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Design and Synthesis of Pentahydroxylhexylamino Acids and Their Effect on Lead Decorporation

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A series of enantiopure pentahydroxylhexylamino acids **4a**−**t** were synthesized via an improved one-pot-three-step procedure. Their potential as antagonists for lead intoxication was investigated both *in vitro* and *in vivo*. Lead decorporation assays *in vivo* confirmed that after treatment with **4a**−**t**, the levels of lead in treated mice were significantly reduced in the liver, kidney, bone, and brain compared to those in the control group. In addition, the lead levels in feces and urine were significantly higher after treatment with **4a**−**t** than those of the control group. In particular, the lead decorporation potency of compounds **4b**, **4i**, **4j**, and **4s** were comparable or better than that of DL-penicillamine. Furthermore, new chelating agents did not affect the levels of endogenous essential metals. The stability constants of the formed lead complexes of **4a**−**t** were determined by potentiometric titration. It seems that the therapeutic efficiency of the lead chelating agents depends on factors that affect the stability constants of the formed lead complexes. The membrane permeability of representative compounds was evaluated in a Caco-2 cell monolayer. A good correlation between *in vitro* results and *in vivo* lead decorporation capacity of the chelating agents was observed. Some of these new pentahydroxylhexylamino acids (**4b**, **4i**, **4j**, and **4s**) may be developed as effective lead chelating agents.

Introduction

There is increasing concern with regard to lead exposure in young children and pregnant women. Even at very low levels, lead may affect the development of fetuses and children. There is strong evidence that exposure to lead, even at blood levels as low as 10−15 μg/dL or possibly even lower, can cause undesirable development outcomes in human fetuses and children. Specifically, elevated levels of lead may lead to impairments of the central nervous system, such as delayed cognitive development, reduced IQ scores, impaired hearing, and neurobehavioral deficiencies (*1*). In general, chelation therapy is usually considered if the lead levels in the blood are above normal.

Amino-carboxyl and mercapto-based compounds are two types of drugs used in current lead chelation therapy. The amino-carboxyl compounds include calcium disodium ethylenediamine tetraacetic acid ($CaNa_2EDTA$) and diethylenetriaminepentaacetic acid (DTPA) (*2*). The mercapto compounds include DL-penicillamine (DL-PA) and *meso*-2,3-dimercaptosuccinic acid (DMSA) (*3*). Most of these chelating agents have side effects, such as poor selectivity, high toxicity, and weak ability to cross the cellular membrane (*4*−*10*). As a result, it greatly limits their therapeutic effects on lead poisoning. Therefore, it is of practical significance to develop new, highly effective and safer chelating agents to solve the problem of worldwide lead pollution.

Previous studies in our laboratory have demonstrated that the exogenous glycosylamines, which resulted from reducing glycosylation, are characteristic in lead decorporation. We have demonstrated that as lead decorporation substances, glycoamino acids may be important. (*11*, *12*) Nearly 50 years ago, Borsook and co-workers were able to isolate some fructose-amino acids from hog liver by using $Pb(C_2H_3O_2)_2 \cdot 3H_2O$ as a precipitant. They discovered the characteristic chemical and physical properties and their stimulation of amino acid incorporation *in vitro* into proteins of rabbit reticulocytes. Borsook's observation suggested that as endogenous substances with lead decorporation activity, the fructose-amino acids exhibit low toxicity and cell membrane permeability (*13*). To simulate the effect of natural fructose-amino acids on lead decorporation, in this study, we developed a new class of lead chelating agents, *N*-(2,3,4,5,6-pentahydroxylhexyl)amino acids. Because both glucose and amino acids are endogenous substances, the new chelating agents are expected to have minimal toxicity. Herein, we report the design, synthesis, and biological evaluations of pentahydroxylhexyl amino acid as an antagonist of lead intoxication.

Experimental Procedures

Materials and Methods. All of the reactions were carried out under nitrogen (1 bar). ^1H (300 and 500 MHz) and ^{13}C (75 and 125 MHz) NMR spectra were recorded on Bruker AMX-300 and AMX-500 spectrometers for solutions D_2O, DMSO-d_6, or $CDCl_3$

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with tetramethylsilane as the internal standard. IR spectra were recorded with a Perkin-Elmer 983 insturment. ESI/MS was determined on Waters ZQ2000 LC-MS, and TOF-MS was recorded on MDS SCIEX QSTAR. Melting points were measured on a XT5 hot stage microscope (Beijing Key Elector-Optic Factory). Optical rotations were determined on a Schmidt and Haensch Polartronic D instrument at 20 °C. All L-amino acids and α-D-glucose were purchased from China Biochemical Corp. TLC was made with Qingdao silica gel GF_{254}. Chromatography was performed with Qingdao silica gel H_{60} or Sephadex-LH_{20}. All solvents were distilled and dried before use by referring to literature procedures.

