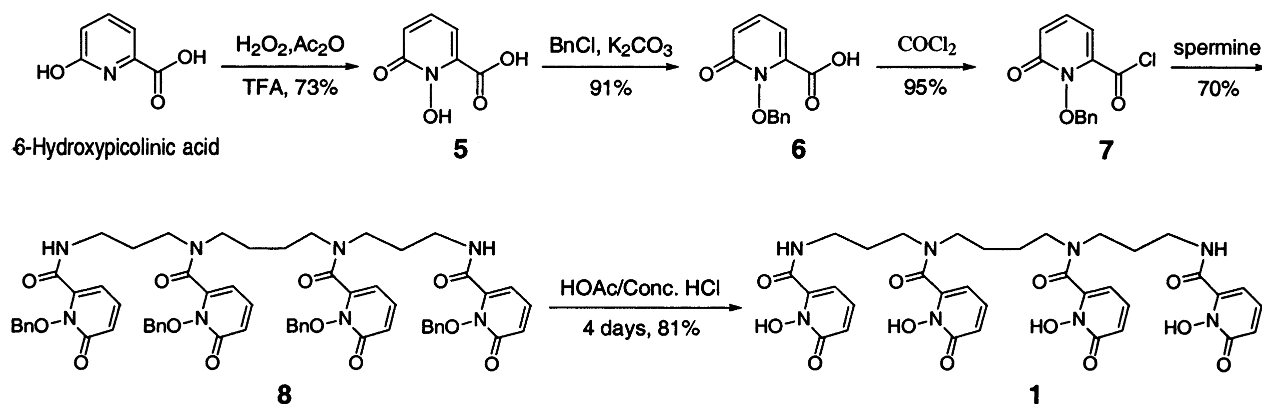
in all low dose evaluations in small laboratory animals.[18,23,26,27] Subsequently, we developed a strategy[25] for the design and synthesis of powerful HOPO-based metal sequestering agents that functionalizes the HOPO ring carbon atom adjacent to the HOPO hydroxyl group with a carboxyl group and then attaches the HOPO units through amide linkages to a suitable molecular scaffold. Structural characterization of these functionalized 1,2-HOPO and 3,2-HOPO ligands as well as their metal complexes indicated that these ligands are not only predisposed toward complexation, but their metal complexes are also further stabilized by the amide hydrogen bonding as shown in Figure 2.[22,24,28]

Because the 1,2-HOPO ligands, in particular 3,4,3-LI(1,2-HOPO) **1**, are potentially useful sequestering agents for hard metal ions including the actinides, research was directed toward improving the initial synthesis. The original synthesis of multidentate 1,2-HOPO ligands, reported a decade ago,[23] involves several low yield steps and difficult purifications: 2,6-dibromopyridine was converted into 6-bromopicolinic acid, which was oxidized and then hydrolyzed to 1-hydroxy-2(1*H*)-pyridinone-6-carboxylic acid (6-carboxy-1,2-HOPO) **5**. Compound **5** was activated by phosgene and then coupled to a suitable amine scaffold.[23] Separation and purification of the multidentate 1,2-HOPO products are tedious, often requiring high-performance liquid chromatography (HPLC) purification.

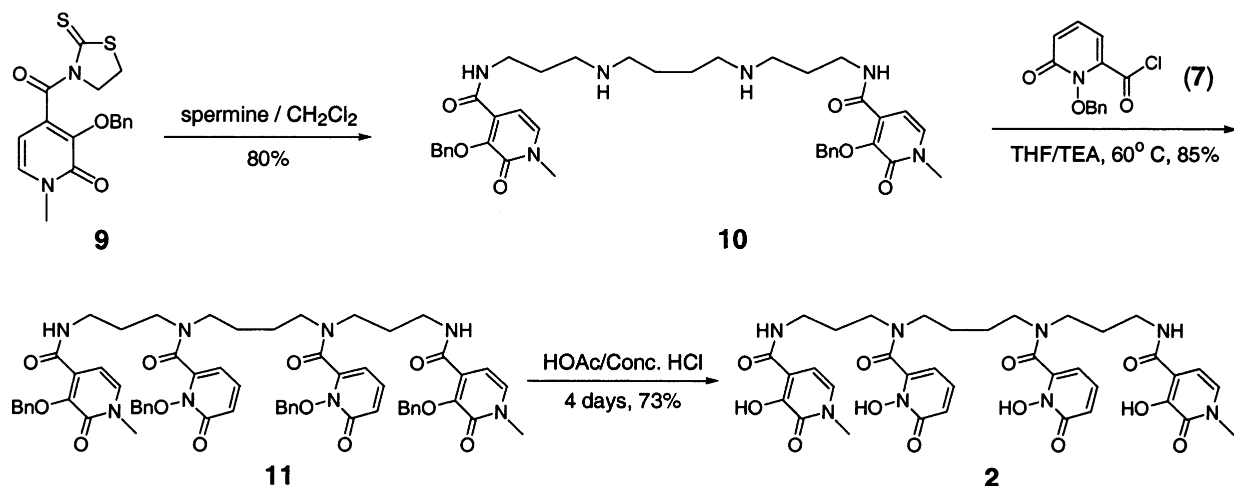
In 1992, Uhlir and Raymond[29] reported that following benzyl protection of the N-hydroxyl group of **5**, the protected species 1-benzyloxy-2(1*H*)-pyridinone-6-carboxylic acid (**6**) could be activated and coupled to an amine scaffold. It was shown that like other aromatic carboxylic acids, the HOPO carboxyl group could be activated by various activation procedures used widely in peptide synthesis.[29,30] Those procedures include the N-hydroxysuccinimide (NHS)/1,3-dicyclohexylcarbodiimide (DCC), HOBT/DCC, and 2-mercaptothiazoline/DCC, as well as acid chloride activations. Bailly and Burgada[31] independently improved the synthesis of **1** by using 1-benzyloxy-2(1*H*)-pyridinone-6-carbonyl chloride (**7**) as the activated species, which was reacted with the spermine to afford the benzyl-protected ligand in good yield. We developed the new synthesis for **5** reported herein, synthesized a series of multidentate 1,2-HOPO ligands with different topologies, and evaluated these ligands as Pu(IV) sequestering agents in Pu-injected mice.[26,32] Transition metal complexes of these multidentate ligands were also synthesized and structurally characterized.[32]

