Decorporation of Aged Americium Deposits by Oral Administration of Lipophilic Polyamino Carboxylic Acids

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Several new powerful chelating agents, suitable for the removal of a variety of certain heavy-metal ions from the body by oral application, have been synthesized and tested. Structurally, these compounds are partially lipophilic polyamino carboxylic acids (PACA). They were synthesized in nonaqueous media from triethylenetetramine (TT) by monoalkylation of a primary amino group, followed by exhaustive carboxymethylation of the remaining amino groups using ethyl bromoacetate and subsequent alkaline hydrolysis of the ester. Compounds were characterized using IR, ^{1}H NMR, ^{13}C NMR, and mass spectrometry. Synthesis and testing of two of these compounds, C_{12}- and C_{22}-triethylenetetraminepentaacetic acid (C_nTT), is described in detail. Gel permeation chromatography of a mixture of the PACA and actinide elements have shown these substances to be strong chelating agents similar to EDTA or DTPA. They were capable of removing plutonium from contaminated liver cytosol in vitro. In contrast to their nonlipophilic counterparts EDTA and DTPA, the model substances exhibited appreciable absorption from the intestine and, therefore, can be administered orally. With increasing length of the alkyl chain, the chelons can be directed primarily to the liver, one of the target organs for actinide contamination. In vivo, absorption from the ligated duodenum and jejunum of rats after 2 h was 27% of the amount introduced. Compared to untreated controls, daily feeding of 200 μmol of the chelons (C_{12}TT or C_{22}TT)/kg of body weight to rats for 10 days reduced the whole body Am by 29% and 44%, respectively. Am was eliminated most efficiently from the liver, with a reduction of 71% and 89% ($p < 0.001$). However, the skeletal retention also was reduced by 17% and 32% from the femora ($p < 0.001$) and 20% and 37% from the carcass for the C_{12}TT and C_{22}TT compounds, respectively. No weight loss or other obvious signs of blood, kidney, liver, or intestinal toxicity were observed after the 10-day treatment. These new chelators are promising as agents for oral chelation therapy to remove actinides and possibly other elements from body stores.

Introduction

The most commonly used agent for the removal of 5f elements and several other heavy metals from the body is DTPA (diethylenetriaminepentaacetic acid). The literature describing the use of DTPA for the decorporation of internally deposited actinides is abundant. An excellent review of actinide chelation therapy was presented by Volf.[1] DTPA usually is applied either as the Ca or Zn chelate. The soluble Na salt and the chelates formed are strongly hydrophilic. Therefore, the preferred route of administration is by intravenous (iv) injection, although inhalation[2-4] and oral administration[5,6] have been proposed or used in animal studies. More recently, a new group of powerful chelating agents, based on some naturally occurring, bacterially produced iron chelators have been developed under the direction of Raymond[7-9] and tested by Durbin[10,11] and others.[12-16] These compounds structurally are linear catechoylamides that preferentially bind the tetravalent plutonium. The status of development has recently been reviewed by Durbin.[17] Most, but not all, of these compounds also are strongly hydrophilic. In general, strongly hydrophilic properties result in relatively rapid, primarily urinary excretion and limit entry of the

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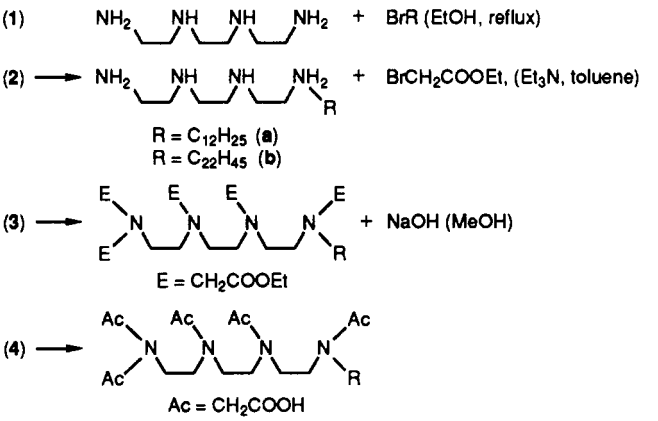
drug into cells, the site of a large fraction of heavy-metal deposition within the target organs. This property limits the effectiveness of the drug.[18,19] Hydrophilic compounds usually are poorly absorbed from the intestine. Therefore, they require parenteral application which, in general, necessitates the presence of a physician or other certified health professional for their administration.[20,21] The effectiveness of the treatment diminishes rapidly with time after exposure,[22] and the necessity of parenteral administration often increases the critical time period between exposure and first treatment. Very often, long treatment periods are required to achieve the desired reduction of the heavy-metal burden.[23] Parenteral application repeated over extended periods is inconvenient to the patient and sometimes difficult to maintain. Many of these shortcomings can be eliminated by oral administration of the decorporation agent.

Pilot experiments carried out at this laboratory have shown that basic chelating properties of the polyamino carboxylic acids (PACAs) as EDTA (ethylenediaminetetraacetic acid) and DTPA may be maintained in spite of partial exchange of reactive hydrophilic sites (carboxymethyl groups of EDTA or DTPA) by nonreactive lipophilic substituents. At the same time, some of the pharmacological properties may be improved because increased lipophilicity may facilitate the passage of such an agent through the intestinal barrier and permit a greater penetration through cellular membranes.[18] These properties would allow oral administration and initiation of treatment very early after the contaminating event, i.e. at a time when treatment appears most effective.[22] They also offer considerable convenience for treatment protracted over long time periods, which in some cases may be necessary to minimize the toxic effects of the nuclide deposition. This paper describes the design, synthesis, characterization, and in vitro and in vivo testing of two such compounds whose basic structure is closely related to DTPA or EDTA. The new substances were expected to be suitable for oral application, to have the ability to penetrate bilipid cellular membranes, and to result in higher uptake by certain critical organs as well as higher cellular uptake than the parent compounds.