General Procedure for the Synthesis of 4a−t. To a mixture of 0.40 g (10 mmol) of NaOH in 3 mL of MeOH/H_2O (1:1), 10 mmol of L-amino acid was added. The mixture was then stirred at room temperature until L-AA was completely dissolved. Then, 1.80 g (10 mmol) of D-glucose was added to the above mixture. The mixture continued stirring at room temperature until D-glucose completely dissolved. Under argon protection, the mixture continued stirring at 50−60 °C for 5 h, and compound **2** that formed was reduced *in situ* by 1.14 g (30 mmol) of sodium borohydrate at room temperature for 96 h. The reaction mixture was then cooled to 0 °C and the pH was adjusted to 2.5 using concentrated HCl to afford intermediate compound **3**. The precipitates were removed by filtration, and the filtrate was concentrated under vacuum. The residue was diluted with anhydrous ethanol and the precipitates formed were again removed by filtration. The procedure was repeated five times, and the residue was dissolved in 10 mL of water, which was then loaded onto the column of acidic ion-exchange resin and eluted with 3% aqueous solution of *N*-methylmorpholine to give the desired compounds **4a−t**. (NMR data are included in Supporting Information.)

Lead Decorporation Assay. The lead decorporation assay (*11*, *12*, *14*, *15*) was performed as follows. Twenty-two groups (10 each of male Kunming mice weighing 23 ± 2 g) of mice were loaded with lead via i.p. injections of 8.2 mg/kg of $Pb(C_2H_3O_2)_2 \cdot 3H_2O$ in 0.2 mL of water per day for seven consecutive days. After a 2-day interval, one group served as the control and was daily given i.p. injections of 0.2 mL of 0.9% saline instead of the chelating agent. The positive control group was daily given i.p. injections of 0.4 mmol/kg of DL-PA in 0.2 mL of water. The remaining groups were daily given i.p. injections of compounds **4a−t** (0.4 mmol/kg in 0.2 mL of water) for five consecutive days.

On each day, 2 h after the administration of chelating agents, urine samples of each group were continually collected for 5 h. After the administration of chelating agents, the fecal samples of each group were continually collected for 24 h. Twenty-four hours after the last administration of the chelating agent, all animals were sacrificed by diethyl ether anesthesia and immediately dissected to obtain liver, kidney, brain, and left femur samples.

All biosamples were digested in $HClO_4$ and HNO_3 (1:3) on a heating block, dried at 80 °C, and redissolved in 1% nitric acid to determine the content of lead using a Varian SpectrAA-220Z atomic absorption spectrometer in a graphite furnace. The variety of trace metals was determined by synchrotron X-ray fluorescence.

Stability Constants. The potentiometric pH titrations were carried out with a PHS-2 pH meter in water solution at 25 ± 0.1 °C and 0.20 ionic strength (maintained with KCl) under high purity N_2 flow. The acidic formation constants of the chelating agents were determined by preparing 15 mL of 0.01 M ligand solutions (2.00 mL of 0.107 M hydrochloric acid, 6.00 mL of 0.5 M potassium chloride, and 7.00 mL of water) and carrying out pH titrations with a 0.060 M carbonate-free sodium hydroxide solution. With the same procedures, 0.70 mL of 0.109 M of $Pb(NO_3)_2$ solution was added to the solution system (final volume of 15 mL) to determine the stability constants of the lead−chelating agent complex. The concentration ratio of the metal to the ligand was 1:2 in each solution. The TITFIT Basic program was used to calculate the acidic formation constants of the ligand and the stability constants of the lead−chelating agent complex (*25*). The activity of the hydrogen ion was determined by titrating 15 mL of standard hydrochloric acid solution. The stability constants were calculated by the equation $n = \{TL - [L] \times (1 + \beta_{1H} \times [H] + 2 \times \beta_{2\,H} \times [H])\}/TM$, wherein $[L] = \{(2 - a) \times TL + [OH] - [H]\}/(\beta_{1H} \times [H] + 2 \times \beta_{2H} \times [H](2))$, and [L] = concentration of free ligand, n = formation function, a = neutralization ration, TL = total concentration of the ligand, TM = total concentration of metal ion, [H] = concentration of H^+, [OH] = concentration of OH^-, β_{1H} = first-order constant of proton addition, and β_{2H} = second-order constant of proton addition. [L] and n were calculated using the of origin 6.0, pK_1, pK_2, log K_1, and log K_2.

Apparent Permeability Coefficients. Apparent permeability coefficients (*24*, *26*) were calculated as described. Caco-2 cells were purchased from the American Type Culture Collection, Rockville, MD. The cells were cultivated on polycarbonate filters (transwell cell culture inserts; diameter, 12 mm; mean pore size, 3.0 μm) as described elsewhere (*24*, *26*). Caco-2 cells grown on filter supports for 21 days were used for all transport studies, and the integrity of monolayers was routinely checked by measurements of transepithelial electrical resistance (approximately 700 Ω cm^2).

All absorption studies were performed in Hank's balanced salt solution (HBSS). The stock standard solution of **4a**,**b**,**i**,**j**,**m**,**n**,**s**,**t** with a concentration of 10^{-2} mmol/mL was prepared by dissolving **4a**,**b**,**i**,**j**,**m**,**n**,**s**,**t** in HBSS and storing at 4 °C. The stock standard solution of **4a**,**b**,**i**,**j**,**m**,**n**,**s**,**t** was diluted by HBSS to prepare a series of standard solutions (final concentrations of 8.0×10^{-3} mmol/mL, 5.0×10^{-3} mmol/mL, 3.0×10^{-3}mmol/mL, 1.8×10^{-3} mmol/mL, 1.2×10^{-3}mmol/mL, 8×10^{-4} mmol/mL, and 8×10^{-5} mmol/mL). Onto the HPLC system, 10 μL of standard solution was injected to establish the linear equation of peak area and concentration for **4a**,**b**,**i**,**j**,**m**,**n**,**s**,**t**.