Concerns about the acute toxicity of **1** at high injected doses and its difficult original synthesis[23] stimulated the design and synthesis of multidentate HOPO ligands based on a new set of ligand scaffolds and containing the less acidic Me-3,2-HOPO isomer.[33] In initial evaluations in mice at the standard ligand dose (30 μmol/kg), most of the Me-3,2-HOPO ligands, when promptly injected and nearly all when orally administered, were significantly more effective for in vivo Pu chelation than an equimolar amount of $CaNa_3DTPA$. When injected promptly, six of the Me-3,2-HOPO ligands were as effective as **1**.[33] It was then reasoned that introduction of the Me-3,2-HOPO unit into a spermine-based octadentate 1,2-HOPO ligand such as **1** would result in new ligands **2**−**4** with enhanced affinity for Pu(IV) (Figure 1). In this paper, we present the improved syntheses (Scheme 1) of the precursor **5** and of the octadentate ligand **1** and the synthesis (Scheme 2) and initial biological evaluation of a new octadentate mixed HOPO ligand, 3,4,3-LI(1,2-Me-3,2-HOPO) (Figure 1, **2**). The mixed ligand **2** has been evaluated in mice for acute toxicity and for in vivo Pu chelation with the same Pu removal protocols used to evaluate and compare 3,4,3-LI(1,2-HOPO) and $CaNa_3DTPA$.[26]

Scheme 1. Improved Synthesis of 3,4,3-(LI-1,2-HOPO)



Scheme 2. Synthesis of 3,4,3-LI(1,2-Me-3,2-HOPO)



Results

Ligand Syntheses. The improved synthesis of **1** is shown in Scheme 1. The protection of 1,2-HOPO-6-carboxylic acid (**5**) with benzyl chloride (Scheme 1) is easily accomplished if potassium carbonate is used as the base in methanol. The benzyl-protected acid (1,2-HOPOBn acid **6**) is now isolated in 91% yield after removing the solvent and acidifying the aqueous solution of the residue.[31]

1,2-HOPOBn acid (**6**) is a versatile compound for synthesizing multidentate 1,2-HOPO ligands. Compound **6** can be converted to a number of activated intermediates and then coupled to a variety of amines. For example, **6** reacts with 2-mercaptothiazoline in the presence of DCC and DMAP (1,4-(dimethylamino)-pyridine) to give 1,2-HOPO/thiazolide,[29,32] which selectively reacts with aliphatic primary amines, like its 3,2-HOPO analogue, 3,2-HOPO-thiazolide.[33] Compound **6** can also be converted to NHS or other activated esters for amine coupling. In most cases, the active esters are not separated but are reacted with amines in situ.[29,32] Like 2,3-dibenzyoxybenzoic acid[35] and 1-methyl-3-benzyloxy-2(1*H*)-pyridinone-4carboxylic acid,[28] **6** can be converted to the acid chloride, a very active species. The acid chloride **7** reacts with not only aliphatic primary and secondary amines but also with aromatic amines. Selective coupling with a variety of amines can be easily accomplished by using different activated 1,2-HOPOBn acid species. Coupling of spermine with **7** yields the benzyl-protected 3,4,3-LI(1,2-HOPOBn) (**8**), which is deprotected under acidic conditions to give an 81% yield of ligand **1** (as compared to the 15% reported for the original synthesis).[23]

The mixed HOPO ligand, 3,4,3-LI(1,2-Me-3,2-HOPO) (**2**), is synthesized in three steps (Scheme 2): spermine is reacted with 2 equiv of 3,2-HOPOBn-thiazolide (**9**) to form only the N^1,N^{14}-disubstituted species **10**, as confirmed by NMR spectroscopies since **9** only reacts with primary amine moieties. Compound **10** is then coupled with 1,2-HOPOBn acid chloride to yield the protected mixed ligand **11**. Because hydrogenation would reduce the 1,2-HOPO moieties, acidic deprotection is used to produce the free mixed HOPO ligand **2**. The yield, 73% based on spermine, was also favorable. Efficient syntheses of these octadentate chelators strongly indicate that useful amounts of these promising compounds can be prepared at reasonable cost.

Ligand Efficacy for Pu Removal. Injected Ligands. Excretion and distribution of retained Pu are shown in Table 1 for mice treated with the octadentate HOPO ligands or $CaNa_3DTPA$ (30 μmol/kg, ip). In these trials, **1** was as effective for reducing Pu retention in the skeleton and soft tissue as in the original mouse trials and significantly more effective for reducing Pu in liver and whole body.[18,23] Both octadentate HOPO ligands **1** and **2**, injected at 1 h, greatly reduce Pu in the skeleton and all soft tissues, significantly compared with Pu-injected controls, and with minor exceptions,

Table 1. Removal of ^{238}Pu(IV) from Mice by Injected and Orally Administered Octadentate HOPO Ligands and $CaNa_3DTPA$