Experimental Section

Synthesis of Lipophilic Chelators. Several new bifunctional agents have been synthesized that are based structurally on triethylenetetramine (TT) containing a single unbranched alkyl group of various chain lengths. The notation for these compounds used in this text is C_nTT, where n denotes the length of the alkyl chain. Several methods for the preparation of alkyl, acyl, or aryl derivatives, not necessarily monosubstituted, of polyethyleneamines have been reported.[24-29] Monoalkylation of a single primary amino group of triethylenetetramine followed the same principle by reacting a large excess of the amine in absolute alcohol with the alkyl halide. This was followed by carboxymethylation of the product using ethyl bromoacetate with triethylamine as a base for binding the hydrogen bromide produced during the reaction. The resulting ester was then hydrolyzed to the respective polyamino carboxylic acids. The carboxymethylation reaction was carried out at room temperature with toluene as the solvent. The choice of using the bromoethyl ester for the carboxymethylation instead of a haloacetic acid reported earlier[26,28] was very important because the products were dissolved in organic solvents and were easily purified using flash chromatography. In our experience, the success of preparing these compounds in a pure and well-characterized state depends on the application of a nonaqueous scheme of synthesis. The preparation, characterization, and application of two of these compounds containing a dodecyl or docosyl chain are described in this paper. However, synthesis of homologues with chain length between 8 and 22 carbon atoms follows the same principles.

The general scheme of the synthesis is presented by the following outline:



Melting points were determined on a Reichert hot stage apparatus. Flash chromatography on silica gel was used for purification. Thin-layer chromatography was performed using polyester sheets with silica gel 60 F254 and the following solvent mixtures: (A) chloroform, methanol, propanol, ammonia, 5:2:2:1; (B) chloroform, benzene, propanol, methanol, ammonia, 290:100:12:2:1; (C) propanol, ammonia, water, 5:1:1; (D) chloroform, hexane, 1:1; (E) chloroform, benzene, propanol, methanol, ammonia, 690:100:12:2:1. Compounds were characterized using IR, NMR, and MS spectroscopy. Infrared spectra were obtained from KBr disks or films using a Beckman 2100 spectrometer. The proton NMR spectra were recorded on an IBM NR200 spectrometer in deuteriochloroform ($CDCl_3$) with tetramethylsilane as an internal standard or in deuterium oxide containing some NaOD at pH 13. FAB mass spectra at 7 kV were determined in

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xenon gas using a Matt 112 S or Matt 731 mass spectrometer. All samples were dissolved in glycerol/ammonia.

For the preparation of the Ca and Zn complexes, the title compounds were dissolved in base to give a solution between pH 7.4 and 8, and an equivalent amount of $CaCl_2$ or $ZnCl_2$ was added. The necessary quantity of Ca^{2+} or Zn^{2+} had been determined by prior titration of an aliquot of the chelator with these metal ions in the presence of Cal-Red as the indicator. This amount corresponded to a 1:1 chelate. The resulting complex was crystallized, dried, and used in D_2O to obtain the desired IR spectra.

1-*N*-Dodecyltriethylenetetramine (*N*-Dodecyl-3,6-diazaoctane-1,8-diamine) (2a). Triethylenetetramine (1) (10.4 g, technical grade 70%, 50 mmol) was dissolved in 50 mL of ethanol, and dodecyl bromide (2.5 g, 10 mmol) was added dropwise at room temperature. Excess of triethylenetetramine serves as the base necessary for trapping hydrogen bromide released during the reaction. The reaction mixture was refluxed for 4 h and then evaporated to dryness in vacuo. The oily residue was mixed with 20 mL of 1-butanol and 20 mL of toluene and washed with water. The two phases were separated, the organic layer was dried over sodium sulfate, and the solvents were evaporated. The residue was dissolved in methanol, and the C_{12}-amine precipitated with HCl. The hydrochloride was finally crystallized from wet methanol to yield 2.1 g (45%) of white crystals of the HCl salt of **2a**: mp 220–222 °C; TLC (A) R_f = 0.38. Anal. ($C_{18}H_{42}N_4 \cdot 4HCl$) C, H, N; Cl: calcd, 30.87; found, 30.07. The following ^1H NMR ($CDCl_3$) spectrum was obtained: alkyl chain protons ($(CH_2)_{11}CH_3$), δ 0.8–1.6 (m, 30 H); 5 amine protons (NH_2 or =NH), 12 ethylene protons (CH_2CH_2), δ 2.5–2.8 (m, 12 H).

1-*N*-Docosyltriethylenetetramine (*N*-Docosyl-3,6-diazaoctane-1,8-diamine) (2b). The reaction was carried out as for **2a**, using triethylenetetramine (1) (52.2 g, 70%, 250 mmol), EtOH (200 mL) and docosyl bromide (19.45 g, 50 mmol). Crystallization of the hydrochloric acid salt of **2b** from aqueous MeOH (75%) yielded 12.0 g (40%) of colorless crystals: mp 216–219 °C; TLC (A) R_f = 0.49. Anal. ($C_{28}H_{62}N_4 \cdot 4HCl$) C, H, N, Cl. The ^1H NMR ($CDCl_3$) spectrum was composed of the following: alkyl chain protons ($(CH_2)_{21}CH_3$), δ 0.8–1.6 (m, 45 H); 5 amine protons (NH_2 or =NH), 12 ethylene protons (CH_2CH_2), δ 2.5–2.8 (m, 12 H).