HPLC-grade acetonitrile was purchased from Fisher Chemicals. Deionized water was prepared with a Milli-Q water purification system. Methanol, ammonium formate, and formic acid were purchased from Beijing Chemical Co. (China). The HPLC column used in this study is an Agilent Zorbax SB-C_{18} reverse-phase column (2.1×150 mm, 5 μm). A LC flow rate of 0.35 mL/min was used. The mobile phase consisted of deionized water (A) and acetonitrile (B). The gradient program was that after 3 min, 15% B was increased to 45% within 15 min, increased to 75% within 3 min, and held for another 5 min.

The stock standard solutions of **4a**,**b**,**i**,**j**,**m**,**n**,**s**,**t** were diluted with HBSS to prepare test solutions at a final concentration of 4×10^{-3} M. In the apical to basolateral direction, transport was initiated by adding the test solutions (total AP volume, 0.5 mL) to the apical compartment of inserts held in transwells containing 1.5 mL of HBSS (basolateral compartment). For transporting in the basolateral to apical direction, 1.5 mL of the test solution was added to the basolateral compartment, and 0.5 mL of HBSS as the receiving solution was added into the apical side of the monolayers.

The monolayers were incubated in air at 37 °C under 95% humidity. At 30, 60, 90, and 120 min, samples were withdrawn from the receiving side. The concentrations of the samples were determined by HPLC analysis under the conditions mentioned above and using the corresponding linear equation. Then the curve of concentration versus time was obtained. Through the curve and by using the initial rate method, dQ/dt was obtained. The resistance of monolayers was checked at the end of each experiment. Apparent permeability coefficients (P_{app}) were calculated according to the following equation:

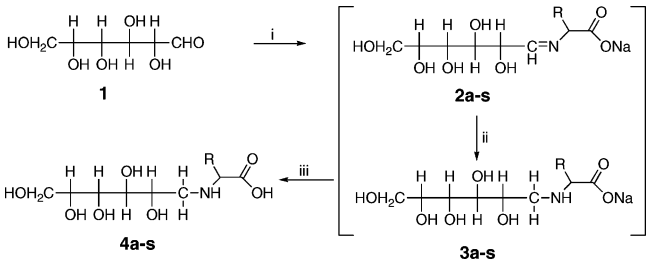
$$P_{app} = dQ/dt \cdot 1/(AC_0)$$

wherein Q represents the total amount of the drug that permeated, dQ/dt is the permeability rate, C_0 is the initial concentration in the domer chamber, and A is the surface area of the monolayer (1 cm^2).

Results and Discussion

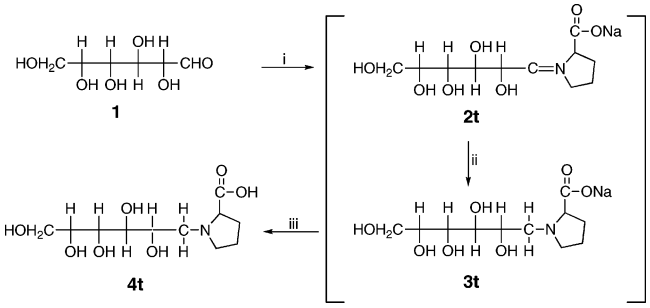
Synthesis of 4a−t via a One-Pot-Three-Step Procedure. The synthetic route of pentahydroxylhexylamino acids **4a−t** was straightforward as shown in Schemes 1 and 2. Pentahydroxylhexylamino acids **4a−s** were prepared via a one-pot-three-step

Scheme 1. Synthetic Route to 4a−s[a]



[a] (i) L-amino Acid, NaOH, Argon, 50−60 °C; (ii) $NaBH_4$, room temperature; (iii) hydrochloric acid. For **2**−**4a** R═$CH_2CH_2CO_2H$; **2**−**4b** R═$CH_2CH_2CH_2CO_2H$; **2**−**4c** R═CH(OH)CH_3; **2**−**4d** R═4-hydroxylphenylmethyl; **2**−**4e** R═CH_2OH; **2**−**4f** R═CH_2CONH_2; **2**−**4g** R═$CH_2CH_2CONH_2$; **2**−**4h** R═H; **2**−**4i** R═CH_2SH; **2**−**4j** R═$CH_2CH_2SCH_3$; **2**−**4k** R═CH_3; **2**−**4l** R═$C_6H_5CH_2$; **2**−**4m** R═$CH_2CH(CH_3)CH_3$; **2**−**4n** R═CH(CH_3)CH_2CH_3; **2**−**4o** R═1*H*-indole-3-methyl; **2**−**4p** R═CH(CH_3)CH_3; **2**−**4q** R═$CH_2CH_2CH_2CH_2NH_2$; **2**−**4r** R═$CH_2CH_2CH_2NHC(NH)NH_2$; **2**−**4s** R═1*H*-imidazole-4-methyl.

Scheme 2. Synthetic Route to 4t[a]



[a] (i) L-Pro, NaOH, argon, 50−60 °C; (ii) $NaBH_4$, room temperature; (iii) hydrochloric acid.

procedure: (i) condensation of glucose and amino acids; (ii) reduction of the Schiff bases **2a**−**s**; and (iii) acidification of the sodium carboxylates **3a**−**s**. It was known that the glucosylation of amino acids generates an equilibrium mixture of glucosyl-amino acids because of the Amadori rearrangement with a complex's spectra of the equilibrium mixture on ^{1}H NMR. However, in our case, ^{1}H NMR spectra were quite clean and simple with a single product, *N*-(2,3,4,5,6-pentahydroxylhex-1-yl)-L-amino acid, suggesting that no Amadori rerrangement occurred during glucosylation. This result was further confirmed by HPLC analysis.