		percent of injected ^{238}Pu ± SD[a]						
		tissues					excreta[b]	
ligand	no. of mice	skeleton	liver	kidneys	soft tissue	whole body	urine	feces and GI contents
Ligand Injected at 1 h[d,f]								
3,4,3-LI(1,2-Me-3,2-HOPO)	10	9.1 ± 1.2	11 ± 2.6	0.2 ± 0.1	1.0 ± 0.9	22 ± 2.4	27	51
3,4,3-LI(1,2-HOPO) old[e]	5	7.5 ± 0.7	8.9 ± 1.7	0.2[b]	1.6 ± 0.7	18 ± 1.7	25	57
3,4,3-LI(1,2-HOPO) new	20	8.0 ± 1.9	5.4 ± 1.6[g]	0.1 ± 0.06	0.8 ± 0.7	14 ± 2.3[g]	21± 0.7	65 ± 2.6
$CaNa_3DTPA$	14	14 ± 1.6	19 ± 5.8	1.2 ± 0.9[d]	2.9 ± 0.6	37 ± 5.1	55 ± 6.1	8.4 ± 2.9
Ligand Orally at 3 min, Normally Fed[d,f]								
3,4,3-LI(1,2-Me-3,2-HOPO)	10	7.4 ± 2.5	2.4 ± 1.7[g]	0.1 ± 0.06	0.9 ± 0.5	11 ± 4.3	31	58
3,4,3-LI(1,2-HOPO)	10	8.8 ± 3.7	5.9 ± 3.2	0.2 ± 0.1	1.5 ± 0.7	16 ± 5.9	27	57
Pu-injected fed controls	35	37 ± 7.6	45 ± 7.2	1.5 ± 05	7.2 ± 1.4	90 ± 1.1	6.5 ± 0.8	3.7 ± 0.6
Ligand Orally at 3 min, Fasted[d,f]								
3,4,3-LI(1,2-Me-3,2-HOPO)	20	7.8 ± 1.3	2.2 ± 1.6[g]	0.09 ± 0.04	0.8 ± 0.6	11 ± 2.7	28 ± 6.4	61 ± 6.1
3,4,3-LI(1,2-HOPO) old[e]	5	12 ± 2.4	11.0 ± 4.9	0.1[b]	1.3 ± 0.7	24 ± 7.7	26	51
3,4,3-LI(1,2-HOPO) new	20	8.0 ± 2.0	4.2 ± 2.7	0.1 ± 0.05	1.1 ± 0.7	14 ± 4.6	26 ± 3.2	60 ± 1.4
$CaNa_3DTPA$	5	40 ± 6.8[d]	33 ± 5.4	0.7 ± 0.1	5.1 ± 1.3[d,]	79 ± 1.3	15	6.5
Pu-injected fasted controls	20	38 ± 5.2	46 ± 5.0	1.3 ± 0.3	5.4 ± 1.3	90 ± 1.1	5.6 ± 0.8	4.0 ± 0.4

[a] Data for each mouse, expressed as %ID, were normalized to 100% material recovery; discrepancies are due to rounding. [b] No SD is shown for kidney (pooled for each five mouse group in the original 3,4,3-LI-(1,2-HOPO) studies) or excreta of single experiments (five mice) or experiments replicated only once (10 mice), because the excreta in each cage of five mice were pooled for radioanalysis; SDs were calculated for excreta in multiple replicated experiments, where n = one-fifth (number of mice). [c] Ligands (30 μmol/kg) were injected ip at 1 h or given orally (gastric tube) at 3 min after iv injection of ^{238}Pu(IV) citrate; kill at 24 h. [d] Means for ligand-treated mice are significantly less than for Pu-injected controls (t-test, $p \leq 0.01$),[29] except five designated means. [e] Previously reported.[18] [f] Mean for HOPO ligand-treated mice is significantly less than for mice similarly treated with $CaNa_3DTPA$. [g] Mean is significantly less than for mice given the different HOPO ligand.

also significantly compared with mice similarly treated with $CaNa_3DTPA$. Injected **2** reduces skeleton, kidney, and bulk soft tissue Pu as much as **1**, but significantly more Pu remained in the livers of mice treated with the mixed ligand (**2**).

Orally Administered Ligands. Excretion and distribution of retained Pu are shown in Table 1 for mice gastrically intubated with the octadentate HOPO ligands or $CaNa_3DTPA$ (30 μmol/kg, by gastric tube (po) at 3 min to fasting or normally fed mice). In these trials, **1**, given orally, is as effective for reducing kidney and soft tissue Pu and significantly more effective for reducing skeleton, liver, and whole body Pu than was observed in the original animal testing.[18,23] Both octadentate HOPO ligands, given orally, markedly reduce Pu retention in the skeleton and all soft tissues of fasting mice, significantly, as compared with fasted Pu-injected controls, and also, significantly, as compared with mice treated orally or injected intraperitoneally (ip) with 30 μmol/kg of $CaNa_3DTPA$ (Table 1). Given orally to fasting mice, **1** is as effective for reducing tissue Pu as the same ligand dose injected ip at 1 h. Orally administered **2** is as effective in fasting mice for reducing skeleton, kidney, and soft tissue Pu as the same ligand dose injected at 1 h and significantly more effective for reducing liver Pu. In contrast to their relative efficacies when injected (Table 1), **2** given orally to fasting mice is somewhat more effective overall than **1** and significantly more effective for reducing liver Pu.

The great efficacies of the orally administered octadentate HOPO ligands for reducing tissue Pu in fasting mice (empty upper gastrointestinal (GI) tract) suggested that those ligands might also remove useful amounts of Pu, if given orally to normally fed mice (variable mass of contents in upper GI tract). The efficacies of both orally administered octadentate HOPO ligands for reducing tissue Pu in normally fed mice exceeded expectations (Table 1). The presence of food in the upper GI tract somewhat reduced the oral efficacy of **1** but did not lessen the effectiveness of orally administered **2**. The greater reductions of liver and whole body Pu obtained with the mixed ligand, as compared with **1**, are significant at the 95% level of confidence ($p = 0.05$).