1-*N*-Dodecyltriethylenetetraminepentaacetic Acid Pentaethyl Ester (Pentaethyl *N*-Dodecyl-3,6-diazaoctane-1,8-diamine-*N*,*N*′,*N*′,3,6-pentaacetate) (3a). Ethyl bromoacetate (75.0 g, 450 mmol) was added dropwise at room temperature to a mixture of 1-*N*-dodecyltriethylenetetramine hydrochloride (**2a**) (20.6 g, 45 mmol), triethylamine (50.8 g, 503 mmol), and 400 mL of toluene. The triethylamine again serves as the base necessary for trapping released hydrogen bromide; it does not react with the dodecyl bromide. This mixture was stirred for 24 h. The resulting soft solid, which consisted mostly of triethylamine–HBr, was filtered off and washed with 50 mL of chloroform. Solvents from the combined filtrates were evaporated under reduced pressure, and the residue was again dissolved in chloroform and washed with water. After drying over sodium sulfate, the chloroform was evaporated. The product was, finally, purified by flash chromatography (eluting successively with solvent D, chloroform, and solvent E) and obtained as a colorless oil (8.3 g, 25%) of **3a**: n^{20}_D = 1.4850; TLC (B) R_f = 0.53; IR (film) 1734, 1462 cm^{-1} corresponding to the ester and amino groups. Anal. ($C_{38}H_{72}N_4O_{10}$) C, H, N. The following ^1H NMR ($CDCl_3$) spectrum was obtained: alkyl chain protons ($(CH_2)_{11}CH_3$), δ 0.84–1.43 (m, 25 H); methyl protons of five ester groups (OCH_2CH_3) δ 0.84–1.43 (m, 15 H); protons of one methylene group (NCH_2CH_2N-alkyl) δ 2.59 (t, 2 H, J = 7.9 Hz); protons of five methylene groups (NCH_2CH_2N) δ 2.75–2.84 (m, 10 H); methylene protons of acetic acid residue (NCH_2COOEt) δ 3.39 (s, 2 H), 3.45 (s, 2 H), 3.46 (s, 2 H), 3.57 (s, 4 H); methylene protons of five ester groups (OCH_2CH_3) δ 4.09–4.21 (m, 10 H).

The methylene protons of carboxymethyl groups were observed as four singlets: three as two-protons signals and only one as a four-proton signal. This indicates that the alkyl group of **3a** binds to the terminal nitrogen N^1. If the alkyl group had been attached to N^2, two four-protons signals or one eight-proton signal should be observed.[29] The two-proton signal at 2.59 ppm, separated from the pattern of methylene protons of the trimethylenetetramine chain, confirmed the proposed structure of a terminally attached alkyl group. Only one methylene group has a different attachment and is shifted upfield.

1-*N*-Docosyltriethylenetetraminepentaacetic Acid Pentaethyl Ester (Pentaethyl *N*-Docosyl-3,6-diazaoctane-1,8-diamine-*N*,*N*′,*N*′,3,6-pentaacetate) (3b). 1-*N*-Docosyltriethylenetetramine hydrochloride (12 g, 20.0 mmol) and ethyl bromoacetate (33.4 g, 200 mmol), in toluene (200 mL) and triethylamine (28.3 g, 280 mmol) were stirred at room temperature for 4 days. Then the temperature was raised to 40 °C and agitation was continued for an additional 3 h. The reaction mixture was separated by flash chromatography (using successively the solvents: D, chloroform, and finally solvent E), and the product **3b** was purified by crystallization from a mixture of MeOH and ether: yield 9.4 g (53.0%); mp 82–83 °C; TLC (B) R_f = 0.49; IR (film) 1730, 1462 cm^{-1}. Anal. ($C_{48}H_{92}N_4O_{10}$) C, H, N.

The proton NMR spectrum corresponded to that of **3a** except for the integration of the signal between 0.85 and 1.42 which had a 60-proton multiplet instead of 40 protons. Thus, the following ^1H NMR ($CDCl_3$) spectrum was obtained: alkyl chain protons ($(CH_2)_{21}CH_3$) δ 0.85–1.42 (m, 45 H); methyl protons of five ester groups (OCH_2CH_3) δ 0.85–1.42 (m, 15 H); protons of methylene group (NCH_2CH_2N-alkyl) δ 2.57 (t, 2 H, J = 7.9 Hz); protons of five methylene groups (NCH_2CH_2N) δ 2.73–2.84 (m, 10 H); methylene protons of acetic acid residue -NCH_2COOEt δ 3.36 (s, 2 H), 3.43 (s, 2 H), 3.45 (s, 2 H), 3.57 (s, 4 H); methylene protons of five ester groups (OCH_2CH_3) δ 4.09–4.21 (m, 10 H). Again, the alkyl group was attached to the terminal nitrogen.

1-*N*-Dodecyltriethylenetetraminepentaacetic Acid (C_{12}TT, *N*-Dodecyl-3,6-diazaoctane-1,8-diamine-*N*,*N*′,*N*′,3,6-pentaacetic Acid, 4a). The dodecyl ester **3a** (1.2 g 1.7 mmol) was dissolved in 19 mL of MeOH, and aqueous 2 M NaOH (15 mL) was added in small portions. The reaction mixture was stirred at room temperature for 1 h, acidified with concentrated HCl, and left overnight in the refrigerator. The product **4a** was filtered off and crystallized from wet MeOH as colorless crystals (0.8 g, 82%): mp 202–203 °C; TLC (C) R_f = 0.45; IR 1730, 1630 cm^{-1}, indicating partially protonized carboxyl groups; mass spectrum ($M^+ - 1$) 603. The fragments observed were consistent with the molecular structure of the title compound above (**4a**). Anal. ($C_{28}H_{52}N_4O_{10} \cdot 4HCl$) C, H, N; Cl: calcd, 18.93; found, 18.06. ^1H NMR ($NaOD/D_2O$, pH = 13): alkyl chain protons ($(CH_2)_{11}CH_3$) δ 0.77–1.16 (m, 25 H); protons of ethylene groups (NCH_2CH_2N) δ 2.4 (br s, 12 H); methylene protons of acetic acid residue (NCH_2COO^-) δ 2.94 (br s, 10 H). ^{13}C NMR ($NaOD/D_2O$, pH = 13): alkyl chain carbons ($(CH_2)_{11}CH_3$) δ 16.6, 25.1, 31.9, 34.3; carbons of ethylene groups (NCH_2CH_2N) δ 53.5, 54.2, 54.7; methylene carbons of acetic acid residue (NCH_2COO^-) δ 57.2, 60.8, 61.4, 61.8; carboxylic carbons of acetic acid residue (NCH_2COO^-) δ 173.8, 181.7, 181.6, 182.2.

