Conjugates of Gadolinium Complexes to Bile Acids as Hepatocyte-Directed Contrast Agents for Magnetic Resonance Imaging

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Received October 20, 2003

A series of structurally different Gd(III) conjugates incorporating a bile acid moiety have been prepared. Polyaminopolycarboxylic ligands such as diethylenetriaminepentaacetic acid (DTPA) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetracetic acid (DOTA) have been selected as chelating subunit for the Gd(III) ion. Cholic acid, cholylglycine, and cholyltaurine have been incorporated as the bile acid moieties. In first generation conjugates the Gd(III) complex is linked to the carboxyl group of cholic acid. Second generation conjugates feature the attachment of the Gd(III) complex to the 3 position of the steroidic backbone of the bile acid. Finally, in third generation conjugates the Gd(III) complex is attached to the ϵ nitrogen atom of cholyllysine. The conjugates are eliminated through the biliary route to a various extent (7.5 to 77% in rats) according to their structural features. Among the most promising terms, a second generation conjugate in which the Gd(III) complex is linked to cholic acid through the 3α hydroxy group seems to enter hepatocytes using the Na^+/taurocholate transporter. Noticeably, some of the second generation conjugates are characterized by very high tolerabilities (LD_{50} up to 9.5 mmol/kg) after intravenous administration in mice.

Introduction

In the past decade the use of gadolinium complexes of simple polyaminopolycarboxylic ligands (e.g. diethylenetriaminepentaacetic acid = DTPA or 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid = DOTA) as extracellular MRI contrast agents has become more and more widespread.[1] Such agents immediately after intravenous administration equilibrate between blood and the interstitial fluid; however, they do not enter cells.[2] In the meantime, the search for complexes, which are taken up into hepatocytes, has been quite active.[3] Noticeably, Gd(III) complexes in which a lipophilic residue was introduced in the basic unit of DTPA have been developed as hepatospecific agents (e.g. Gd-BOPTA,[4] Gd-EOB-DTPA[5]). Compared to the extracellular contrast agents, these "lipophilic" complexes are taken up to a variable extent by hepatocytes in different animal species[6] as well as in humans.[7,8] However, it has been shown that the transport of such agents through the basolateral membrane of hepatocytes occurs by passive diffusion.[9]

More recently, we became interested in contrast agents which enter hepatocytes by means of active transport mechanisms. Conjugation of the DTPA moiety to a triiodinated carrier (i.e. iopanoic acid)[10] led to a contrast agent which was taken up by hepatocytes through a carrier-mediated mechanism operated by the organic anion transporting polypeptide (OATP).[9] Indeed, it has been shown that the Na^+/taurocholate transporter is not expressed in the basolateral membrane of some human hepatoma cell lines.[11] Therefore, the discovery of a contrast agent which is transported into hepatocytes by such a transporter could, in principle, allow clear-cut diagnoses of specific hepatic diseases (e.g. hepatocellular carcinoma). Since it is known that several other bile acids are taken up by hepatocytes through the Na^+/taurocholate transporter, we investigated whether bile acids can be successfully used as address moieties to confer specificity to Gd(III) complexes.

In this paper we discuss (i) the structural features that were taken into account in the design of the Gd(III) complex/bile acid conjugates, (ii) the synthetic approaches for the preparation of the conjugates, and (iii) pharmacokinetic and toxicology evaluation of the conjugates.[12]

Results and Discussion

Design of the Conjugates. We studied conjugates in which a Gd(III) complex is linked to a bile acid through a spacer of different length. Various chelating subunits for the coordination of Gd(III) ion were selected. As a general requirement they had to afford conjugates characterized by thermodynamic and kinetic stabilities for the Gd(III) complex high enough to allow in vivo administration. We incorporated in the structure of the conjugates, DTPA, DTPA monoamide, DOTA monoamide, and 10-hydroxypropyl-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (HP-DO3A) subunits. On the basis of thermodynamic studies carried out on the parent or closely related structures,[13] we can assume that all these chelating subunits are characterized by log $K_{ML} > 19$.

Among the bile acids for which hepatocyte uptake is demonstrated to be mediated by the Na^+/taurocholate transporter,[14] we chose cholic acid, cholylglycine (glycocholic acid), and cholyltaurine (taurocholic acid).

The site of conjugation of the chelating subunit on to the bile acid moiety was quite thoroughly explored.

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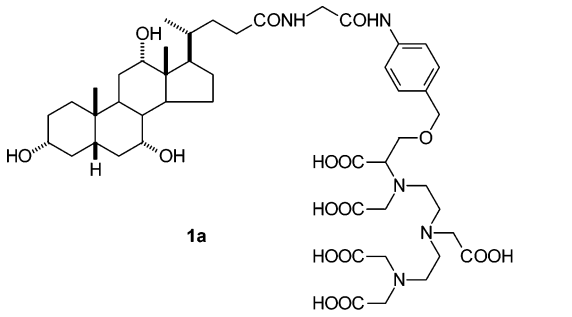
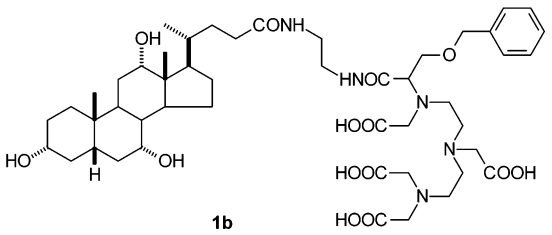
10.1021/jm0310683 CCC: $27.50
Published on Web 06/05/2004

Chart 1



1a



1b

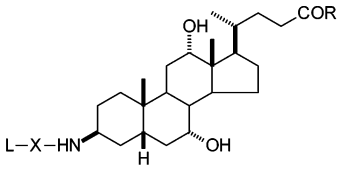
Accordingly, three different generations of conjugates can be recognized.

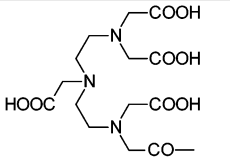
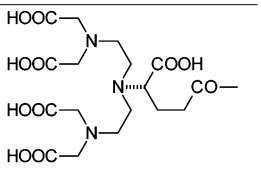
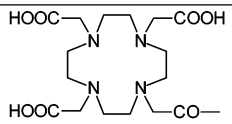
First Generation Conjugates. From a purely synthetic perspective the easiest way to functionalize cholic acid takes advantage of its carboxylic moiety. Indeed, a cholic acid conjugate of EDTA has been already reported.[15] We therefore planned the synthesis of the two ligands (Chart 1) in which cholic acid is linked to the chelating subunit through an amide bond. It is worth noting that upon complexation with Gd(III) such DTPA and DTPA-monoamide ligands give rise to complexes characterized by a residual charge of −2 and −1, respectively. On the basis of previous studies, a negative charge on the chain linked to C17 on the steroid moiety seems crucial for bile acids to be taken up by the Na^+/taurocholate transport system.[16]

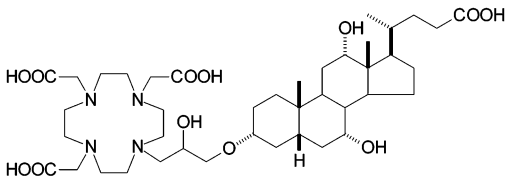
Second Generation Conjugates. In light of the importance of a negative charge on the side chain, the second approach involved the insertion of the chelating moiety on to the steroid skeleton of the bile acid. In particular, we focused on the hydroxy group in position 3, which is more reactive and accessible if compared to the other two hydroxy groups in positions 7 and 12. This strategy, already reported by Kramer and co-workers[17] for the synthesis of bile acid−drug conjugates for the specific targeting of liver, was successfully applied to achieve the DTPA, DOTA, and HP-DO3A derivatives (Chart 2). A homogeneous series of DTPA monoamides conjugated to cholic acid, cholylglycine and cholyltaurine, i.e. **1c**−**e,** was also synthesized in order to assess the role played by the bile acid.