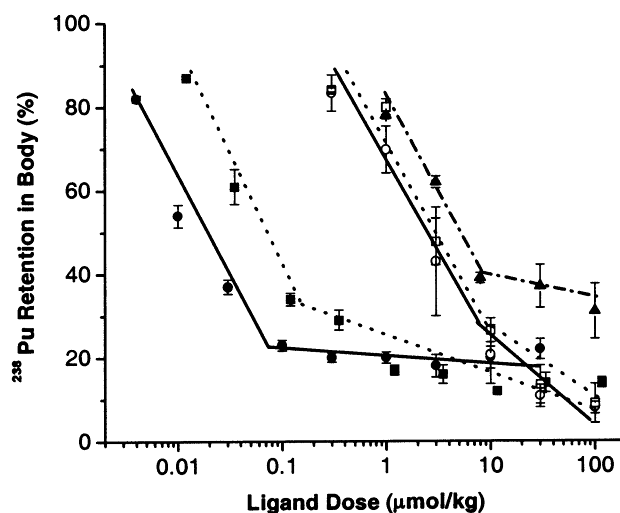


Figure 3. Dose-dependent removal of ^{238}Pu from mice with 3,4,3-LI(1,2-HOPO), 3,4,3-LI(1,2-Me-3,2-HOPO), and $CaNa_3$-DTPA. Key: —●—, 3,4,3-LI(1,2-Me-3,2-HOPO), ip; ···■···, 3,4,3-LI(1,2-HOPO), ip; —○—, 3,4,3-LI(1,2-Me-3,2-HOPO), oral; ···□··· 3,4,3-LI(1,2-HOPO), oral; – · –▲– · –, $CaNa_3DTPA$, ip.

Ligand Dose-Effectiveness. Figure 3 shows, in comparison with injected $CaNa_3DTPA$, the efficacies for reducing total body Pu of **1** and **2**, injected ip or administered orally to fasting mice, over the dose range of 0.003–100 μmol/kg. Ligand dose-effectiveness is described by pairs of intersecting straight lines relating Pu retention (%ID) to the logarithm of the ligand dose (μmol/kg). Each curve segment for the individual ligands

and treatment modes was fitted independently by regression analysis.[26] The remarkable efficacy, as compared with $CaNa_3DTPA$, of low doses of injected **1** has been reported.[18,26,36] Similarly, **2**, injected at doses as low as 0.3 μmol/kg, reduces body Pu significantly more than the injected standard clinical dose of $CaNa_3DTPA$ (30 μmol/kg). Injected **1** is most efficient (%ID Pu removed/μmol/kg ligand) at doses $\leq$0.4 μmol/kg; larger doses promote little additional Pu excretion. The mixed HOPO ligand is most efficient at injected doses $\leq$0.1 μmol/kg; larger doses gradually reduce skeletal Pu, but there is no additional reduction of body Pu. At injected doses of $\leq$0.3 μmol/kg, the mixed HOPO ligand (**2**) is significantly more effective for reducing whole body Pu than **1**.

Even though their GI absorption is estimated to be less than 5%ID,[18,27,37] oral doses of the octadentate HOPO ligands as low as 3 μmol/kg reduce body Pu of fasting mice as much as or more than 30 μmol/kg of injected $CaNa_3DTPA$. The mixed HOPO ligand **2** is somewhat more effective than **1**, when given orally at doses from 0.1 to 100 μmol/kg, but the differences were statistically significant only for the reduction of liver Pu.

Ligand Toxicity. The acute toxicity of injected multidentate ligands containing 1,2-HOPO or Me-3,2-HOPO is caused primarily by chemical tubular nephrosis, the severity of which depends on both ligand structure and dose.[23,25,34] There was no evidence of toxicity in mice given **1** in 10 daily 30 μmol/kg injections[26] or in baboons given eight 30 μmol/kg injections over 26 days.[37] In mice given 10 daily 100 μmol/kg injections, mortality was 2.5%, and the BUN (blood urea nitrogen) was consistently and significantly elevated. On day 11, liver weight was elevated and body weight was depressed (both significant); 13 of 19 kidneys were moderately nephrotic. By day 21, body and tissue weights were normal; 10 of 20 kidneys contained foci and/or radial strands of regenerating tubular epithelium.[26]

There was no evidence of acute toxicity in mice given 10 daily 30 μmol/kg injections of **2**. There were no deaths among mice given 10 daily 100 μmol/kg injections, and body, spleen, and kidney weights and the BUN were consistently within control limits. On day 11, liver weight was significantly elevated; three of 10 kidneys were moderately nephrotic, and five were mildly nephrotic. On day 21, liver weight was the same as the controls; five of 10 kidneys contained rare small foci or radial strands of regenerating tubular epithelium. The acute toxicity of the mixed ligand appears to be somewhat less than that of **1**.

Discussion

Design of the Octadentate Mixed HOPO Ligand. The design of mixed ligands was motivated by the desire to develop new highly effective chelators by introducing Me-3,2-HOPO moieties into a multidentate 1,2-HOPO chelator. Our solution thermodynamic studies reveal extraordinarily high pM values ($\approx$37) for Ce(IV) complexes with tetradentate Me-3,2-HOPO ligands. With the same ionic charge and radius, Ce(IV) is generally accepted as a good model for Pu(IV). The stabilities for Pu(IV) complexes are therefore expected to be substantially the same as those for Ce(IV) complexes with Me-3,2-HOPO ligands.[24] Because the affinities of Me-3,2-HOPO for Fe(III) and Th(IV) at physiological pH exceed those of 1,2-HOPO, multidentate Me-3,2-HOPO ligands were expected to be more effective in vivo Pu chelators than 1,2-HOPO ligands of the same denticity.[22,33] However, direct comparisons could not be made, because the backbones of the available 1,2-HOPO and Me-3,2-HOPO ligands differed.[23,33] To allow for direct comparison of 1,2-HOPO and Me-3,2-HOPO ligands, four pairs of HOPO ligands with identical backbones containing the different HOPO units were synthesized as shown in Figure 4.[26] The new multidentate 1,2-HOPO ligands **12**−**15** were prepared with the same backbones as the four most effective Me-3,2-HOPO ligands **16**−**19** (Figure 4). Each set included an octadentate ligand with the H-shaped PENTEN backbone. Compound **1** was synthesized in the revised procedure reported here. Compound **1** and $CaNa_3DTPA$ were evaluated in mice for in vivo Pu chelation, with emphasis on oral activity and effectiveness at low dose.[26] The four Me-3,2-HOPO ligands were overall more effective than their structural 1,2-HOPO analogues, in agreement with their relative