One unique feature of our simplified one-pot-three-step synthesis is that it can be applied to a variety of natural amino acids, and product yields slightly vary with the nature of the amino acid side chain. In this study, we have successfully demonstrated a concise and efficient procedure by using one-pot synthesis to combine a three-step reaction without tedious separation steps.

Lead Decorporation Study *in Vivo*. To evaluate the potential of pentahydroxylhexylamino acids **4a**−**t** as antagonists for lead intoxication, the structure−activity relationship studies were evaluated via a series of *in vivo* and *in vitro* assays according to our previously reported methods (*11*, *12*, *14*, *15*). The results are summarized in Table 1−4.

The administration of $Pb(C_2H_3O_2)_2{\cdot}3H_2O$ for 7 days caused elevated lead levels in rat liver, kidney, brain, and bone (*11*, *12*, *14*, *15*). Treatment with chelating agent DL-penicillamine (DL-PA) and pentahydroxylhexylamino acids **4a**−**t** for 5 consecutive days reduced lead levels in the liver, kidney, bone, and brain (Table 1). After the treatment with **4a**−**t**, compared with control group, compounds **4b**,**e**,**f**,**j**,**i**,**m**,**q**,**s**,**t** significantly reduced the hepatic lead levels and the lead mobilization

Table 1. Tissue Lead Levels ($X \pm$ SE μg/g) of Mice after Chelating Treatment[a]

group	bone	brain	kidney	liver
control	20.33 ± 3.75	2.10 ± 0.45	9.14 ± 1.84	7.14 ± 2.63
DL-PA	16.44 ± 3.90[b]	1.31 ± 0.84[b]	7.98 ± 2.49	3.76 ± 0.45[c]
4a	18.90 ± 2.69	2.65 ± 0.46	7.98 ± 1.90	5.92 ± 1.76
4b	11.32 ± 3.35[c,e]	1.53 ± 0.22[b]	7.38 ± 2.78[b]	3.83 ± 0.67[b]
4c	17.20 ± 3.43	1.17 ± 0.15[c]	6.31 ± 1.13[b]	7.66 ± 1.21
4d	15.88 ± 3.18[c]	0.99 ± 0.55[c]	8.93 ± 2.29	7.46 ± 1.72
4e	20.97 ± 2.54	1.94 ± 0.16	7.67 ± 1.75	4.44 ± 1.00[c]
4f	14.60 ± 3.97[c]	1.43 ± 0.27[b]	8.77 ± 2.67	4.55 ± 1.45[b]
4g	17.57 ± 3.04	1.06 ± 0.60[c]	8.57 ± 3.38	5.24 ± 1.95
4h	20.22 ± 2.96	2.19 ± 0.26	7.54 ± 1.71	5.88 ± 1.43
4i	10.38 ± 1.59[c,e]	1.11 ± 0.13[c]	2.97 ± 0.47[c,e]	5.80 ± 1.12
4j	16.37 ± 2.77[c]	0.93 ± 0.10[c,d]	6.10 ± 0.85[c]	4.18 ± 0.55[c]
4k	15.11 ± 3.19[c]	1.19 ± 0.17[c]	6.09 ± 1.94[c]	6.78 ± 0.86
4l	13.88 ± 3.93[c,d]	1.09 ± 0.65[b]	7.13 ± 2.49	3.16 ± 0.90[c]
4m	14.70 ± 1.25[c]	1.16 ± 0.64[b]	6.50 ± 1.98[b]	4.27 ± 1.17[b]
4n	16.68 ± 2.82[b]	1.22 ± 0.12[c]	4.95 ± 1.50[c]	6.69 ± 1.45
4o	14.42 ± 2.92[c]	1.29 ± 0.49[c]	7.69 ± 2.23	6.80 ± 2.08
4p	20.24 ± 1.97	1.93 ± 0.29	6.82 ± 1.09[b]	5.38 ± 1.26
4q	18.54 ± 4.28	1.15 ± 0.33[c]	8.69 ± 2.10	4.68 ± 1.70[b]
4r	19.00 ± 6.08	1.37 ± 0.53[c]	6.94 ± 2.37[b]	5.63 ± 1.94
4s	16.70 ± 2.53[b]	1.06 ± 0.23[c]	4.11 ± 1.96[c,e]	2.99 ± 0.64[c,e]
4t	16.61 ± 2.33[b]	1.63 ± 0.69	8.95 ± 3.73	4.45 ± 1.23[b]

[a] The data are represented by micrograms per gram of tissue weight ($X \pm$ SE), where $n = 10$. [b] Data are significantly less than that with the control $P < 0.05$. [c] Data are significantly less than that with the control $P < 0.01$. [d] Data are significantly less than that with DL-PA $P < 0.05$. [e] Data are significantly less than that with DL-PA $P < 0.001$.