Third Generation Conjugates. Generally speaking, the last approach involved the coupling of the bile acid carboxylic moiety to the α-amino group of a functionalized α-amino acid. Accordingly, cholic acid was coupled to lysine and after further functionalization led to the conjugates depicted in Chart 3. This strategy is very attractive and straightforward because the steroid fragment of the bile acid is not modified and a free carboxylic moiety in the side chain is still retained. This synthetic approach has already showed promising results in the enhancement of the hepatoselectivity of rhodamine,[18] thyroxine,[19] and peptide[20] conjugates.

Chart 2

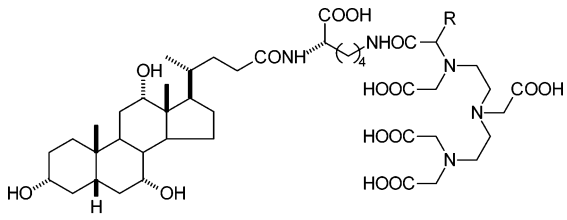


	R	X	L
1c	OH	$NHCH_2CO$	
1d	$NHCH_2COOH$	$NHCH_2CO$	
1e	$NHCH_2CH_2SO_3H$	$NHCH_2CO$	
1f	OH	$NH(CH_2)_5CO$	
1g	OH	none	
1h	$NHCH_2CH_2SO_3H$	none	
1i	$NHCH_2CH_2SO_3H$	$NHCH_2CO$	



1j

Chart 3



1k R = CH_2OBzl

1l R = H

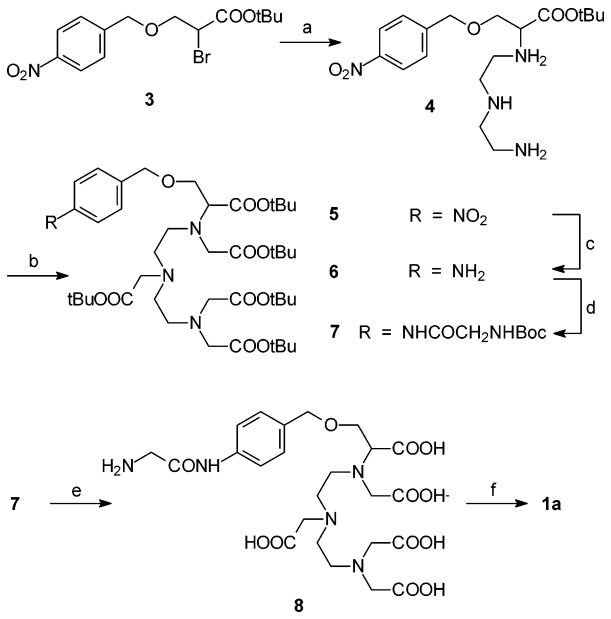
Chemistry

The synthesis of **1a** is reported in Scheme 1. A 5-fold molar excess of diethylenetriamine was reacted with the bromo derivative **3**[21] in order to maximize the yield in monoalkylated product **4**, which was then fully alkylated with *tert*-butyl bromoacetate. The nitro group of **5** was reduced to amino and acylated with Boc-glycine, using diethyl cyanophosphonate (DEPC) as coupling reagent.[22] Deprotection of Boc and *tert*-butyl esters with trifluoroacetic acid gave compound **8**, which was coupled to cholic acid *N*-hydroxysuccinimidyl ester **9**[23] (Chart 4) to obtain ligand **1a**.

Ligand **1b** was synthesized by reaction of monoacid **10**[24] with cholic acid *N*-(2-aminoethyl) amide **11**[25] (Chart 4) in the presence of DEPC, followed by hydrolysis of the methyl esters with NaOH at pH 12 (Scheme 2).

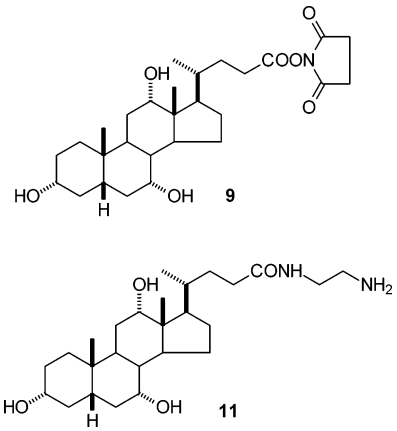
The synthesis of the bile acid intermediates **15c**−**f**, which are necessary for the achievement of the second

Scheme 1[a]



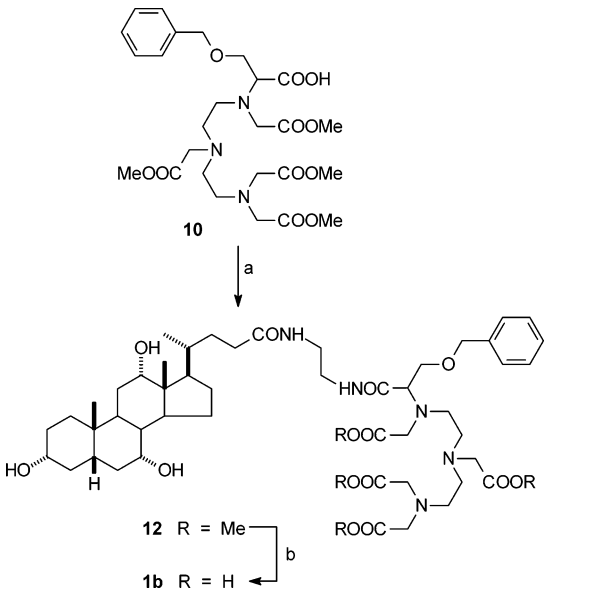
[a] Reagents and conditions: (a) $(NH_2CH_2CH_2)_2NH$, MeCN; (b) $BrCH_2COOtBu$, $ClCH_2CH_2Cl$, DIEA; (c) H_2, Pd/C, EtOH; (d) Boc-Gly, DEPC, Et_3N; (e) CF_3COOH, PhOMe, CH_2Cl_2; (f) **9**, H_2O–DMF.

Chart 4



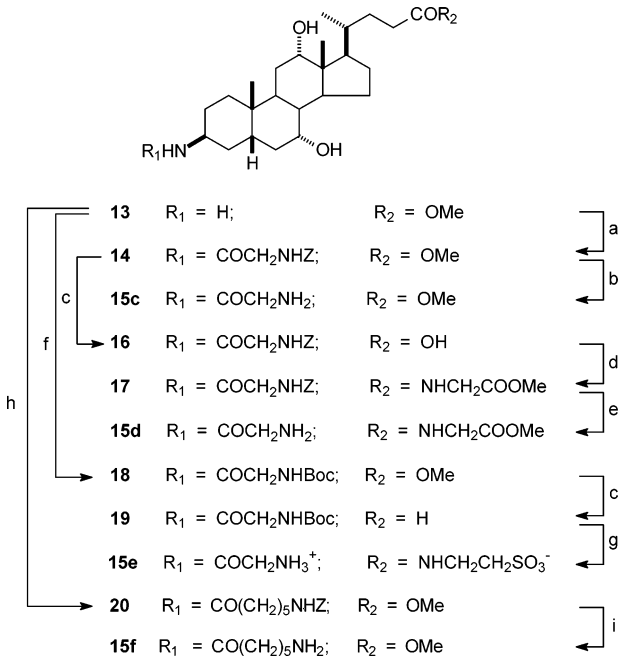
generation conjugates, is depicted in Scheme 3. The starting key intermediate **13** was synthesized according to a previously published procedure.[26] Glycine and 6-aminohexanoic acid were chosen as spacers between the polyaminopolycarboxylic moiety and the bile acid. In this way, Z-glycine and *N*-Z-6-aminohexanoic acid were activated by means of isobutyl chloroformate and coupled to compound **13** to give, after hydrogenolysis of the Z-group, derivatives **15c** and **15f**, respectively. To achieve the whole series of bile acid derivatives (i.e. with cholic acid, cholylglycine and cholyltaurine) the side chain of compound **13** was modified. The cholylglycine derivative **15d** was obtained from **14** by hydrolysis of the methyl ester, coupling to glycine methyl ester, and final hydrogenolysis of the Z-group. The cholyltaurine derivative **15e** was synthesized by coupling Boc-glycine to **13**, hydrolysis of the methyl ester, coupling to taurine using 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ),[27] and deprotection of the Boc group with HCl in MeOH. The ligands **1c**–**f** were obtained as shown in Scheme 4. A four molar excess of bis-anhydride **21**[28] (Chart 5) was suspended in DMF and