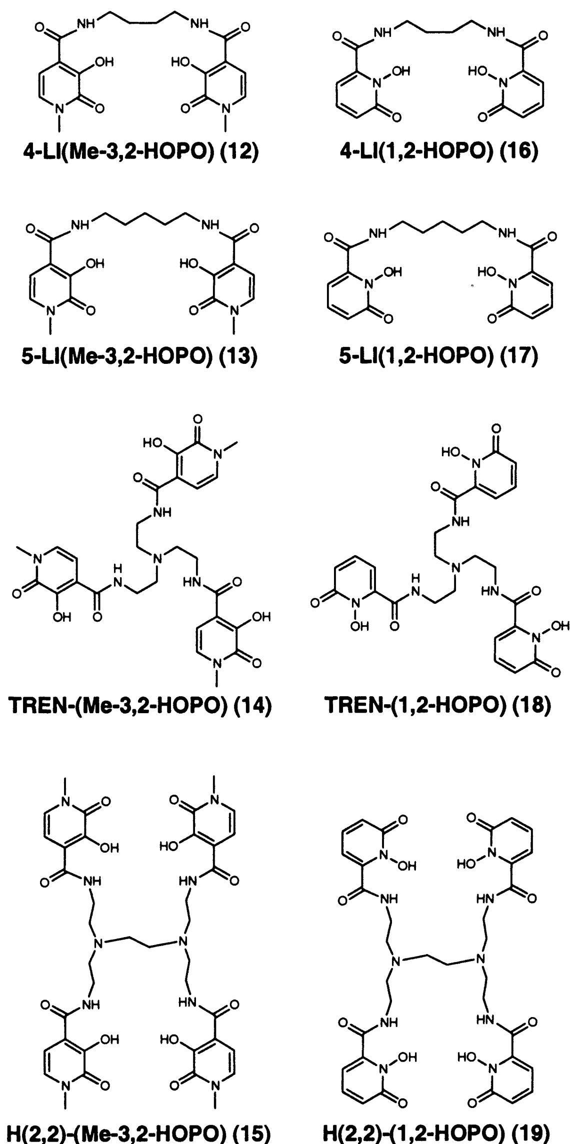


Figure 4. Multidentate 1,2-HOPO and Me-3,2-HOPO ligands.

metal binding affinities. However, octadentate **1**, with its linear spermine backbone, was more effective than either the octadentate H(2,2)-Me-3,2-HOPO (**15**) or the octadentate H(2,2)-1,2-HOPO ligand (**19**) with the H-shaped branched backbone. Superior in vivo Pu complexation by linear **1** is attributed mainly to the linear backbone, which appears to be more suitable for eight coordination as required by Pu(IV).[26]

On the basis of initial evaluation, the effectiveness for in vivo Pu chelation, in particular oral activity, could be improved if Me-3,2-HOPO are introduced into the spermine backbone, thereby incrementally increasing the stability of the Pu complex and the lipophilicity of the ligand.[34] Because 4-LI(Me-3,2-HOPO) (**12**) (Figure 4) has poor solubility in water and is toxic in mice, one would expect 3,4,3-LI(Me-3,2-1,2-HOPO) (**3**) and 3,4,3-LI(Me-3,2-HOPO) (**4**) (Figure 1) be less soluble and probably toxic, because they contain the 4-LI(Me-3,2-HOPO) moiety as the central part. On the other hand, 4-LI(1,2-HOPO) (**16**) (Figure 4) is much more soluble in water and much less toxic than 4LI(Me-3,2-HOPO).[26] The combination of the 4-LI(1,2-HOPO) moiety with Me-3,2-HOPO moieties, 3,4,3-LI(1,2- Me-3,2-HOPO) (Figure 1, **2**), was likely to be superior overall to that of the 4-LI-(Me-3,2-HOPO) moiety with 1,2-HOPO moieties, 3,4,3-LI(Me-3,2-1,2-HOPO) (**3**) (Figure 1). Therefore, the mixed ligand, **2**, was prepared, while mixed ligands **3** and **4** (Figure 1) were not synthesized. In ligand **2**, the Me-3,2-HOPOs are attached by amide linkages to the two terminal primary amines and 1,2-HOPO is similarly attached to the two secondary amines of the spermine scaffold (Figure 1).

Previous Experience with Octadentate Mixed Ligands. Several ligands containing different metal binding units (mixed ligands) have been synthesized in this laboratory. When ligand denticity was increased, for example, by addition of various bidentate binding units to hexadentate desferrioxamine B (DFO B) to make octadentate ligands, the formation constants (log K_f) of the respective Th(IV) and Pu(IV) complexes[38] and their abilities to remove Pu from mice[20,23] were also greatly increased. In contrast, when binding units with greatly differing pH-dependent formation constants for "hard" metal complexes were combined in a single macromolecule with denticity held constant, Pu removal efficacies were usually disappointing. The Pu removal efficacy of DTPA-DX, a mixed ligand containing two hydroxamic acid and three amino acetate groups, was only marginally greater than that of native $CaNa_3$-DTPA.[18] In vivo Pu chelation by TREN-bisacetate-bisMe-3,2-HOPO, a mixed octadentate ligand containing two Me-3,2-HOPO and two amino acetate groups,[33] or by 3,4,3-LI(diCAM-diHOPO), a mixed octadentate ligand containing two 1,2-HOPO and two catecholamide (CAM) groups,[39] was significantly less than that of the structural analogues containing only HOPO units. Clearly, the contributions of all of the individual binding units to the overall pH-dependent formation constant of the Pu(IV) complex must be taken into account. An increase in the overall formation constant and improved effectiveness for Pu chelation can be achieved only by replacing an effective binding group (e.g., 1,2-HOPO) with a more effective unit (e.g., Me-3,2-HOPO).