Methylene carbons of five carboxymethyl groups were observed as four signals, with one shifted upfield 3.6 ppm. Five carboxylic carbons also appeared as four signals, with one shifted upfield 8.0 ppm. This also confirmed that the alkyl group was bound to N^1. If the alkyl group had been attached to N^2, two or a maximum of three signals should have been observed at the same region.[30]

1-*N*-Docosyltriethylenetetraminepentaacetic Acid (C_{22}TT, *N*-Docosyl-3,6-diazaoctane-1,8-diamine-*N*,*N*′,*N*′,3,6-pentaacetic Acid, 4b). The docosyl ester **3b** (1.8 g, 2 mmol) was hydrolyzed at room temperature with 2 M NaOH in MeOH (26 mL). After 4 days, water (40 mL) was added and the mixture was acidified to pH 3 with concentrated HCl. The title compound was then crystallized from water, yielding 1.4 g or 92% of **4b**: mp 212–213 °C; TLC (C) R_f = 0.5; IR 1730, 1630 cm^{-1}, indicating partially protonized carboxyl groups; mass spectrum ($M^+ - 1$) 743. Again, the fragments observed were consistent with the molecular structure of the title compound (**4b**). Anal. ($C_{38}H_{72}N_4O_{10} \cdot 4HCl$) C, H, N, Cl. ^1H NMR ($NaOD/D_2O$, pH = 13): alkyl chain protons ($(CH_2)_{21}CH_3$) δ 0.77–1.16 (m, 45 H); protons of ethylene groups (NCH_2CH_2N) δ 2.4 (br s, 12 H); methylene protons of acetic acid residue (NCH_2COO^-) δ 2.94 (br s, 10 H). ^{13}C NMR ($NaOD/D_2O$,

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pH = 13): alkyl chain carbons ($(CH_2)_{21}CH_3$) δ 16.7, 25.4, 32.7, 34.7; carbons of ethylene groups (NCH_2CH_2N) δ 53.5, 54.1, 54.7; methylene carbons of acetic acid residue (NCH_2COO^-) δ 57.6, 60.8, 61.4, 61.8; carboxylic carbons of acetic acid residue (NCH_2COO^-) δ 173.6, 181.1, 182.0, 182.2. This confirmed again that the alkyl group was attached to the terminal nitrogen.

Application of the Lipophilic Chelators. The aim of this research was to determine the degree of effectiveness of the alkylated PACAs for the removal of Pu, Am, other actinide, or perhaps lanthanide elements from the body and to detect signs indicative of possible toxicity.

Plutonium and Am have somewhat similar in vivo distribution patterns and, in general, it may be assumed that a decorporation agent may be effective for Pu if found effective for Am, although not necessarily to the same degree. However, this assumption may hold only for the relatively nonspecific PACAs. It does not, for instance, hold for the linear catechoylamine chelates which mostly are very specific for Pu only. For most of the in vivo and in vitro studies, Am was selected as the test nuclide because of the convenience of its detection. ^{241}Am decays by emission of an α-particle that is accompanied in 36% of the decay events by emission of a γ-ray of 60 keV. The γ-ray permits detection of this nuclide by external γ-counting without further processing of the samples and tissues, an essential requirement for the nuclide determination in the intact animal by whole-body counting. However, some in vitro experiments produced specimens of a much smaller mass which, without extensive sample preparation, permitted the determination of α-emitting radionuclides by liquid scintillation counting. With those samples, ^{239}Pu instead of ^{241}Am was used as the nuclide of primary interest to study the effectiveness of some of the chelators for nuclide removal.

In Vitro Studies. A number of in vitro tests were performed to ascertain the existence and relative stability of the desired actinide chelates and the necessary pharmacological properties of the new chelons and to detect signs of overt toxicity. These tests include (a) a comparison of the elution patterns of Am–citrate (a moderately stable complex with $K_1 = 7.74$), ^{241}Am–EDTA or ^{241}Am–DTPA (two very stable complexes with $K_1 = 16.91$ and $K_1 = 24.03$, respectively), and ^{241}Am–$C_{12}TT$ (a complex of unknown stability) after gel chromatography on Sephadex G-15, and a comparison of the IR spectra of the open C_nTT chain with that of the chelate configuration; (b) a comparison of the ability of ^{241}Am–citrate and ^{241}Am–$C_{12}TT$ to pass the intestinal barrier; (c) some simple blood tests that were considered useful preliminary indicators of possible chelon toxicity; (d) a test for the elimination of Pu from in vivo tagged canine liver cytosol using $C_{12}TT$.