Scheme 2[a]



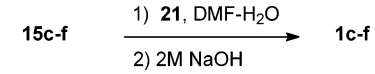
[a] Reagents and conditions: (a) **11**, DEPC, Et_3N, DMF; (b) NaOH, H_2O–MeOH.

Scheme 3[a]



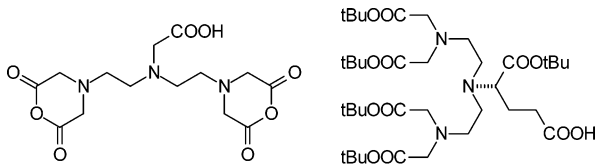
[a] Reagents and conditions: (a) Z-Gly, IBCF, NMM, THF; (b) H_2, Pd/C, MeOH–H_2O; (c) i: NaOH, H_2O–MeOH; ii: HCl; (d) GlyOMe, IBCF, Et_3N, THF; (e) H_2, Pd/C, MeOH; (f) Boc-Gly, IBCF, Et_3N, THF; (g) i: $NH_2CH_2CH_2SO_3H$, EEDQ, Et_3N, DMF; ii: HCl, MeOH; (h) $ZNH(CH_2)_5COOH$, IBCF, Et_3N, THF; (i) H_2, Pd/C, EtOH.

Scheme 4



partially hydrolyzed at 80°C by addition of water then reacted with amino derivatives **15c**–**f**. This convenient procedure allows to increase the yield of the corresponding DTPA monoamides, overcoming the problem faced when using bis-anhydride **21** alone in DMF. In this case, even with a large excess of **21**, the formation of DTPA bisamide derivatives is predominant.

Chart 5



21 22

After completion of the amidation reaction, NaOH was added to hydrolyze the methyl ester moiety (when present) and residual anhydride moieties. The crude was purified by elution through an Amberlite XAD-16 resin column with a MeCN/H_2O gradient. Elution with only water enables elimination of the excess of DTPA and the residual content of DMF, while the DTPA monoamides **1c**−**f** are usually eluted with 30−50% MeCN in water.

The useful and versatile pro-ligand **22**[29] (Chart 5) was employed for the synthesis of both DTPA ligands **1g** and **1h**. In particular, **22** was coupled to 3β-aminocholic acid methyl ester **13** by means of DEPC to give the hexaester **23** which was fully deprotected to **1g** with H_2SO_4 in dioxane (90 °C). With the same strategy the coupling of **22** to 3β-aminocholic acid benzyl ester **24** gave derivative **25** which was hydrogenolysed, coupled to taurine, and finally deprotected to ligand **1h** (Scheme 5).

The ligand **1i** was obtained by coupling the DOTA tris-*tert*-butyl ester **27**[30] (Scheme 6) to the cholyltaurine derivative **15e** in the presence of benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) and deprotection of the *tert*-butyl esters in a mixture of water, acetic acid, and 2-propanol at reflux.

The ligand **1j** was the last second generation product and its synthesis is shown in Scheme 7. Cholic acid *tert*-butyl ester[31] was reacted with an excess of epichlorohydrin under phase transfer conditions to give **29** as the main product. Reaction of 1,4,7,10-tetraazacyclododecane-1,4,7-triaacetic acid tris(1,1-dimethylethyl) ester (DO3A ^{t}Bu ester)[32] with the epoxy derivative **29** in refluxing ethanol gave compound **30** which was hydrolyzed in 0.5 M sulfuric acid at 90 °C to the ligand **1j**.

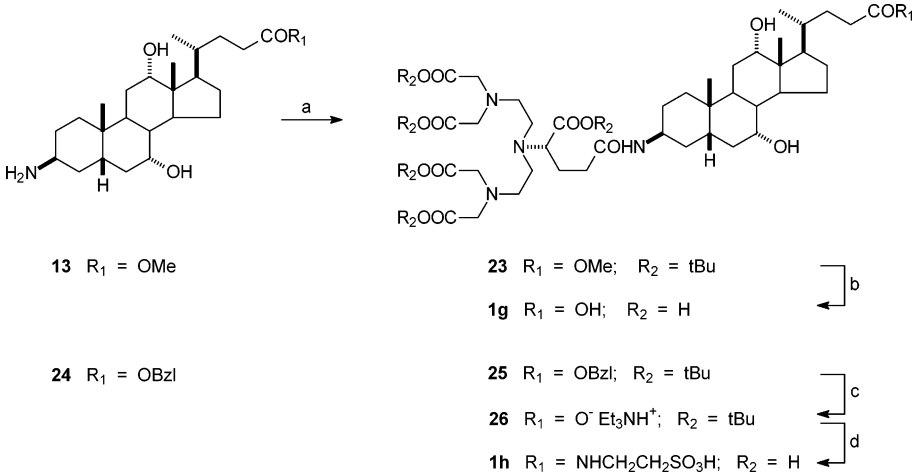
The synthesis of the third generation products **1k** and **1l** is depicted in Scheme 8. The key intermediate **32** was prepared by activation of cholic acid by means of isobutyl chloroformate, coupling to N^6-Z-lysine, esterification with MeOH/PTSA, hydrogenolysis of the Z group, and treatment with HCl/MeOH. Interestingly, if the acidic treatment is not performed just before evaporation, the only product obtained is the lactam **33** (Chart 6). Coupling of **32** with the monoacid **10** followed by complete hydrolysis of the methyl esters with NaOH gave ligand **1k**. Ligand **1l** was achieved by reaction of **32** with bis-anhydride **21**[28] (Chart 5) and successive hydrolysis with NaOH, using the same procedure previously described for ligands **1c**−**f**.

Ligands **1a**−**l** were converted into the corresponding gadolinium complexes **2a**−**l** using two different procedures. According to procedure A (see Experimental Section), which is the most simple and straightforward, Gd_2O_3 is added to a water solution of the ligand and a suitable amount (according to the stoichiometry of the final complex) of NaOH, and the suspension is heated at 50 °C for several hours. The complex is then simply recovered after filtration of the excess Gd_2O_3 and evaporation of the aqueous solution.

If the ligand is not stable to prolonged heating, procedure B, which employs $GdCl_3$ as a source of Gd(III) ions, must be adopted. In this case a solution of $GdCl_3$ is added dropwise to a solution of the ligand, while the pH is constantly maintained at 7 by addition of a suitable base, usually NaOH. A desalting step is then necessary in order to eliminate the produced "byproduct" salt (e.g. NaCl).