Comparison of the Mixed HOPO Ligand with 3,4,3-LI(1,2-HOPO). The stability of the Pu(IV) complex with **2** was predicted to be somewhat greater than that of Pu(IV) with **1**. Therefore, the mixed ligand could be expected to be more effective for in vivo chelation of Pu, especially in the low dose range. The biological results agree with that expectation. Compound **2** is more effective than **1** when injected in mice at doses ≤ 0.3 μmol/kg, given orally to fasting mice from 0.3 to 100 μmol/kg, or given orally to normally feeding mice at the standard dose of 30 μmol/kg. This mixed ligand **2** also appears to be somewhat less acutely toxic than **1**. Compounds **2** (0.1 μmol/kg injected or 5 μmol/kg orally) and **1** (0.3 μmol/kg injected or 10 μmol/kg orally) remove significantly more Pu from mice than 30 μmol/kg of injected $CaNa_3$DTPA. The effective injected doses of the two HOPO ligands are about 1% of the standard dose (30 μmol/kg ip), a dose level that did not induce detectable acute toxicity in mice when given daily for 10 days. Thus, both HOPO ligands meet the criterion of low toxicity at doses that are more effective than the standard dose of $CaNa_3$DTPA. Their great effectiveness at low dose and great oral effectiveness (despite low GI absorption) imply that effective treatment regimens can be developed using the HOPO ligands alone or as adjuncts to $CaNa_3$DTPA therapy, which will greatly exceed the amount of Pu excretion that is achievable with $CaNa_3$DTPA alone.

Experimental Section

Synthesis. General. All chemicals were obtained from commercial suppliers (Aldrich or Fisher) and were used as received. 6-Hydroxypicolinic acid was purchased from Fluka. Me-3,2-HOPOBn-thiazolide (**9**) was prepared as previously described.[33] Reactions were carried out under an atmosphere of nitrogen. Flash silica gel chromatography was performed using Merck 40−70 mesh silica gel. Unless otherwise specified, all NMR spectra were recorded at ambient temperature on Bruker DRX 500, AMX 400, or AMX 300 spectrometers in the University of California, Berkeley, NMR laboratory. HPLC analyses were performed on a Varian Pro Star System with a Dynamax-60A C18-reversed phase column using a mobile phase of 65% methanol in water. Microanalyses were performed by the Microanalytical Services Laboratory, College of Chemistry, University of California, Berkeley. Mass spectra were recorded at the Mass Spectrometry Laboratory, College of Chemistry, University of California, Berkeley.

6-Carboxy-1-hydroxy-2(1*H*)-pyridinone (5). Acetic anhydride (100 mL) was mixed with 30% H_2O_2 solution (25 mL) in an ice bath. The mixture was stirred for 4 h until a homogeneous peracetic acid solution formed. This peracetic acid solution was added slowly with stirring to a solution of 6-hydroxy-picolinic acid (Fluka, 25.0 g, 0.18 mol) in a mixture of trifluoroacetic acid (150 mL) and glacial acetic acid (100 mL). (CAUTION! Any solid particles in the mixture will cause vigorous oxygen evolution and lead to an uncontrolled reaction.) The mixture was stirred at room temperature for 1 h and then heated slowly to 80 °C and kept at 80 °C for 10 h. A white precipitate formed during this period, which was collected by filtration, washed with cold methanol, and dried. It was dissolved in aqueous 10% KOH, heated to 80 °C for 6 h, and reprecipitated with concentrated HCl. The product was collected by filtration, washed with water, and dried in a vacuum oven to yield 20.5 g (0.132 mol, 73%); mp 176−177 °C. Anal. Calcd (Found) for $C_6H_5NO_4$: C, 46.46 (46.31); H, 3.25 (3.45); N, 9.03 (9.12). ^{1}H NMR (500 MHz, DMSO-d_6): δ 6.63 (dd, J = 7.0, 1.5 Hz, 1H), 6.71 (dd, J = 9.2, 1.5 Hz, 1H), 7.43 (dd, J = 9.0, 7.1 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 106.9, 120.5, 134.9, 137.3, 157.4, 163.3. IR (KBr pellet): ν 1734 cm^{-1} (br, C=O); 1616 cm^{-1} (m, C=O).

1-Benzyloxy-6-carboxy-2(1*H*)-pyridinone (6).[29] Compound **5** (15.50 g, 0.10 mol) and anhydrous potassium carbonate (27.60 g, 0.20 mol) were mixed with benzyl chloride (15.20 g, 0.12 mol) in methanol (250 mL). The mixture was refluxed for 16 h and filtered, and the filtrate was evaporated to dryness. The residue was dissolved in water (50 mL) and acidified with 6 N HCl to pH 2. The white precipitate was isolated by filtration, washed with cold water, and dried in vacuo to yield 22.3 g (91%) of compound **6**; mp 176−177 °C. Anal. Calcd (Found) for $C_{13}H_{11}NO_4$: C, 63.66 (63.75); H, 4.53 (4.55); N, 5.71 (5.52). ^{1}H NMR (500 MHz, DMSO-d_6): δ 5.26 (s, 2H, CH_2), 6.54 (dd, J = 6.7, 1.1 Hz, 1 H), 6.73 (dd, J = 9.2, 1.6 Hz, 1 H), 7.39−7.51 (m, 6 H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 77.9, 106.0, 124.1, 128.5, 129.1, 129.6, 133.8, 138.7, 140.4, 157.6, 161.7.

1,2-HOPOBn Acid Chloride (7). To a suspension of 1,2-HOPOBn acid (5.0 g, 20 mmol) in toluene or benzene (50−70 mL), excess oxalyl chloride (5.0 g) was added while stirring. Gas bubbles evolved, and the suspension became clear upon the addition of a drop of dimethyl formamide (DMF) as a catalyst. The mixture was then warmed to 60 °C for 4 h, and the solvent was removed by rotary evaporation to leave a pale brown oil. After it was coevaporated twice with toluene (5 mL), the residue was dissolved in dry tetrahydrofuran (THF), passed through a silica gel plug, and eluted with dry THF. The 1,2-HOPOBn acid chloride was obtained as a thick pale yellow oil after the solvent was removed under reduced pressure with a raw yield of 5.0 g (95%). It was used directly for the next reaction without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.32 (s, 2H, CH_2), 6.88 (d, J = 7.0 Hz, 1 H), 6.726 (d, J = 9.0 Hz, 1 H), 7.32−7.51 (m, 6 H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 78.5, 112.2, 128.5, 128.6, 129.4, 130.3, 132.7, 136.4, 140.1, 158.1, 158.8.