(a) Column Chromatography. Gel permeation chromatography was performed on buffer-equilibrated, degassed Sephadex G-15 (column size 1.5 cm × 85 cm, 0.1 N TRIS buffer of pH 7.4 containing 0.01% sodium azide). The void volume was determined by elution of Dextran Blue-2000. Columns were loaded with a known quantity of ^{241}Am–citrate, ^{241}Am–EDTA, ^{241}Am–DTPA, or ^{241}Am–$C_{12}TT$ and eluted under constant hydrostatic pressure with buffer. The $C_{22}TT$ compound was omitted from this test because of micelle formation which would have interfered with normal sorption properties. The ^{241}Am in each of the collected fractions was counted via the 60-keV γ-emission. The resulting elution spectra are shown in Figure 1 with the amount of ^{241}Am in individual fractions expressed as percent of the total nuclide loaded. Elution of ^{241}Am–citrate was characterized by the relatively low stability of this complex. During the initial phase of the migration, excess citrate was separated from the ^{241}Am–citrate. The ^{241}Am–citrate complex dissociated, and most of the nuclide hydrolyzed and did not migrate through the resin. Total recovery of ^{241}Am was only 15%. Addition of a small amount of DTPA to the column followed by elution with buffer increased the recovery to 93%. The elution spectrum of ^{241}Am was very broad, but the peak appeared essentially at the same position as obtained after gel permeation of ^{241}Am–DTPA. Both ^{241}Am–EDTA and ^{241}Am–DTPA produced sharp peaks separated by five fractions. Recoveries were 96% for ^{241}Am–EDTA and 100.2% for ^{241}Am–DTPA. The elution of ^{241}Am–$C_{12}TT$ was consistent with the view that a very small fraction of the chelate was present as micelles, and this fraction was eluted at the position of the exclusion MW. The remaining ^{241}Am–$C_{12}TT$ migrated as expected for a true solution. Recovery was >99%. Thus, Am forms a chelate with the alkylated PACA that will not dissociate under the experimental conditions. No value has yet been determined for the thermodynamic stability constants of the new compounds.

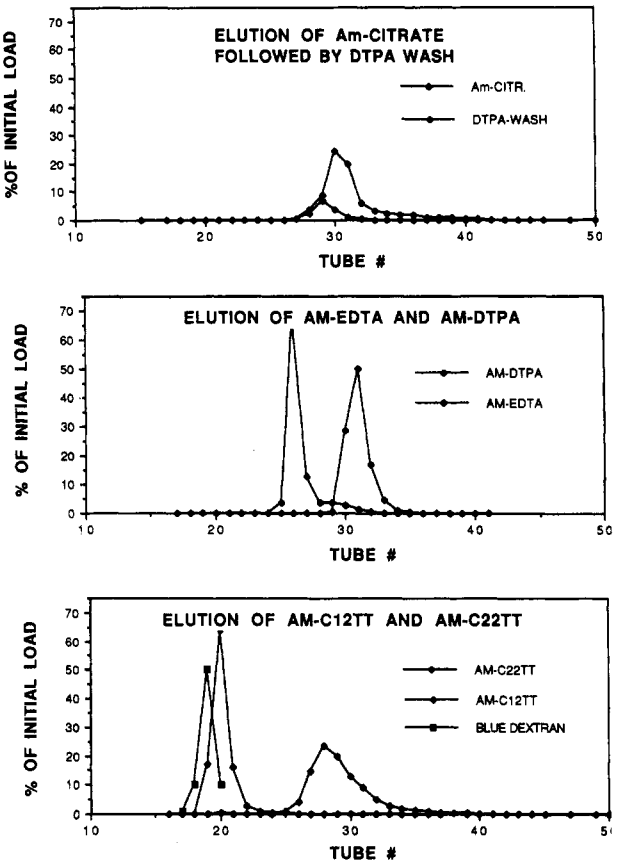

Figure 1. Elution spectra of ^{241}Am applied to a column of Sephadex G-15 in three chemical forms. ^{241}Am–citrate dissociates under the elution conditions, and migration through the gel is inhibited because of hydrolysis. The nuclide can be eluted by subsequent addition of DTPA (top). ^{241}Am–EDTA and ^{241}Am–DTPA form stable chelates which are eluted at their expected relative positions (center). ^{241}Am–$C_{12}TT$ and ^{241}Am–$C_{22}TT$ also form stable chelates, but increasing micelle formation, especially with $C_{22}TT$, causes partial exclusion from the gel and elution near the void volume (bottom).

The existence of the two chelates is corroborated by the changes observed in the IR spectra as the complexes or chelates are formed. The absorbance maxima near 1600 and 1400 cm^{-1} of the metal-free $C_{22}TT$ compound are separated by about 160 cm^{-1}, in the Ca chelate by about 190 cm^{-1}, and in the Zn chelate by approximately 220 cm^{-1}. At the same time, the corresponding relative peak heights change from 0.895 to 1.664 and 1.761, respectively. The minimum between these two peaks in the uncomplexed form has no special features, but in the Ca–$C_{22}TT$ it shows two shoulders, and these develop into two distinct peaks in the Zn–$C_{22}TT$. The frequency of a minor peak at 880 cm^{-1} (Na–$C_{22}TT$) changes to about 920 cm^{-1} in the chelated form, and the relative peak heights of this to another peak at 720 cm^{-1} change from 2.357 to 0.772 and 0.905, respectively. Similar shifts of frequencies and relative peak heights have been observed previously for the IR spectra of Na–EDTA vs alkaline earth–EDTA complexes.[31] Although, this is not a proof for the formation of a chelate structure of the metal–$C_{22}TT$, the probability of having a bond analogous to the respective EDTA chelates is given. A more rigorous verification will be provided at a later time.

(b) The Ability of ^{241}Am–Citrate and ^{241}Am–$C_{12}TT$ To Pass the Intestinal Barrier. The ability of Am–citrate and ^{241}Am–$C_{12}TT$ to cross the intestinal mucosa in situ was measured using

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the intestinal tied-loop technique of Trechsel.[32] Rats were anesthetized and a 18–20-cm segment of the duodenum and proximal jejunum was isolated and rinsed with cold saline. The most proximal portion of this segment was 1 cm distal to the pyloric junction. With the stability of the ^{241}Am complex established, known quantities of the corresponding complexes were introduced in vivo directly into the isolated and ligated segment. The animals remained anesthetized to allow for passage of the lipophilic compounds through the intestinal barrier into the body. After 3 h, the rats were euthanized, the ligated section of the gut was excised, and its lumen flushed with saline. Lumen wash, cleaned gut section, combined viscera, liver, and the remaining carcass were counted separately. The ^{241}Am found in the various parts was considered as representative of the distribution of the chelon. ^{241}Am given as the citrate was retained almost entirely in the lumen and the gut section. Less than 1% of the total was found in the combined carcass, liver, and viscera. Of the C_{12}TT only 73% was left in the lumen and gut section, 20% was in the carcass, 3% in the liver, and 4% in the viscera. Thus passage of the alkylated compound through the intestinal wall was considerably greater than for ^{241}Am-citrate.