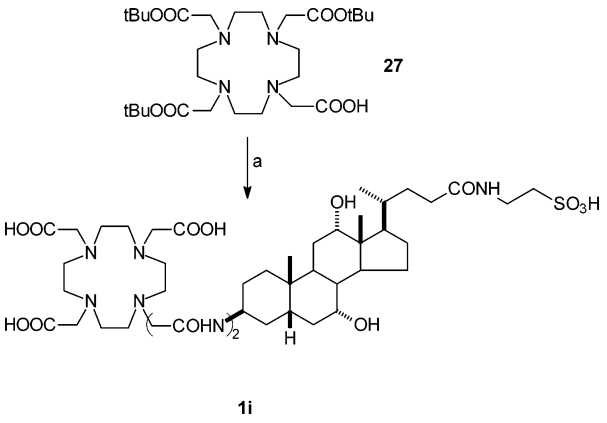
Tolerability. Animal tolerability tests were performed in mice by the intravenous route, aimed at assessing at least the low tolerability limit. When available, survival at the dose of pharmacokinetic assay in rats was used as toxicity estimate. Further investigations also assessed the toxicity by the intracerebroventricular route on the most promising candidates, as this test was recognized to be predictive for chemotoxicity of iodinated contrast agents.[33] To allow for a comparison, LD_{50} values for two of the extracellular ionic gadolinium complexes which are used clinically,

Scheme 5[a]



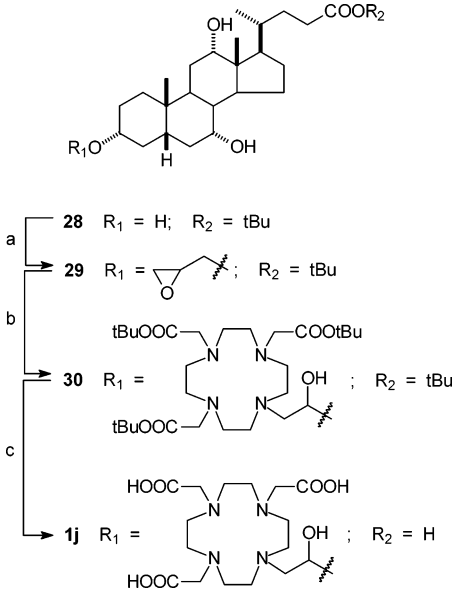
[a] Reagents and conditions: (a) **22**, DEPC, Et_3N, DMF; (b) aq H_2SO_4, dioxane, Δ; (c) i: H_2, Pd/C, EtOH; ii: column chromatography with CH_2Cl_2/MeOH/Et_3N; (d) i: $NH_2CH_2CH_2SO_3H$, DEPC, Et_3N, DMF; ii: aq H_2SO_4, dioxane, Δ.

Scheme 6[a]



[a] Reagents and conditions: (a) i: **15e**, BOP, DIEA, DMF; ii: H_2O/AcOH/*i*-PrOH, Δ.

Scheme 7[a]

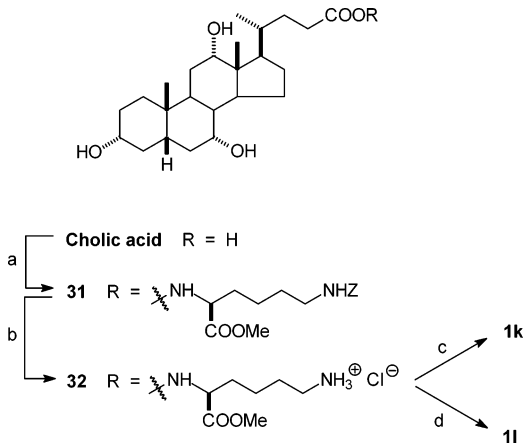


[a] Reagents and conditions: (a) epichlorohydrin, Bu_4NHSO_4, 50% NaOH−CH_2Cl_2; (b) DO3A ^{t}Bu ester,[32] EtOH, Δ; (c) aq H_2SO_4, Δ.

namely Gd-DTPA-dimeg and Gd-DOTA-meg, are also reported in Table 1.

First generation conjugates, i.e. **2a**,**b**, proved too toxic to deserve further investigation. Differently, several second generation conjugates showed more than promising preliminary results. Noticeably, all terms of the homogeneous series **2c**−**e**, i.e. featuring the same chelate subunit coupled to cholic acid, cholylglycine, and cholyltaurine, respectively, show iv LD_{50} > 3.0 mmol kg^{-1}. This seems to indicate that this class of conjugates are characterized by good tolerability independently of the nature of the bile acid. Furthermore, conjugates **2c** and **2e** show a more than acceptable degree of neurotoxicity. Also, conjugate **2g**, which differs from **2c** because all five carboxylate groups of DTPA are involved in the complexation of the gadolinium ion, featured high tolerability (i.e. iv LD_{50} 7.6 mmol kg^{-1}). The tolerability data gathered for third generation conjugates, i.e., **2k**,**l**, are only preliminary and do not allow us to draw any definitive conclusion. Owing to their safety levels, compounds **2c** and **2g** were selected for further safety assessment and underwent cardiovascular safety and mutagenicity studies.

Scheme 8[a]



[a] Reagents and conditions: (a) i: N^6-Z-Lys, IBCF, Et_3N, THF; ii: PTSA, MeOH; (b) H_2, Pd/C, MeOH·HCl; (c) i: **10**, BOP, DIEA, DMF; ii: NaOH, H_2O−MeOH; (d) i: **21**, DMF−H_2O; ii: 2 M NaOH.

Chart 6

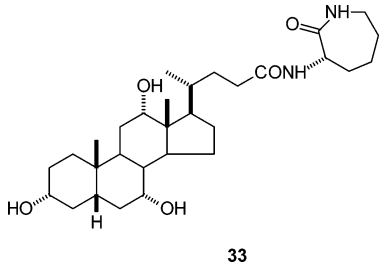


Table 1. Tolerability of Complexes **2a**−**l**

compound	iv LD_{50} (mmol/kg)[a]	ic LD_{50} (mmol/kg)[a]
2a	<2.0[b]	0.0512
2b	<0.25[b]	n.p.[c]
2c	>7.0[d]	0.30 (0.22−1.13)
2d	>3.0[d]	n.p.[c]
2e	9.5 (8.2−20)[d]	0.21 (0.16−0.27)
2f	>0.25[b]	n.p.[c]
2g	7.6 (5.9−16.4)[d]	n.p.[c]
2h	>0.25[b]	n.p.[c]
2i	>2.0[d]	<0.00125
2j	1.19 (0.93−1.45)[d]	0.003−0.006
2k	>0.25[b]	n.p.[c]
2l	>0.25[b]	n.p.[c]
Gd-DTPA-dimeg	6[e]	-
Gd-DOTA-meg	10.6[f]	-

[a] LD_{50} = median lethal dose; iv = intravenous, ic = intracerebroventricular administration (95% CL). [b] In rats, values express mortality at the dose of pharmacokinetic assay (see Experimental Section). [c] Not performed. [d] In mice. [e] From ref 34. [f] From ref 35.

Neither **2c** nor **2g** showed significant activity on cardiovascular system in anaesthetized rabbits. The cardiovascular changes induced by these compounds were short lasting: all the examined parameters recovered the baseline values in 10−15 min after the injection. Moreover, the effects observed with both compounds were not qualitatively different from those observed after injection of saline and mannitol solutions used as control articles.