Table 2. Excretion Lead Levels of Mice after Chelating Treatment[a]

group	fecal lead	urinary lead
control	1.60 ± 0.54	0.29 ± 0.18
DL-PA	6.15 ± 2.87[b]	2.34 ± 0.89[b]
4a	5.28 ± 1.72[b]	1.17 ± 0.32[b]
4b	5.43 ± 1.92[b]	4.37 ± 1.17[b,d]
4c	5.93 ± 0.85[b]	1.27 ± 0.27[b]
4d	4.59 ± 3.17[b]	4.20 ± 1.41[b,d]
4e	5.21 ± 1.71[b]	0.95 ± 0.35[b]
4f	4.80 ± 1.12[b]	2.65 ± 0.97[b]
4g	4.83 ± 2.74[b]	4.37 ± 1.45[b,d]
4h	5.88 ± 1.43[b]	0.97 ± 0.17[b]
4i	8.32 ± 2.53[b]	1.60 ± 0.26[b]
4j	5.31 ± 1.75[b]	1.32 ± 0.20[b]
4k	6.42 ± 0.80[b]	1.43 ± 0.23[b]
4l	7.48 ± 1.69[b]	4.83 ± 1.16[b,d]
4m	7.48 ± 2.99[b]	4.03 ± 0.75[b,d]
4n	6.32 ± 2.53[b]	1.21 ± 0.22[b]
4o	3.24 ± 1.35[b]	1.52 ± 0.32[b]
4p	5.24 ± 1.87[b]	1.52 ± 0.32[b]
4q	5.01 ± 1.56[b]	3.69 ± 1.27[b,c]
4r	4.51 ± 1.77[b]	1.97 ± 1.18[b]
4s	5.35 ± 1.89[b]	3.04 ± 1.26[b]
4t	5.42 ± 2.91[b]	2.41 ± 1.36[b]

[a] The data are represented by micrograms per gram of tissue weight ($X \pm$ SE μg/g), where $n = 10$. [b] Data are significantly less than that with the control $P < 0.001$. [c] Data are significantly less than that with DL-PA $P < 0.05$. [d] Data are significantly less than that with DL-PA $P < 0.001$.

efficiency. In particular, compound **4s** exhibited greater activity in the liver than DL-PA, causing a rapid decrease in the hepatic lead levels (**4s** with 2.99 ± 0.64 μg/g vs DL-PA with 3.76 ± 0.45 μg/g; $p < 0.001$). Similarly, after the treatment with **4a**−**t**, compared with control group, compounds **4b**,**c**,**i**,**j**,**k**,**m**,**n**,**p**,**r**,**s** significantly reduced the renal lead level, suggesting that these agents are effective for the removal lead in kidneys. Particularly, compounds **4i** and **4s** were more potent than DL-PA in terms of the removal of the lead burden in kidneys (**4i** with 2.97 ± 0.47 μg/g and **4s** with 4.11 ± 1.96 μg/g vs DL-PA with 7.98 ± 2.49 μg/g; $p < 0.001$). Compared with the control, 12 of the newly synthesized compounds **4a**−**t** significantly decreased bone lead levels. In particular, **4b**, **4i**, and **4l** were more potent

Table 3. Stabilities of Pb^{2+}−Chelating Agent Complexes

complex	log K_1	log K_2
Pb^{2+}−**4a**	3.40	2.21
Pb^{2+}−**4b**	4.67	3.69
Pb^{2+}−**4c**	3.85	3.58
Pb^{2+}−**4d**	4.33	3.50
Pb^{2+}−**4e**	4.12	3.28
Pb^{2+}−**4f**	4.91	3.67
Pb^{2+}−**4g**	4.45	2.31
Pb^{2+}−**4h**	3.66	2.13
Pb^{2+}−**4i**	11.87	8.11
Pb^{2+}−**4j**	5.01	3.78
Pb^{2+}−**4k**	4.78	3.72
Pb^{2+}−**4l**	4.96	3.72
Pb^{2+}−**4m**	4.63	3.44
Pb^{2+}−**4n**	4.56	3.40
Pb^{2+}−**4o**	4.00	3.52
Pb^{2+}−**4p**	3.85	3.48
Pb^{2+}−**4q**	3.83	3.50
Pb^{2+}−**4r**	3.92	3.55
Pb^{2+}−**4s**	5.59	4.13
Pb^{2+}−**4t**	3.24	2.39

Table 4. Apparent Permeability Coefficients of the Chelating Agents[a]

	$P_{app} \times 10^{-6}$ (cm/s)		
chelating agents	A → B	B → A	A → B/B → A
DL-PA	14.22	12.75	1.12
4a	5.48	4.96	1.10
4b	15.22	5.30	2.87
4i	17.54	5.46	3.21
4j	13.38	5.55	2.41
4m	14.05	4.88	2.88
4n	13.96	4.70	2.97
4s	16.31	5.27	3.09
4t	7.00	5.26	1.33

[a] The standard deviations were generally less than 10% ($n = 4$). A → B is from the apical side to the basolateral side, and B → A is from the basolateral side to the apical side.

than DL-PA in the removal of lead in the bone (**4b** with 11.32 ± 3.35 μg/g, **4i** with 10.38 ± 1.59 μg/g, and **4l** with 13.88 ± 3.93 μg/g vs DL-PA with 16.44 ± 3.90 μg/g; $p < 0.001$ and $p < 0.05$, respectively). Compared with control, 16 of the new synthesized compounds significantly reduced brain lead levels. These compounds displayed a potency comparable to that of DL-PA in lead burden removal in the brain. For example, **4j** was more potent than DL-PA in the removal of lead in the brain (**4j** with 0.93 ± 0.10 μg/g vs DL-PA with 1.31 ± 0.84 μg/g; $p < 0.05$).