(c) Blood Test for Chelon Toxicity. Toxicity studies were limited to a few preliminary tests that were expected to be indicative of early gross toxicity. During initial pilot experiments, chelons of similar structure produced hemolysis and some crenation of red cells when incubated with fresh canine blood. Therefore, the crenation–hemolysis tests were repeated with the highly purified C_{12}- and C_{22}-PACAs. Two-milliliter aliquots of fresh canine blood, drawn with a heparinized syringe, were incubated at 37 °C with an equal volume of a 0.1 M TRIS buffer containing 10 and 20 mg, respectively, of the Ca chelate of the two compounds whose osmolarity had been adjusted to 270–300 mosM. After 1 and 4 h of incubation, respectively, blood smears were prepared and examined microscopically and cells were sedimented. No red cell crenation was observed. All sera were slightly pink in appearance, but there was no visible difference in color intensity between the two concentrations of the agents.

(d) Elimination of Pu by C_{12}TT from in Vivo Tagged Canine Liver Cytosol. A crude liver homogenate in 0.25 M sucrose was obtained from a canine injected with Pu-citrate 7 days before sacrifice. Endogenously bound Pu in this homogenate was bound either to subcellular organelles or high molecular weight proteins and was not ultrafilterable. Centrifugation of this material for 20 min at 40000*g* produced a Pu-tagged liver cytosol that, mixed 1:1 by weight with glycerol, could be stored at a temperature of −20 °C for extended periods of time. Removing the glycerol by dialysis in 50 mM TRIS, pH 7.5, yielded a medium that was suitable for the in vitro testing of the effectiveness of soluble chelating compounds in the removal of Pu from endogenous ligands.

The frozen cytosol was thawed and dialyzed exhaustively against 50 mM TRIS-HCl. Five milliliters of this material was mixed with 5 mL of a 4 mM solution of the test compound in 0.1 M TRIS-HCl adjusted to 270–300 mOsm and pH 7.5. The sample was then incubated at 40 °C for 2 h and subsequently filtered under pressure through a molecular filter with a cutoff at 10 000 Da. The ratio of the Pu activity in the filtrate to the residue on the filter served as a measure of the chelator effectiveness. Since no clear solution could be obtained from the C_{22}TT because of micelle formation, another compound with an eight-carbon alkyl chain (C_8TT, not described in detail in this text) was substituted in order to verify the general effectiveness of *n*-alkyl PACAs in more than a single compound. Filtration of the cytosol carried through the same procedure but without the addition of any of the test compounds served as the control. The following filtrate to residue activities were obtained: control, 0.03; C_8TT, 0.16; and C_{12}TT, 0.26. Thus, the alkylated compounds were capable of reacting with endogenously bound Pu.

In Vivo Studies. Animal care at the Radiobiology Division of the University of Utah fully complies with NIH guidelines for use of laboratory animals. The facility also operates under the direction of the Animal Resource Personnel of the University of Utah. Animals are housed in approved facilities under the supervision of veterinarians. Consistent with recommended policies, animals are anesthetized when necessary. If any adverse side effects are noted that may cause pain, distress, or discomfort, the animals are removed from the study.

The effect of oral treatment on rats given an injection of ^{241}Am was studied by (a) comparing the reduction of an internal burden of ^{241}Am from rats fed C_{12}TT or C_{22}TT with the natural biological retention of ^{241}Am under condition of no chelation treatment, (b) examination of histologic sections of tissues obtained from treated rats, and (c) comparing the relative weight gain of treated rats and controls during the chelon feeding period.

The effect of feeding C_{12}TT or C_{22}TT on the retention of ^{241}Am was tested in three groups of nine rats each. Sprague–Dawley rats (Simonson Labs., Gilroy, CA), aged 60 days, were used in these studies. The animals were housed in groups of three or four until treatment was initiated. ^{241}Am was given via the jugular vein as a single injection in citrate buffer of pH 3.5. One week later, rats were transferred to individual cages and feeding of the chelator was initiated. This delay allowed the nuclide to be incorporated firmly in the target organs, mostly liver and bone. The first group served as untreated controls, the second and third groups received 50 μmol of C_{12}TT or C_{22}TT/rat or about 200 μmol/kg of body weight, respectively, in their daily ration of 25 g of rodent chow. On days 1 and 7 after injection and on days 3, 6, and 10 of chelation treatment, the total body retention of ^{241}Am was measured using a whole-body counter. The animals were necropsied after 10 days of oral chelation therapy, and the femora, liver, 1 kidney, viscera, and carcass with integument were counted for ^{241}Am content. The other kidney and a sample of the small and large intestine were prepared for histological evaluation. Whole-body retentions as a function of time were evaluated. Terminal organ retentions were measured after necropsy only. The reduction in whole-body or organ retention due to oral chelation treatment was expressed as

$$\text{reduction} = 1 - \frac{\text{fractional retention in treated specimen}}{\text{fractional retention in control specimen}}$$

both taken at the same time after treatment initiation. Results are shown in Table I. Both chelons were highly effective, resulting with C_{12}TT in a 29% and C_{22}TT in a 44% reduction of the whole-body ^{241}Am. The biggest fractional reduction was seen in the liver burden with 71% and 89%, respectively. However, there also was 20% and 36%, respectively, less in the carcass and 17% and 32% less in the femora, indicating that the loss from bone also was accelerated. The insignificance of the loss from the kidney may have been due to the small amount of ^{241}Am found in this organ. There certainly was no increased retention of ^{241}Am in the kidneys.