3,4,3-LI(1,2-HOPO)Bn (8). To a solution of crude 1,2-HOPOBn acid chloride (**7**) (5.0 g, 19 mmol) and triethylamine (2.5 mL) in dry THF (60 mL), spermine (0.8 g, 4 mmol) was added in three portions while stirring. The mixture was heated to 60 °C (oil bath temperature) in a stoppered 100 mL round flask overnight. The solvent was then removed with a rotary evaporator, and the residue was partitioned into a mixture of water (50 mL) and dichloromethane (50 mL). The organic phase was then washed successively with 1 M NaOH (100 mL), 1 M HCL (100 mL), and saline solution (100 mL) and loaded onto a flash silica column. Elution with 2−6% methanol in dichloromethane allowed separation of the benzyl-protected precursor 3,4,3-LI(1,2-HOPOBn) as white foam (yield 70%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.40−1.85 (m, 16H), 2.81−3.66 (m, 24H), 4.81−5.13 (m, 2H), 4.88−5.05 (m, 2H), 5.15−5.30 (m, 4H), 5.30−5.45 (m, 2H), 6.00−6.46 (m, 4H), 6.55−6.70 (m, 4H), 7.25−7.55 (m, 24H), 8.72−8.95 (m, 2H, NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 23.4, 24.2, 24.5, 24.6, 26.4, 26.6, 26.7, 27.3, 27.7, 36.6, 36.7, 41.8, 42.0, 42.4, 43.7, 46.2, 47.4, 47.7, 48.1, 79.2, 102.7, 104.7, 123.1, 128.3, 128.4, 128.7, 129.2, 129.3, 129.4, 130.0, 130.1, 130.2, 130.3, 130.4, 132.8, 132.9, 133.0, 133.1, 138.2, 142.0, 142.5, 142.6, 143.3, 157.9, 158.1, 158.3, 160.4, 160.6, 161.2, 161.3. MS (FAB$^+$): 1111.5 (MH$^+$).

3,4,3-LI(1,2-HOPO) (1). The precursor **8** was deprotected at room temperature with 1:1 HCl (37%)/glacial HOAc for 4 days. All of the volatiles were removed in vacuo. The residue was dissolved in a minimum amount of water, filtered, and evaporated to dryness (yield 81%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.25−1.87 (m, 8H), 2.81−3.63 (m, 24H), 6.11−6.22 (m, 3H), 6.29−6.34 (m, 2H), 6.48−6.58 (m, 4H), 7.31−7.42 (m, 4H), 8.82 (q, J = 7.2 Hz,1H), 8.91 (q, J = 7.2 Hz, 1H). ^{13}C NMR (125 MHz, D_2O): δ 23.1, 23.7, 24.2, 25.4, 26.7, 36.6, 36.8, 41.9, 44.0, 45.8, 47.7, 48.1, 106.2, 106.5, 108.7, 109.0, 118.9, 120.1, 138.8, 139.0, 139.6, 140.0, 140.5, 159.2, 161.1, 161.2, 162.2. MS (FAB$^+$): 751 (MH$^+$). Anal. for $C_{34}H_{38}N_8O_{12}\cdot H_2O\cdot 2HCl$ (FW = 841.68), Calcd (Found): C, 48.52 (48.16); H, 5.03 (4.82); N, 13.31 (13.23).

3,4,3-LI-Bis(Me-3,2-HOPOBn) (10). To a solution of spermine (0.50 g, 2.5 mmol) in dry dichloromethane (60 mL), Me-3,2-HOPO-thiazolide (**9**) (2.0 g, 5.5 mmol) was added while stirring. The mixture was stirred at room temperature overnight and then washed with 1 M KOH solution (30 mL × 3). The organic phase was then dried in vacuo to afford a pale brown oil as raw product (yield 85%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.43 (m,br, 4H), 1.50 (qint, J = 6.7 Hz, 4H), 2.44 (t, J = 6.7 Hz, 8H), 3.25 (q, J = 6.7 Hz, 4H), 3.57 (s, 6H), 5.34 (s, 4H, OCH_2), 6.72 (d, J = 7.2 Hz, 2H), 7.10 (d, J = 7.2 Hz, 2H), 7.21−7.55 (m, 10H), 8.09 (t, J = 5.3 Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 27.2, 28.7, 37.4, 37.6, 46.8, 49.2, 74.5, 104.6, 128.5, 128.6, 128.8, 130.7, 132.0, 136.1, 146.0, 159.4, 163.1. MS (FAB$^+$): 685.3 (MH$^+$).

3,4,3-LI(1,2-Me-3,2-HOPO)Bn (11). The raw 3,4,3-LI-Bis-(Me-3,2-HOPO Bn) (**10**) (0.82 g, 1.2 mmol) was dissolved in dry THF (50 mL) containing triethylamine (1.2 mL) and slowly added to a solution of raw 1,2-HOPOBn acid chloride (1.7 g, 6.4 mmol) in dry THF (60 mL) over 4 h while stirring. The reaction mixture was heated at 60 °C overnight. After the solvents were removed, the residue was partitioned into a mixture of water (50 mL) and dichloromethane (50 mL). The organic phase was washed successively with 1 M NaOH (100 mL), 1 M HCl (100 mL), and saline water (100 mL) and loaded onto a flash silica column. Elution with 3−8% methanol in dichloromethane allowed the separation of the benzyl-protected precursor as white foam (yield 75%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.91−1.64 (m, 16H), 2.65−3.45 (m, 24H), 3.54 (s, 6H), 4.90−5.02 (m, 4H), 5.20−5.6 (m, 8H), 5.80−6.10 (m, 4H), 6.54−6.80 (m, 8H), 7.07−7.11 (m, 4H), 7.15−7.51 (m, 20H), 7.75 (m, br, 2H), 7.99 (m,br, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 24.9, 26.3, 26.6, 27.6, 36.4, 36.6, 37.3, 41.8, 43.6, 45.6, 47.8, 74.3, 74.4, 74.7, 74.8, 78.8, 79.0, 104.1, 104.2, 104.3, 104.4, 122.5, 128.1, 128.2, 128.4, 128.5, 128.7, 129.8, 129.9, 131.9, 133.1, 142.8, 157.8 157.9, 159.1, 159.2, 161.4, 163.0, 163.12. MS (FAB$^+$): 1139.8.