When **2c** and **2g** underwent the Ames test, although toxicity was observed with **2c** at just 5000 μg/plate, no evidence of mutagenic activity was seen for both compounds in all tested strains, either in the presence or in the absence of metabolic activation. The tolerability

Table 2. Pharmacokinetic Evaluation of Complexes **1a**−**l**. Elimination in Rats, after 8 h (dose level 0.25 mmol kg^{-1})

conjugate	biliary (%)	renal (%)
2a	25.4 ± 3.6	76.1 ± 3.6
2b[a]	71−77 ($n = 2$)	0−2.8 ($n = 2$)
2c	45.6 ± 2.5	50 ± 13
2d	20.3 ± 2.9	71.32 ± 0.42
2e	19.9 ± 7.9	71 ± 18
2f	52 ± 11	36.6 ± 4.2
2g	46.4 ± 2.7	56.9 ± 1.0
2h	12 ± 6.8	89 ± 14
2i	18.4 ± 2.0	66 ± 10
2j	63.5 ± 5.0	18.0 ± 6.7
2k	34.0 ± 3.5	57.4 ± 3.1
2l	7.5 ± 3.8	84 ± 12

[a] Too toxic to be evaluated after administration at a dose level of 0.25 mmol kg^{-1}, tested at 0.1 mmol kg^{-1}.

data so far collected indicate that **2c** and **2g** are safe enough candidates for possible development.

Pharmacokinetic Evaluation. Conjugates **2a**−**l** underwent pharmacokinetic evaluation, and results, in terms of biliary vs renal elimination, are reported in Table 2 and deserve some comments. Among the first generation conjugates, **2a** showed a moderate biliary elimination, whereas **2b**, although more toxic than **2a**, showed an higher biliary elimination. This difference in the elimination pattern could be related to the global charge of the conjugates (i.e. −2 for **2a** and −1 for **2b**) and/or to the presence of the benzyloxymethyl residue in **2b**. More interesting are the results found for the second generation conjugates. A comparison among structurally analogous conjugates **2c**−**e** indicates that cholic acid (compound **2c**) directs transport of gadolinium conjugates from blood into bile two times more effectively than cholylglycine and cholyltaurine. This evidence is somehow unexpected. Indeed, after intraportal administration in rats of bile acids, it was observed that cholyltaurine is the bile acid most efficiently transported into bile followed by cholylglicine and cholic acid.[36] These results were further confirmed in rat liver perfusion experiments. Attempts to explain this trend in terms of different extent of binding to serum albumin failed since each of the three bile acids have a similar fraction bound to albumin as determined by equilibrium dialysis.[37]

Our observations with **2c**−**e** can be explained on the basis of two hypotheses: (i) **2d** and **2e**, possibly due to their lower binding to rat albumin, are excreted more quickly through the kidneys than **2c**; (ii) conjugation to gadolinium complexes of cholyltaurine and cholylglycine influences their nature to such an extent that the corresponding conjugates are less efficiently recognized by the systems responsible for transport through hepatocytes than the conjugate containing cholic acid.

The introduction of a spacer between cholic acid and the chelating mojety, see **2f** in comparison with **2c**, did not exert any change in the biliary elimination. Cholyltaurine conjugates **2e** and **2i**, which contain a subunit of Gd-DTPA-monoamide and Gd-DOTA-monoamide, respectively, show very similar elimination patterns. This indicates that for second generation conjugates, a variation of the global charge from −2 to −1 is without major effects on the elimination route. The cholic mojety is again more effective toward the biliary elimination than the cholyltaurine moiety, also among conjugates bearing −3 global charge (**2g** vs **2h**). The third generation conjugates (**2k** and **2l**) showed biliary elimination of 34% and 7.5%, respectively. This could likely reflect the importance of the benzyloxymethyl moiety in facilitating the biliary elimination of these conjugates.

Compound **2j**, which contains a subunit of cholic acid linked to a neutral macrocyclic chelating mojety and features a global charge of −1, showed an high biliary excretion. Moreover, for the compound **2j**, the hepatobiliary elimination was inhibited by coadministration of sodium cholyltaurine (see Experimental Section) from 39% in control animals to 23% in animals with sodium cholyltaurine coadministration (0−30 min). For other conjugates (e.g. **2d**, **2h**) no significant inhibition was noticed. This seems to indicate that the latter conjugates do not use the Na^+/taurocholate carrier to enter hepatocytes, whereas that carrier is, at least in part, involved in the uptake of **2j**. This also suggests that the stereochemical configuration has to be considered in the design of contrast agents which may enter hepatocytes using the Na^+/taurocholate transporter.

Conclusions

It has been shown that bile acids can be used as carrier units for the preparation of MRI contrast agents which are eliminated through the biliary route.[38] The extent to which this is achieved depends on several structural features such as (i) nature of the bile acid, (ii) site of conjugation of the Gd(III) complex to the bile acid moiety, (iii) global charge of the conjugate. For example cholic acid appears to be a much more effective carrier than cholylglycine and cholyltaurine. Several second generation conjugates (i.e. conjugates in which the Gd(III) complex is linked to the position 3 of the steroid moiety of the bile acid) show high biliary elimination as well as good tolerabilities. Noticeably, for such conjugates, the 3α stereochemical configuration on the steroid moiety, such as in the case of conjugate **2j**, seems to play a peculiar role in addressing the Na^+/taurocholate transport system.

Experimental Section

Chemistry. General Methods. Melting points were determined with a Büchi 540 apparatus and are uncorrected. TLC analyses were carried out using Merck KGaA silica gel plates 60 F_{254}. Detection: AcOH/concentrated H_2SO_4/*p*-anisaldeide, 100:2:1; 1% $KMnO_4$ in 1 M NaOH. Flash chromatography was performed using Merck KGaA silica gel 60 (230−400 mesh ASTM). ^1H and ^{13}C NMR spectra were recorded on a Bruker AC 200 spectrometer, equipped with a 5 mm dual probe, using deuterium as lock signal in $CDCl_3$ as the solvent when not noted. The ^1H NMR spectral data are not reported, being useless for the assignment of the structure due to the broadness and extreme overlapping of the signals. Elemental analyses were performed by Redox laboratories, Monza, Italy. Mass spectra were recorded on a TSQ 700 (Finnigan MAT) spectrometer equipped with an ESI source.

Analytical HPLC was performed on a Merck KGaA apparatus: Hitachi high-pressure gradient pump system (L6200 and L6000), Hitachi AS 2000 autosampler, T 6300 column thermostat, Hitachi L 4250 UV detector (210 nm). Flow rate of 1 mL min^{-1}. Analytical HPLC methods: (A) stationary phase: Lichrosorb Select B 5 μm, 250 × 4 mm column packed by Merck KGaA; mobile phase: eluent A = 0.01 M KH_2PO_4 and 0.017 M H_3PO_4 in H_2O, eluent B = MeCN, gradient elution: $t = 0$ min (5% B), $t = 30$ min (80% B), $t = 45$ min (80% B); (B) stationary phase: Kromasil C4 5 μm, 250 × 4

mm column packed by Bischoff; mobile phase: eluent A = 0.017 M H_3PO_4 in H_2O, eluent B = 2-propanol, gradient elution: t = 0 min (20% B), t = 30 min (35% B), t = 40 min (35% B); (C) stationary phase: Inertsil C8 5 μm, 250 × 4 mm column packed by GL Sciences; mobile phase: eluent A = 0.01 M KH_2PO_4 and 0.017 M H_3PO_4 in H_2O, eluent B = MeCN, gradient elution: t = 0 min (5% B), t = 30 min (80% B), t = 45 min (80% B); (D) stationary phase: Lichrospher 100 RP-8 5 μm, 250 × 4 mm column packed by Merck KGaA; mobile phase: isocratic elution with premixed mobile phase prepared as follows: 1 g of *n*-octylamine is added to a solution of MeCN and H_2O (35:65; 1 L), and the resulting solution was buffered to pH 6 with H_3PO_4; (E) stationary phase: Lichrospher 100 RP-18 5 μm, 250 × 4 mm column packed by Merck KGaA; mobile phase: isocratic elution with premixed mobile phase, same as method D. Retention times are in minutes.