At the age used, rats were growing vigorously. After 10 days of oral chelation treatment, there was no statistical difference in weight gain between the three groups. At the end of the feeding period, controls showed a weight gain of 61.1 ± 8.8 g, rats receiving the C_{12} compound gained 55.7 ± 11.1 g and those fed the C_{22} compound also showed a gain of 70.2 ■ 11.6 g. All animals appeared active and alert.

A segment of the duodenum, the large intestine, and kidney were fixed in formalin and embedded in paraffin, and histological

Table I. Reduction of ^{241}Am Organ Burden by Oral Chelon Administration[a]

organ	C_{12}TT treated: % reduction ± fsd[b]	C_{12}TT treated: *p* value	C_{22}TT treated: % reduction ± fsd	C_{22}TT treated: *p* value
whole body	29 ± 0.07	<0.001	44 ± 0.08	<0.001
carcass	20 ± 0.14	<0.001	37 ± 0.13	<0.001
both femora	17 ± 0.12	<0.001	32 ± 0.11	<0.001
liver	71 ± 0.46	<0.001	89 ± 0.41	<0.001
one kidney	23 ± 0.57	>0.1	26 ± 0.51	>0.1

[a] Reduction = 100 − (% of pretreatment activity in treated rats at death)/(% of pretreatment activity in controls at death); nine rats/group; 10 days of treatment. [b] FSD = fractional standard deviation = variance/(% reduction value).

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sections were prepared. The sections were stained with hematoxylin and eosin, and examined microscopically. There was no histological evidence of any tissue toxicity associated with the chelon administration after the 10 days of treatment.

Discussion

Attempts to use chelation therapy for removal of actinides from humans were started in the mid 1940s[33] with the first success noted about 1950 using EDTA.[34] DTPA given as the Ca or Zn chelate also has proven to have some effect on reducing body burdens of metals in humans and is currently approved as an experimental drug.[20] However, DTPA and EDTA require frequent parenteral administrations, have limited cellular and tissue penetration, can result in metal redistribution rather than decorporation, and can have toxic side effects. The emphasis of the present work was to overcome these detrimental properties, to use the experience gained with EDTA and DTPA, and to maintain their beneficial properties seen with their use as a drug for the removal of certain radioactive elements and other toxic heavy metals. It was our aim to synthesize new compounds with chelation characteristics similar to EDTA and DTPA but different pharmacological properties that, as a primary objective, eliminated the obstacle of parenteral administration and poor cellular penetration. It was also our goal to produce a compound that is effective orally and can be given quickly after a contamination event as well as over long periods of time. The results described in the present report indicate that compounds with these desired properties can be obtained. This report is intended as a first communication describing only the synthesis, chemical characterization, and some of the in vivo and in vitro properties of these new compounds. These new, partially lipophilic PACAs also pass the intestinal barrier and thus can be given orally. The compounds appeared to penetrate the sites of primary-metal deposition which are, in part, intracellular. They interact with the actinides, are excreted without redeposition in other organs, as for instance the kidneys, and have no obvious signs of toxicity using the doses and times as described in this report. An extension of the work in which the efficacy and the physicochemical, pharmacological, and toxicological properties are evaluated in detail and compared with those of the parent compounds EDTA and DTPA and other chelators is in progress.

Commonly in the past, carboxymethylation of the alkyl-substituted amine obtained in the first step of the synthesis was carried out in aqueous media using digestion with bromoacetic acid at a basic pH. In our hands, the products usually were not very well defined and were extremely difficult to purify. For most purposes, therefore, the resulting material was not suitable for in vivo use. The nonaqueous synthesis method described in this paper produced the desired compounds with a reasonable yield and in the highly purified form that made both a rigorous chemical characterization and in vivo testing possible.

The compounds described in some more detail in this report, C_{12}TT and C_{22}TT, are only two representatives of a series of PACAs with chain lengths ranging from C_6 to C_{22} that have been synthesized from triethylenetetramine and diethylenetriamine (DT). Due to the availability of two additional donor groups, chelators synthesized from TT proved to be stronger for the 8-coordinate Am than those based on DT. However, it remains to be determined if DT derivatives may have some therapeutic advantage on certain other types of metal deposits.

Altering the length of the alkyl side chains and consequently the lipophilicity of the chelators is expected to change several of their pharmacological properties. Preliminary data suggest that the length of the alkyl chain will influence the intestinal absorption of the compounds, their selective uptake by individual organs or tissues, and their transport across cellular and subcellular membrane barriers. This property might be used to direct the compounds to metal depot sites in the cell. This is important, for instance, as actinide deposits in the liver are known to be located initially in the hepatocytic ferritin with subsequent migration to secondary lysosomes and then to macrophages associated with sinusoidal lining cells. It is expected that for fresh deposits of the nuclides, compounds with shorter side chains may be more effective, whereas removal from lysosomes and macrophages may be more effective using longer chain, micelle-forming compounds. Further studies are necessary to determine the pharmacokinetics of these partially lipophilic compounds with their less lipophilic counterparts, i.e. EDTA and DTPA, and their relative efficacy in the removal of actinides from cells, tissues, and organs.

The effectiveness of the oral administration of the two tested model compounds, C_{12}TT and C_{22}TT, to remove aged Am deposits from the body was demonstrated in a rodent model. The power of these chelons to greatly accelerate the removal of established deposits of Am by this route of administration relates to their ability to traverse cellular barriers and membranes. The longer chain may form micelles and be more readily incorporated into the liver. As discussed above, such a mechanism may also help explain the greater loss of Am from the bone, where the longer side chain may increase the uptake of the chelator by bone marrow macrophages, also a site of Am deposition. The use of an oral chelator offers considerable improvement over existing methods.