3,4,3-LI(1,2-Me-3,2-HOPO) (2). The benzyl-protected **11** was deprotected in the same manner as 3,4,3-LI(1,2-HOPO). The raw product was dissolved in a minimum amount of distilled water, pure 3,4,3-LI(1,2-Me-3,2-HOPO) was precipitated as beige solid upon cooling, and it was filtered and dried under vacuum (yield 73% based on spermine). Analytical purity (≥99%) was determined by HPLC (Dynamax RP-C18, 15 cm × 4.6 mm ID, 8 μm particles, mobile phase 65% MeOH/35% H_2O). ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.21−1.85 (m, 16H), 2.85−3.35 (m, 24H), 3.45 (s, 6H), 6.10−6.52 (m, 6H), 7.10−7.41 (m, 4H), 8.32 (m, 1H), 8.48 (m, 1H). ^{13}C NMR (125 MHz, D_2O/NaOD): δ 23.8, 24.0, 24.1, 24.5, 24.8, 25.0, 26.8, 27.0, 28.1, 28.3, 35.6, 35.7, 36.4, 36.5, 37.5, 42.9, 43.0, 43.1, 44.7, 44.9, 45.1, 46.7, 48.3, 48.4, 48.6, 105.7, 106.1, 106.2, 106.4, 106.9, 107.1, 114.8, 114.9, 115.0, 115.8, 116.1, 119.6, 119.9, 132.7, 132.8, 133.0, 142.8, 143.1, 160.3, 161.9, 162.2, 164.4, 164.4, 165.6, 165.7, 169.4, 169.6. MS (FAB$^+$): 779.4 (MH$^+$). Anal. for $C_{36}H_{42}N_8O_{12}\cdot 3H_2O\cdot 2HCl$ (905.76), Calcd (Found): C, 47.74 (48.05); H, 5.56 (5.22); N, 12.37 (12.16).

Biological Evaluation. Ligand Efficacy. Under anesthesia, groups of five female Swiss−Webster mice (87 ± 9 days old, 34 ± 2 g)[40] were injected intravenously (iv) in a warmed lateral tail vein with 0.2 mL of a solution containing 925 Bq (0.025 μCi) of ^{238}Pu(IV) in 0.008 M sodium citrate plus 0.14 M NaCl, pH 4. Solutions of the HOPO 1igands (0.002 M) were prepared in 0.14 M NaCl, pH 7.4. $CaNa_3$DTPA was obtained as a 25% solution. The standard ligand dose (30 μmol/kg) and dilutions for dose−effectiveness studies were delivered to a 35 g mouse in 0.5 mL of ligand solution. Ligand-to-metal molar ratio at standard ligand dose was 2 × 10^5 for ^{238}Pu. Ligand doses were standardized by adjusting the injected volume to the body weight of each mouse.

In parenteral injection studies, ligands were injected ip at 1 h after iv injection of Pu, and the mice were killed at 24 h. In oral studies, ligands were administered po 3 min after iv injection of Pu to mice previously fasted for 16 h or to normally fed mice, and the mice were killed at 24 h. In studies of dose−effectiveness for Pu removal, Pu-injected mice were injected ip at 1 h or intubated orally at 3 min (mice fasted 16 h) with ligand at doses ranging from 0.001 to 100 μmol/kg, achieving ligand:Pu molar ratios from 7.0 to 7 × 10^5, and the mice were killed at 24 h. Groups of five normally fed or fasted Pu-injected

mice were given 0.5 mL of 0.14 M NaCl ip or orally and killed at 24 h to define the control distribution and excretion of Pu. Each five mouse experimental or control group was housed together in a plastic cage lined with a 0.5 cm layer of highly absorbent low ash pelleted cellulose bedding. Water was available ad lib, and mouse chow was given 4 h after the Pu injection. The combined control groups were representative of the several Pu solutions used and the several shipments of mice. The replicated Pu removal studies are, in addition, representative of the several synthetic batches of the ligands.

Details of autopsy procedures, tissue and excreta collection and processing, and α particle detection (liquid scintillation counting, Packard Tri-Carb model B4430), and methods of data managenent have been previously reported.[14,17,26,33] The Pu distribution data are expressed as mean ± SD percent of injected Pu (%ID) in individual tissues and the whole body (sum of tissues), where $SD = [\sum dev^2(n-1)^{-1}]^{1/2}$.[41] For tissue and body Pu, n is the number of mice in the group. Excreta were pooled for each five mouse group, and SD was calculated for excreta only for experiments and control groups replicated more than twice. "Significant" is used throughout in the statistical sense (Student's t-test, $p \cdot 0.01$).[41] That criterion of statistical significance is sufficiently conservative that application of the Bonferroni correction[42] was not needed for comparing multiple individual test groups to the same group of controls.

Acute Ligand Toxicity. The acute toxicity of **1** in mice has been reported.[26,34] Using the reported methods, 20 mice were injected subcutaneously daily for l0 days with 30 or 100 μmol/kg of **2**. Toxicity was assessed from the combined data, and observations were obtained for 10 mice each on days 11 and 21: survival; body, liver, spleen, and kidney weight; BUN; gross pathology of internal organs; microscopic pathology of liver, kidneys, and injection site.

Acknowledgment. This research was supported by the Director, Office of Energy Research, Office of Basic Energy Science, Chemical Sciences Division, U.S. Department of Energy under Contract No. DE-AC-03-76F00098 and a grant from the National Institute of Environmental Health Science ES.-02698. We thank Emily Dertz for helpful discussions.

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JM010564T