Preparative HPLC was performed on a Merck KGaA Prepbar 100 apparatus. Preparative HPLC methods: (A) stationary phase: Lichroprep RP-8; 45−63 μm; 250 × 50 mm column packed by Merck KGaA; mobile phase: eluent A = 0.01 M KH_2PO_4 adjusted to pH 6.2 with 80:20 NaOH/MeCN, eluent B = 0.01 M KH_2PO_4 adjusted to pH 6.2 with 50:50 NaOH/MeCN, gradient elution from 100/0 to 0/100; flow rate: 70 mL min^{-1}; detection (UV): 210 nm, 280 nm; injection: 100 mL; sample concentration: 10 mg mL^{-1}; (B) stationary phase: Lichroprep RP-8; 25−40 μm; 250 × 50 mm column; mobile phase: eluent A = H_2O, eluent B = MeCN, gradient elution from 95/5 to 0/100; flow rate: 70 mL min^{-1}; detection (UV): 210 nm, 280 nm; injection: 200 mL; sample concentration: 15 mg mL^{-1}. Nanofiltration was performed on a Unit P123C (Celfa, Schwyz, Switzerland) instrument using DRA 4020 (Daicel, Japan) membranes.

CAUTION: For the reactions in which diethyl cyanophosphonate (DEPC) was used, during the aqueous workup attention must be given to HCN evolution. All operations must be carried out under a well ventilated fume cupboard, and the aqueous washings must be treated with aqueous sodium hypochlorite before disposal.

2-Bromo-3-[(4-nitrophenyl)methoxy]propionic acid 1,1-dimethylethyl ester (**3**),[21] cholic acid *N*-hydroxysuccinimidyl ester (**9**),[23] 4-carboxy-5,8,11-tris(2-methoxy-2-oxoethyl)-1-phenyl-2-oxa-5,8,11-triazatridecan-13-oic acid methyl ester (**10**),[24] *N*-(2-aminoethyl)(3α,5*β*,7α,12α)-3,7,12-trihydroxycholan-24-amide (**11**),[25] (3*β*,5*β*,7α,12α)-3-amino-7,12-dihydroxycholan-24-oic acid methyl ester (**13**),[26] *N*,*N*-[2-(2,6-dioxo-4-morpholinyl)-ethyl]glycine (**21**),[28] *N*,*N*-bis[2-[bis[2-(1,1-dimethylethoxy)-2-oxoethyl]amino]ethyl]-L-glutamic acid 1-(1,1-dimethylethyl) ester (**22**),[29] (3*β*,5*β*,7α,12α)-3-amino-7,12-dihydroxycholan-24-oic acid phenylmethyl ester (**24**),[26] 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid tris(1,1-dimethylethyl) ester (**27**),[30] (3α,5*β*,7α,12α)-3,7,12-trihydroxycholan-24-oic acid 1,1-dimethylethyl ester (**28**),[31] and 1,4,7,10-tetraazacyclododecane-1,4,7-triaacetic acid tris(1,1-dimethylethyl) ester (DO3A ^{t}Bu ester)[32] were synthesized following the procedure reported in the literature. Other reagents were commercially available and used as purchased.

***N*-[2-[(2-Aminoethyl)amino]ethyl]-*O*-[(4-nitrophenyl)-methyl]-D,L-serine 1,1-Dimethylethyl Ester (4).** A solution of **3**[21] (14 g; 38.9 mmol) in MeCN (30 mL) was slowly added to a solution of diethylenetriamine (20 g, 194 mmol, 20.1 mL) in MeCN (20 mL) at 0−5 °C under nitrogen. The reaction mixture was warmed to 35 °C and stirred for 4 h. After evaporation of the solvent, brine (100 mL) was added to the oily residue and the mixture was extracted with Et_2O. The organic layer was separated, washed with H_2O, dried (Na_2SO_4), and evaporated to give **4** as a pale yellow oil: yield 12 g, 81%; TLC R_f 0.5 ($CHCl_3$/MeOH/25% NH_4OH, 10:2:0.5); ^{13}C NMR δ 27.7, 41.6, 47.6, 49.0, 51.8, 61.6, 71.6, 71.8, 80.9, 123.1, 127.2, 145.6, 146.9, 171.9; HPLC (Method A) retention time = 12.2, 95.8% (area %).

6,9,12-Tris[2-(1,1-dimethylethoxy)-2-oxoethyl]-2,2-dimethyl-5-[[(4-nitrophenyl)methoxy]methyl]-4-oxo-3-oxa-6,9,12-triazatetradecan-14-oic Acid 1,1-Dimethylethyl Ester (5). *tert*-Butyl bromoacetate (28.3 g, 145 mmol, 21.4 mL) was slowly added into a solution of **4** (11.0 g, 28.8 mmol) and *N*,*N*-diisopropylethylamine (37.5 g, 290 mmol, 49.3 mL) in 1,2-dichloroethane (50 mL) at 0−5 °C under nitrogen. The reaction mixture was stirred at room temperature for 16 h, cooled to 0 °C, and filtered. After evaporation of the solvent, the oily residue was taken up with EtOAc (100 mL) and washed with brine and H_2O. The organic layer was separated, dried (Na_2SO_4), and evaporated. The oily residue was purified by flash chromatography (eluent: *n*-hexane/EtOAc 7:3) to afford **5** as a pale yellow oil: yield 14.8 g, 61%; TLC R_f 0.5 (*n*-hexane/EtOAc 7:3); ^{13}C NMR δ 27.9, 28.0, 52.1, 52.7, 53.4, 54.3, 55.9, 54.6, 70.8, 71.7, 80.1, 80.3, 80.4, 80.9, 123.2, 127.3, 145.9, 147.0, 170.4, 170.7, 171.2; HPLC (Method A) retention time = 33.5, 96% (area %).

5-[[(4-Aminophenyl)methoxy]methyl]-6,9,12-tris[2-(1,1-dimethylethoxy)-2-oxoethyl]-2,2-dimethyl-4-oxo-3-oxa-6,9,12-triazatetradecan-14-oic Acid 1,1-Dimethylethyl Ester (6). To a solution of **5** (13.7 g, 16.3 mmol) in EtOH (200 mL) at room temperature was added 10% Pd/C (1.37 g), and the resulting suspension was stirred under hydrogen atmosphere for 1 h. The reaction mixture was filtered, the solvent evaporated, and the oily residue purified by flash chromatography (eluent: *n*-hexane/Et_2O/*i*-PrOH 70:25:5) to give **6** as a pale yellow oil: yield 10.7 g, 81%; TLC R_f 0.15 (*n*-hexane/Et_2O/*i*-PrOH 70:25:5); ^{13}C NMR δ 28.0, 28.1, 52.1, 52.4, 52.7, 53.2, 54.3, 55.8, 64.6, 70.4, 73.2, 80.0, 80.4, 80.6, 80.7, 114.6, 128.2, 129.1, 147.7, 170.5, 170.7, 170.9, 171.4; HPLC (Method A) retention time = 29.2, 99% (area %).