In the doses given in this report, which were remarkably effective in accelerating the removal of established deposits of Am from the body, there were no obvious toxic reactions to the chelators. There were no significant differences in body weights and no overt histological evidence of pathological damage to the tissues. This is in contrast to expectations based on the destruction of the epithelial lining and related structures of the intestinal tract followed by death after frequent administration of Ca–DTPA in dogs as observed by Taylor.[35] A possible mechanism for this damage is found in the studies of Bohne[36] and Taylor.[37] Some adverse reactions may be expected with different time and/or dose regimens. Such toxicity might be expected because of interference with biochemical pathways due, for example, to inactivation of metal-dependent enzyme systems, toxicity of degradation products, and trace-metal depletion. Complete toxicological testing will be done when the efficacy of these or related compounds for the removal of other actinide elements or

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possibly other toxic heavy elements and optimal conditions for their administration have been established.

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Registry No. 1, 112-24-3; **2a**, 56166-94-0; **2a**·4HCl, 137433-54-6; **2b**, 137203-77-1; **2b**·4HCl, 137433-55-7; **3a**, 137203-78-2; **3b**, 137203-79-3; **4a**, 137203-80-6; **4a**·4HCl, 137433-56-8; **4b**, 137203-81-7; **4b**·4HCl, 137433-57-9; Pu, 7440-07-5; Am, 7440-35-9; ^{239}Pu, 15117-48-3; ^{241}Am, 14596-10-2; $CH_3(CH_2)_{11}Br$, 143-15-7; $CH_3(CH_2)_{21}Br$, 6938-66-5; $BrCH_2COOEt$, 105-36-2.

Supplementary Material Available: Mass spectrum of **4b** (2 pages). Ordering information is given on any current masthead page.

Synthesis and Biological Activities of Fatty Acid Conjugates of a Cyclic Lactam α-Melanotropin

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Four fatty acid conjugates of a cyclic lactam-bridged α-MSH fragment analogue were synthesized and their potencies and biological activities compared in several melanotropin bioassays. Palmitoyl, myristoyl, decanoyl, and hexanoyl conjugates of H-Asp-His-D-Phe-Arg-Trp-Lys-NH_2 were prepared. In the in vitro mouse melanoma cell assay, each of the conjugates was 10–100 times more potent than α-MSH or the substrate peptide in elevating tyrosinase activity. The shorter conjugates of hexanoic and decanoic acid were as potent as α-MSH in the lizard skin bioassay, whereas the longer myristoyl and palmitoyl analogues were about 100 times less potent. The potency of the myristoyl and palmitoyl conjugates increased with time in contact with the skins. These observations may be related to the more lipid-like nature of these peptide–fatty acid conjugates. Each of the conjugates exhibited prolonged melanotropic activity in the lizard skin bioassays and in the mouse S91 melanoma tyrosinase bioassay, since the biological response continued following removal of the conjugates from the incubation media. The prolonged residual melanotropic activity resulted from conjugation of the fatty acids to the MSH fragment analogue since the analogue itself did not exhibit prolonged activity.

Introduction

α-Melanotropin, α-MSH (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2), is a tridecapeptide that is biosynthesized and secreted from the pars intermedia of the pituitary gland.[2] This hormone plays an essential role in the adaptive color changes of many animals through its action on integumental melanocytes.[3] The melanotropic peptide appears to regulate other physiological functions as well.[4-6]

Because of an extensive hepatic first-pass elimination, peptides usually are not orally active and often require parenteral administration to be therapeutically effective. A potential route of administration of peptides is through the skin. It has been shown that the skin lacks the proteolytic enzymes which are responsible for the enzymatic degradation of peptides.[7] In addition, the use of iontophoresis as a facilitating technique in transdermal delivery of peptides shows promising results.[8] The majority of drugs that have been successfully delivered through skin are lipophilic, charged, and of small molecular size. Certain α-MSH analogues can be transdermally delivered through the skin of mice[9-11] and humans.[12]

We have attempted to design α-MSH analogues that possess structural characteristics more favorable for delivery either across the skin or across other epithelia. These peptides are derivatives of a previously reported superpotent melanotropin, Ac[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH_{4-10}-NH_2.[13-15] All of these peptides have a general structure, FA-[Asp^5,D-Phe^7,Lys^{10}]α-MSH_{5-10}-NH_2 (FA = fatty acid), with various chain lengths of lipophilic hydrocarbons at their N-terminal, a fixed ring size (23-membered), and a charge (2+ if His imidazole is counted). The preparation of these cyclic lactam α-melanotropin

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(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC–IUB Commission of Biochemical Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977). All optically active amino acids are of the L variety unless otherwise stated. Other abbreviations include the following: α-MSH, α-melanotropin, α-melanocyte stimulating hormone; Nle, norleucine (2-aminohexanoic acid); Bom, benzyloxymethyl; 2,4-Cl_2-Z, 2,4-dichlorobenzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DIC, diisopropylcarbodiimide; 2,6-Cl_2-Bzl, 2,6-dichlorobenzyl; Fmoc, fluorenyloxymethylcarbonyl; Fmo, fluorenylmethyl ester, pMBHA resin, *p*-methylbenzhydrylamine resin; HOBt, *N*-hydroxybenzotriazole; For, formyl; Tos, tosyl; N^{α}-Boc, N^{α}-*tert*-butyloxycarbonyl; 1-BuOH, 1-butanol; TEA, triethylamine; TFA, trifluoroacetic acid; DIEA, diisopropylethylamine; DCM, dichloromethane; DMAP, 4-*N*,*N*-dimethylpyridine; FA, fatty acid; Pml, palmitic; Mrl, myristic; Dcl, decanoic; Hxl, hexanoic.

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