We noticed lead levels in soft tissues reduced fairly rapidly after the chelation treatment with agents **4a**−**t**; however, lead levels in bone tissue still remained high. This indicates that chelating and removal of lead from the bone is a slow process, and it may require prolonged treatment with the chelating agents and/or more time after chelation. This further emphasized the important need to develop highly potent lead chelating compounds of low toxicity.

A major advantage of the new chelating agents is that they can significantly decrease the lead level in the rat brain. This is a big advantage compared to some existing chelating agents, for example, $CaNa_2EDTA$. It has been reported that after the treatment with $CaNa_2EDTA$, the concentration of lead in the brain equaled almost that of control animals, even though the the level of lead in urine increased, and lead levels in the blood, bone, and kidney decreased (*16*). Because the brain is the critical organ affected by lead poisoning in the fetus and young children, lead removal from the brain is still a difficult issue when chelation therapy with $CaNa_2EDTA$ is considered for children. In this regard, these newly synthesized pentahydroxylhexylamino acids appeared to be more promising than existing compounds.

Another advantage of the new chelating agents is their tissue specificity. On the basis of the tissue-specific potency of some of these chelating agents, it may be beneficial to start chelation therapy using combined treatments with a liver-target chelating agent, for example, **4s**, for rapid lead removal primarily from the liver and then continue the treatment with a kidney-target chelating agent, for example, **4i**, to further decrease lead retention in the kidneys. Experiments with such combined treatments for removing lead from livers and kidneys remain to be determined.

The results for fecal and urinary lead levels after the treatment with pentahydroxylhexylamino acids **4a**−**t** by intraperitoneal injection are presented in Table 2 and Figure 1. Consistent with the tissue depletion result, urinary and fecal excretions of lead were significantly increased after the treatment with **4a**−**t** (compared with the control, $p < 0.001$). In particular, the urinary lead levels after the treatment with **4b**, **4d**, **4g**, **4l**, **4m**, **4q** were significantly higher than that of DL-PA (**4b**, 4.37 ± 1.17 μg/g; **4d**, 4.20 ± 1.41 μg/g; **4g**, 4.37 ± 1.45 μg/g; **4l**, 4.83 ± 1.16 μg/g; **4m**, 4.03 ± 0.75 μg/g; and **4q**, 3.69 ± 1.27 μg/g vs DL-PA, 2.34 ± 0.89 μg/g; $p < 0.001$and $p < 0.05$, respectively).

The third advantage is the biliary routes of lead excretion after treatment with these newly synthesized chelating agents. More specifically, after treatment with pentahydroxylhexylamino acids **4a**−**t**, total lead excretion was significantly increased, and the fecal excretion of lead was obviously higher than that in urine (Figure 1). Fecal (biliary) excretion is important and preferred to avoid renal stress during the management of lead intoxication.

It has long been acknowledged that sulfhydryl-containing compounds have the ability to chelate heavy metals (*17*). It was not surprising to observe that **4j**, bearing the sulfur-containing amino acid methionine, significantly contributed to the chelation and excretion of metals from the body, thus resulting in superior lead decorporation capacity. Similarly, **4i**, with the sulfur atom that binds readily to heavy metal ions, was also very effective in reducing bone, brain, and renal but not hepatic lead levels. These observations were consistent with previously reported results. (*17*)

Interestingly, **4s** with an imidazole ring in the structure preorganized two extra nitrogen atoms to facilitate its simultaneous coordination to the same metal atom. It appeared that the imidazole ring could involve Pb(II) binding, thereby result in enhanced stability and be a very effective lead chelating agent. As shown in Table 1, compound **4s** significantly reduced hepatic, renal, brain, and bone levels of lead and was highly effective for lead intoxication. Compounds **4m** and **4n** bear large aliphatic hydrophobic side chains in their respective amino acid residues. The rigid and hydrophobic side chain might play an important role for the coordination with metal ion; therefore, **4m** and **4n** were endowed with good lead clearance profiles. Compound **4h**, with the smallest amino acid glycine residue, rotates very easily. Perhaps too much flexibility was not desirable for metal complexation; hence, **4h** was not an effective lead chelating agent. Interestingly, **4l**, with the biggest rigid aromatic group on the side chain, has a unique combination of hydrophilic character because of its glucamine moiety and lipophilic character attributable to a benzyl group in the molecule. Therefore, it was more effective in removing extracellularly as well as intracellularly bound Pb. The potent capacity of **4l** was most likely due to its lipophilic property as an