5-[[[4-[[[[(1,1-Dimethylethoxy)carbonyl]amino]acetyl]-amino]phenyl]methoxy]methyl]-6,9,12-tris[2-(1,1-dimethylethoxy)-2-oxoethyl]-2,2-dimethyl-4-oxo-3-oxa-6,9,12-triazatetradecan-14-oic Acid 1,1-Dimethylethyl Ester (7) (**CAUTION:** see General Methods)**.** Diethyl cyanophosphonate (DEPC) (2.19 g, 13.4 mmol, 2.03 mL) was dropped over 15 min into a stirred solution of **6** (4.95 g, 6.12 mmol), *N*-(*t*-butoxycarbonyl)glycine (2.14 g, 12.2 mmol), and Et_3N (1.36 g, 13.4 mmol, 1.86 mL) in DMF (50 mL) at 0 °C under nitrogen. After 16 h, the reaction mixture was concentrated to one-fourth of its initial volume, diluted with EtOAc (100 mL), and washed with brine and H_2O. The organic phase was separated, dried (Na_2SO_4), and evaporated to an oily residue that was purified twice by flash chromatography (eluent: *n*-hexane/Et_2O/*i*-PrOH 50:45:5; second column eluent: *n*-hexane/Et_2O/Et_3N 30:70:2) to yield **7** as a pale yellow oil: yield 3.02 g, 51%; TLC R_f 0.15 (*n*-hexane/Et_2O/*i*-PrOH 70:25:5); ^{13}C NMR δ 28.0, 28.2, 45.0, 52.2, 52.3, 52.7, 53.3, 53.5, 54.2, 56.0, 64.8, 70.2, 72.7, 80.0, 80.1, 80.4, 80.6, 80.7, 119.6, 129.0, 133.9, 137.0, 156.3, 167.9, 170.5, 170.7, 170.8, 171.4; MS *m*/*z* 967 $(M + H)^+$; HPLC (Method A) retention time = 31.9, 98% (area %).

1-[4-[[Aminoacetyl]amino]phenyl]-4-carboxy-5,8,11-tris(carboxymethyl)-2-oxa-5,8,11-triazatridecan-13-oic Acid (8). Trifluoroacetic acid (246 g, 2.16 mol, 167 mL) was dropped over 2 h into a solution of **7** (35.1 g, 36.3 mmol) and anisole (270 mL) in CH_2Cl_2 (270 mL) at 0 °C. The reaction mixture was stirred at room temperature for 3 days. After removal of the solvent, the residue was taken up with CH_2Cl_2 (350 mL) and the solution evaporated. This operation was repeated twice. The residue was suspended in H_2O (400 mL) and the mixture neutralized with 25% NH_4OH (19 mL) to give a clear solution that was washed with Et_2O and evaporated. The oily residue was purified by flash chromatography (eluent: EtOH/25% NH_4OH 8:2) to give **8** as a white solid: yield 13.0 g (the compound is partially salified as the ammonium salt); TLC R_f 0.19 (EtOH/25% NH_4OH 8:2); ^{13}C NMR (D_2O) δ 43.7, 58.7, 59.8, 61.8, 63.5, 70.1, 75.0, 71.0, 123.5, 131.8, 136.8, 139.0, 166.0, 180.5, 182.3; MS *m*/*z* 586 $(M + H)^+$; HPLC (Method A) retention time = 2.42, 98.9% (area %).

4-Carboxy-5,8,11-tris(carboxymethyl)-1-[4-[[[[(3α,5*β*,7α,12α)-3,7,12-trihydroxy-24-oxocholan-24-yl]amino]-acetyl]amino]phenyl]-2-oxa-5,8,11-triazatridecan-13-oic Acid (1a). To a solution of **8** (13.0 g) in DMF/H_2O (8:5, 104 mL) at room temperature was added portionwise **9**[23] (21.9 g, 43.0 mmol). The reaction mixture was stirred at room temperature for 30 h. Evaporation of the solvents gave a solid

residue which was purified by flash chromatography (eluent: CH_2Cl_2/MeOH/25% NH_4OH 6:3:0.7). The purified ligand was dissolved in 1 M HCl (270 mL) and the solution loaded onto an Amberlite XAD-16 resin column and eluted with MeOH/H_2O gradient to afford **1a** as a white solid: yield 10.1 g, 28% (from **7**); mp 154−156 °C; TLC R_f 0.21 (CH_2Cl_2/MeOH/25% NH_4OH 6:3:0.7); ^{13}C NMR (D_2O) δ 15.0, 19.6, 25.0, 25.9, 29.0, 30.4, 30.6, 31.9, 34.2, 34.8, 36.3, 38.0, 37.1, 41.1, 42.3, 44.1, 45.8, 49.8, 51.5, 52.2, 54.2, 55.6, 57.8, 58.6, 59.8, 68.6, 75.3, 69.7, 70.7, 74.0, 75.3, 123.6, 131.9, 138.4, 139.6, 172.3, 172.9, 173.3, 173.6, 180.0, 180.6; MS *m/z* 976 $(M + H)^+$; HPLC (Method A) retention time = 20.3, 98.0% (area %). Anal. ($C_{48}H_{73}N_5O_{16}$) C, H, N.

3,6,9-Tris(2-methoxy-2-oxoethyl)-11-oxo-10-[(phenylmethoxy)methyl]-14-[[(3α,5*β*,7α,12α)-3,7,12-trihydroxy-24-oxocholan-24-yl]amino]-3,6,9,12-tetraazatetradecanoic Acid Methyl Ester (12) (**CAUTION:** see General Methods). This compound was synthesized according to the procedure described for **7**, using **10**[24] (39.2 g, 68.8 mmol), **11**[25] (33.1 g, 73.4 mmol), and DEPC (12.4 g, 76.0 mmol, 11.5 mL) in DMF (400 mL) and Et_3N (7.69 g, 76.0 mmol, 10.5 mL). The solid residue was purified by flash chromatography (eluent: CH_2Cl_2/MeOH/25% NH_4OH 9:1:0.1) to give **12** as a pale yellow solid: yield 25.2 g, 36%; TLC R_f 0.34 (CH_2Cl_2/MeOH/25% NH_4OH 9:1:0.1); ^{13}C NMR δ 12.3, 17.3, 22.3, 23.1, 26.1, 27.4, 28.1, 30.3, 31.5, 33.2, 34.6, 35.4, 38.8, 39.4, 39.9, 41.4, 46.2, 46.6, 50.3, 51.2, 51.3, 51.5, 51.8, 52.2, 52.4, 52.7, 54.3, 54.7, 64.6, 68.1, 68.2, 71.6, 72.8, 73.2, 127.4, 127.7, 128.2, 137.8, 171.4, 171.7, 172.5, 173.3, 174.1; MS *m/z* 570 $(M + H)^+$; HPLC (Method A) retention time = 20.4, 91% (area %).

3,6,9-Tris(carboxymethyl)-11-oxo-10-[(phenylmethoxy)methyl]-14-[[(3α,5*β*,7α,12α)-3,7,12-trihydroxy-24-oxocholan-24-yl]amino]-3,6,9,12-tetraazatetradecanoic Acid (1b). To a solution of **12** (11.5 g, 11.5 mmol) in MeOH/H_2O (1:1; 300 mL) at room temperature was added dropwise 2 M NaOH (5.0 mL) until pH 12 was reached. The reaction mixture was stirred at room temperature for 48 h, maintaining pH 12 by the addition of 1 M NaOH (35 mL) through a pH-stat apparatus. The solution was adjusted to pH 7 with 2 M HCl and evaporated. The residue was dissolved with H_2O/MeOH (7:3, 500 mL) and acidified with 6 M HCl (15 mL), and the solution was loaded onto an Amberlite XAD-16 resin column and eluted with MeOH/H_2O gradient to afford a solid which was further purified by reversed-phase preparative HPLC (Method A) to give **1b** as a white solid: yield 2.13 g, 18%; ^{13}C NMR (D_2O) δ 15.0, 19.5, 25.1, 25.8, 29.9, 30.1, 30.5, 32.0, 34.2, 35.0; 37.1, 38.0, 41.2, 41.5, 42.2, 43.9, 49.5, 49.7, 52.6, 54.2, 55.2, 57.3, 58.1, 60.5, 69.6, 70.6, 74.0, 75.3, 75.7, 130.6, 131.2, 140.4, 174.1, 175.4, 176.9, 179.5, 182.2; MS *m/z* 969 $(M + Na)^+$; HPLC (Method A) 96% (area %).