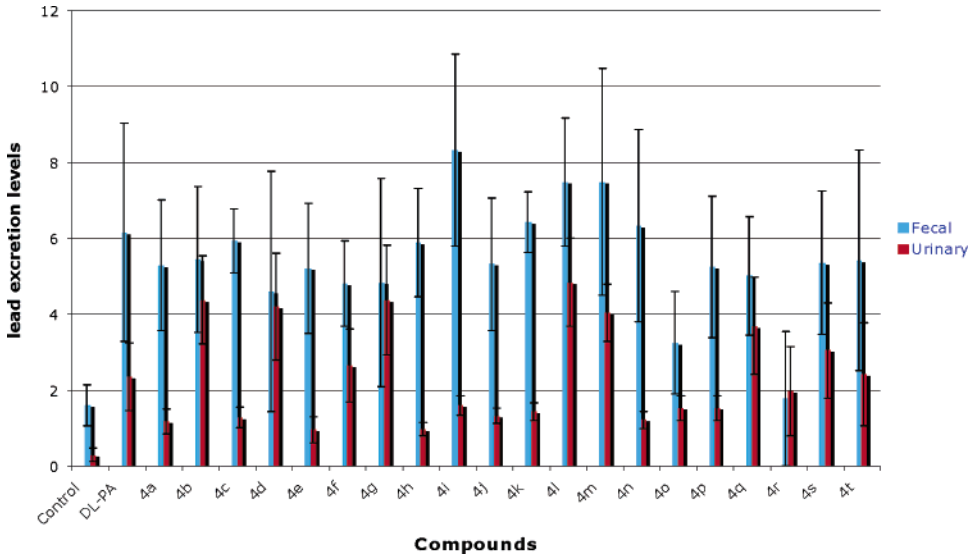


Figure 1. Excretion lead levels of mice after chelation treatment.

important feature.

Compound **4b** contains two widely separated negative charges, which is distinctly different from the neutral or monoanionic systems previously shown, capable of mobilizing lead from the intracellular deposits (*17*). The presence of two widely separated negative charges on **4b** suggested that it may expand our knowledge concerning structural features that may be present on a molecule if it is to be effective in the mobilization of hepatic intracellular lead into bile. Its modest effectiveness in removing renal cadmium suggested that transtubular secretion might be a less important route of excretion than the hepatic route. Its structure feature and behavior further indicated that the design requirements for the removal of lead from the liver might not be identical to those for the removal of lead from the kidney.

The fourth advantage is that treatment with our newly synthesized chelating agents did not deplete endogenous essential trace metals. It is well known that one of the major drawbacks with some existing chelating agents is their potential to deplete endogenous essential trace metals (*18*). In addition, some chelating agents also induce excretion and/or the redistribution of some endogenous essential metals (*18*−*20*). This may cause some serious consequences, particularly upon prolonged chelation therapy in humans. To address this issue, we further investigated the effect of the newly synthesized agents **4a**−**t** on the endogenous essential metals. It was noticed that the concentrations of Fe, Cu, Zn, and Ca in the liver, kidney, and brain of mice treated with **4a**−**t** were the same as those of mice treated with NS, which indicated that treatment with **4a**−**t** did not affect the concentration of the essential metals of mice. In addition, no body weight differences were observed before and after chelation treatment, which implied that the administration of these agents had no effect on the growth of mice (Supporting Information).

Stability Study *in Vitro*. A rational approach is needed to design new chelating agents. The efficiency of therapeutic lead chelating agents depends on the factors that affect the stability constants of formed lead complexes. The calculation values of the stability constants for Pb^{2+}−**4a**−**t** complexes in this experiment are summarized in Table 3.

A critical determining factor for complex stability is the hardness/softness characteristics of electron donors and acceptors. According to the concept of hard/soft acids and bases by Pearson (*21*), the thiol side chain of the Cys residue acts as a soft donor ligand, preferentially binding to soft and moderately soft metal acids, that is, Cd^{2+} and Pb^{2+}, rather than the hard acid metals, that is, Fe^{2+} and Cu^{2+}. Therefore, the stability constants of the Pb^{2+}−**4i** complex (log K_1, 11.87; log K_2, 8.11) were the highest compared to those of other complexes. This further confirmed that the sulfydryl group was very important for forming complexes of chelating agents with heavy metals. On the contrary, the carboxylic acid side chain in the Asp residue acts as a hard donor ligand, preferentially binding with hard acid metals, that is, Cu^{2+} and La^{3+} but not Pb^{2+}; thus, it was not surprising to observe that the stability constants of Pb^{2+}−**4a** were relatively low (log K_1, 3.40; log K_2, 2.21). However, the stability constants of Pb^{2+}−**4b** (log K_1, 4.67; log K_2, 3.69) were higher than those of Pb^{2+}−**4a**, although both have similar hydrophilic acidic side chains. We speculate that the extra stability of Pb^{2+}−**4b** could be attributed to the Pb(II)-induced conformational rearrangement of the molecule. The longer, more flexible side chain of **4b** rendered the molecule with a favorable conformation for metal complexation.

In general, the presence of the N-terminal amino and nearby carbonyl group in these new chelating agents is necessary for lead binding. Nevertheless, the stability of the complex is enhanced with the increase in the number of donor groups. For example, the stability constants of Pb^{2+}−**4s** (log K_1, 5.59; log K_2, 4.13) were relatively higher than those of other complexes. This is most likely due to two extra nitrogen atoms from the histidyl residue that serve as additional ligands for metal ion binding, thereby leading to higher complex stability. Combined with the *in vivo* study, a good correlation between stability constants of the complex and lead decorporation capacity of the chelating agents was observed.

Membrane Permeability Study *in Vitro*. An ideal lead chelating agent should possess greater affinity for the toxic metal, low toxicity, rapid elimination of the metal, high water solubility, and capability to penetrate the cell membrane, oral administration, and minimal metabolism. For successful lead chelation, the chelating agent must be capable of entering the cell and mobilizing the metal and eliminating it without accumulation in the kidney or distribution to other sensitive organs (*22*). The membrane permeability of the chelating agents not only affects the absorption and transportation of the chelating agents but also impacts the metal decorporation potency of chelating agents.

Rapid preclinical screening of lead chelating agents could assist in identifying potential new orally administrated candidates. Caco-2 cells are an epithelial cell line originally derived from a human colorectal adenocarcinoma. When cultured on a permeable membrane filter, it possesses distinct apical, basolateral membranes and tighter junctions between adjacent cells. The apical side has a well-developed brush border with a transport system, enzymes, and ion channels that mimic the characteristics of the small intestine. Because of these properties, the Caco-2 cell model has become an important tool in the *in vitro* assessment or prediction of oral permeability in humans (*23*).

Given the intended oral administration of these new compounds in the future, we chose Caco-2 as a model system to investigate the membrane permeability of several newly synthesized agents representing different potencies of lead chelating capacity. Membrane permeability is difficult to measure; thus, this assay provided a convenient way to measure permeability on the basis of the apparent permeability coefficient (P_{app}).

The assay was initiated by adding the test solution to the apical or basolateral side of the monolayer. The compounds tested across Caco-2 cell monolayers were valuated ($n = 4$) in the apical to basolateral (A → B) and basolateral to apical directions (B → A). P_{app} (A → B) is the permeability from the apical to the basolateral side (intestine to blood), and P_{app} (B → A) is the permeability from the basolateral to the apical side (blood to intestine). The influence of efflux carriers on the permeability of the different compounds was also examined by comparing the permeability ratio of absorptive transport P_{app} (A → B) to the secretory one P_{app} (B → A). Permeability coefficients for the tested compounds are summarized in Table 4.

According to the previous study, a compound with permeability coefficients P_{app} $<1 \times 10^{-6}$, $1-10 \times 10^{-6}$, and $>10 \times 10^{-6}$ cm/s was defined as being poorly, moderately, and well absorbed, respectively (*24*). Using this as a guideline, compounds **4a** and **4t** exhibited moderate permeation through the Caco-2 cell monolayer (**4a**, 5.48×10^{-6} cm/s; **4t**, 7.73×10^{-6} cm/s). In contrast, the permeability for other compounds was increased 2- to 3-fold. Compared with **4a**,**t**, compounds **4b**, **4i**, **4j**, **4m**, **4n**, and **4s** exhibited considerably increased permeation through Caco-2 cells when applied to the apical side of the monolayer, as shown by the 2- to 3-fold increased permeability coefficients P_{app} (A → B)(**4b**, 15.22; **4i**, 17.54; **4j**, 13.38; **4m**, 14.05; **4n**, 13.96; **4s**, 16.31×10^{-6} cm/s). This seemed to be in accordance with the *in vivo* lead decorporation data. Therefore, compounds **4b**, **4i**, **4j**, **4m**, **4n**, and **4s** could potentially exhibit high bioavailability with oral administration, and **4a** and **4t** could be incompletely absorbed. The limited membrane permeation of **4a**,**t** on Caco-2 cells may likely be due to its low lipophilicity.

In addition, among the compounds tested, the absorptive ability of A → B transport was consistently greater than that observed in the opposite direction (Table 4), and with the exception of **4a** and **4t**, the ratio of the apparent permeability coefficients (P_{app}) of these compounds, calculated from the A → B and B → A transport, varied between 2.00 and 3.21. Apparently, these compounds were readily permeable to Caco-2 cell monolayers from the A → B direction. The results suggested that these new compounds have good membrane permeation characteristics. Because the P_{app} (A → B) of these compounds was substantially greater than P_{app} (B → A), the asymmetric permeation profile further implied that the involvement of apically polarized efflux systems was unlikely.

It appears that the chelating agents pass through biological membranes depending on the subtle balance between hydrophilicity and lipophilicity of the agents. A better understanding of the penetration of the chelating agents or their lead complexes through biological membranes is important for successful chelation therapy.

Conclusions

The metal complexation reactions in the human body are influenced by multiple factors, including competing metals and ligands, dynamics of circulation, compartmentalization, and metabolism of the chelating agent. Successful *in vivo* chelation treatment of lead intoxication requires a significant fraction of the administered chelator foming a complex with the toxic metal. In addition, the high selectivity of chelating agents is important because of the risk of depletion of the patient's stores of essential metals. Chelation is not necessarily an equilibrium reaction *in vivo*, determined by the standard stability constant, because rate effects and ligand exchange reactions considerably influence complex formation. Hydrophilic chelators most effectively promote renal metal excretion, but they inefficiently form complexes intracellular metal deposits. Lipophilic chelators can decrease intracellular stores but may redistribute toxic metals to, for example, the brain. It seems that the optimum structure for balancing the lead-mobilizing activity, stability, and bioavailability requires some compromise of lead-mobilizing activity. In this study, compounds **4b**, **4j**, **4m**, and **4s** possess a balanced combination of stability, lipophilic and hydrophilic (glucose chain) groups, and potent cell membrane permeability and were found to be highly effective in mobilizing lead from the liver and kidney with preferential elimination through bile without carrying it to the brain.

Taken together, the significant therapeutic advantages of these newly synthesized lead chelating agents are that they (1) can significantly decrease the lead level in rat brains; (2) have high tissue specificity; (3) have unique lead excretion pathways via biliary routes; and (4) retain endogenous essential trace metals after treatment with our newly synthesized chelating agents. These new compounds seem to hold promise for the development of safer and more effective lead chelating agents suited for long-term oral administration.

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Supporting Information Available: NMR data of compounds **4a**−**t**, concentrations of essential metals in the kidney, liver, and brain of mice after chelating treatment, and the effect of **4a**−**t** on the body weight of mice. This material is available free of charge via the Internet at http://pubs.acs.org.

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