(3*β*,5*β*,7α,12α)-7,12-Dihydroxy-3-[[[[(phenylmethoxy)carbonyl]amino]acetyl]amino]cholan-24-oic Acid Methyl Ester (14). Isobutyl chloroformate (8.00 g, 58.5 mmol, 7.57 mL) was dropped over 10 min into a solution of Z-glycine (12.2 g, 58.5 mmol) and *N*-methylmorpholine (6.00 g, 59.3 mmol) in THF (400 mL) at −4 °C under nitrogen. After 15 min, a solution of **13**[26] (21.8 g, 51.7 mmol) in THF (100 mL) was dropped over 1.5 h into the reaction mixture that was maintained at −4 °C for additional 30 min and then allowed to rise to room temperature and stirred overnight. After evaporation of the solvent, the residue was taken up with Et_2O (500 mL) and washed with H_2O. The organic layer was separated, dried (Na_2SO_4), and evaporated, and the crude was purified by flash chromatography (eluent: CH_2Cl_2/*i*-PrOH 9:1) to afford **14** as a white solid: yield 27.9 g, 88%; mp 87−89 °C; TLC R_f 0.20 (CH_2Cl_2/*i*-PrOH 9:1); ^{13}C NMR δ 12.4, 17.2, 22.9, 23.2, 24.4, 25.7, 27.4, 28.4, 30.8, 31.0, 33.3, 34.4, 35.1, 35.2, 37.1, 39.2, 41.6, 44.8, 45.6, 46.4, 47.0, 51.4, 66.7, 68.1, 73.0, 127.8, 128.0, 129.4, 136.2, 156.8, 168.5, 174.8; MS *m/z* 613 $(M + H)^+$; HPLC (Method A) retention time = 28.5, 95.5% (area %). Anal. ($C_{35}H_{52}N_2O_7$) C, H, N.

(3*β*,5*β*,7α,12α)-3-[(Aminoacetyl)amino]-7,12-dihydroxycholan-24-oic Acid Methyl Ester (15c). To a solution of **14** (1.0 g, 1.6 mmol) and ammonium formate (0.47 g; 7.4 mmol) in MeOH/H_2O (10:1, 11 mL) was added 10% Pd/C (0.2 g). The resulting suspension was refluxed for 10 min. The reaction mixture was allowed to cool to room temperature, filtered, and evaporated. The residue was taken up with Et_2O to give the precipitation of a solid which was filtered to afford **15c** as a white solid: yield 0.67 g, 87%; mp 187−189 °C; TLC R_f 0.46 (CH_2Cl_2/MeOH/Et_3N 5:5:0.1); ^{13}C NMR δ 12.3, 17.1, 22.9, 23.2, 24.7, 25.7, 27.4, 28.4, 30.8, 31.0, 31.1, 33.6, 34.4, 35.1, 35.2, 37.3, 39.2, 41.5, 44.6, 44.7, 46.3, 47.0, 51.3, 68.0, 72.9, 76.4, 77.1, 77.7, 171.8, 174.7; MS *m/z* 479 $(M + H)^+$; HPLC (Method A) retention time = 17, 96% (area %). Anal. ($C_{27}H_{46}N_2O_5$) H, N; C: calcd, 67.75; found, 67.14.

(3*β*,5*β*,7α,12α)-3-[[13-Carboxy-6,9,12-tris(carboxymethyl)-1,4-dioxo-3,6,9,12-tetraazatridecyl]amino]-7,12-dihydroxycholan-24-oic Acid (1c). A solution of H_2O (6.1 mL, 0.34 mol) and DMF (250 mL) was added dropwise over 75 min into a suspension of **21**[28] (92.5 g, 0.26 mol) in DMF (1.2 L) at 80 °C. After 2 h, a solution of **15c** (25 g; 52.2 mmol) in DMF (400 mL) was added dropwise over 30 min. The reaction mixture was cooled to 20 °C, and 2 M NaOH (650 mL) was added dropwise, to afford the precipitation of a sticky solid. After 16 h at room temperature, the pH of the mixture was adjusted to 7 with 12 M HCl (35 mL), and the solution was decanted and evaporated to give a crude product which was taken up with H_2O/MeCN (85:15, 1.4 L) and 6 M HCl (100 mL). The resulting suspension was filtered through paper and the solution was loaded onto an Amberlite XAD-16 resin column and eluted with MeCN/H_2O gradient to give **1c** as a white solid: yield 26.0 g, 59%; mp 280 °C (dec); TLC R_f 0.13 (CH_2Cl_2/MeOH/25% NH_4OH 6:3:1); ^{13}C NMR (D_2O + KOD) δ 15.0, 19.6, 25.3, 25.7, 26.6, 28.7, 30.0, 30.7, 33.2, 35.0, 35.4, 36.2, 37.1, 37.4, 38.3, 39.3, 41.7, 44.3, 45.1, 48.6, 49.1, 53.3, 53.6, 54.4, 54.7, 58.0, 60.7, 60.9, 61.3, 71.1, 75.8, 173.0, 177.1, 177.5, 179.8, 181.5, 186.9; MS *m/z* 840 $(M + H)^+$; HPLC (Method A) retention time = 17.6, 98.9% (area %). Anal. ($C_{40}H_{65}N_5O_{14}$) C, H, N.

(3*β*,5*β*,7α,12α)-7,12-Dihydroxy-3-[[[[(phenylmethoxy)carbonyl]amino]acetyl] amino]cholan-24-oic Acid (16). To a stirred solution of **14** (15.3 g, 25.0 mmol) in MeOH/H_2O (3:1, 240 mL) at room temperature was added 1 M NaOH (35 mL) dropwise over 1 h. After 36 h, the pH of the reaction mixture was lowered to neutrality with 2 M HCl (3.5 mL) to afford the precipitation of **16** as a white solid that was filtered and dried: yield 13.0 g, 87%; mp 228−233 °C; TLC R_f 0.41 (CH_2Cl_2/*i*-PrOH/AcOH 85:15:2); ^{13}C NMR (DMSO-d_6) δ 12.3, 17.0, 22.5, 24.5, 25.6, 27.3, 28.7, 30.5, 30.7, 33.4, 34.3, 34.7, 35.1, 36.5, 39.5, 41.4, 43.4, 44.8, 45.8, 46.1, 65.4, 66.3, 71.1, 127.8, 129.0, 129.6, 136.2; MS *m/z* 599 $(M + H)^+$; HPLC (Method A) retention time = 24.4, 96% (area %). Anal. ($C_{34}H_{50}N_2O_7$) C, H, N.

***N*-[(3*β*,5*β*,7α,12α)-7,12-Dihydroxy-3-[[[[(phenylmethoxy)carbonyl]amino] acetyl]amino]-24-oxocholan-24-yl]glycine Methyl Ester (17).** Isobutyl chloroformate (2.73 g, 20 mmol, 2.59 mL) was dropped over 20 min into a solution of **16** (10 g, 16.7 mmol) and Et_3N (2.02 g, 20 mmol, 2.78 mL) in DMF (150 mL) at − 5 °C under nitrogen. After 15 min, a suspension of glycine methyl ester hydrochloride (2.31 g, 18.4 mmol) and Et_3N (1.86 g, 18.4 mmol, 2.55 mL) in DMF (15 mL) was added over 1.5 h into the reaction mixture that was maintained at −4 °C for 30 min and then was allowed to rise to room temperature and stirred overnight. The suspension was filtered, and the solution was evaporated. The residue was taken up with CH_2Cl_2 (100 mL) and washed with H_2O. The organic layer was separated, dried (Na_2SO_4), and evaporated to give a crude product which was purified by flash chromatography (eluent: EtOAc/MeOH 9:1) to afford **17** as a white solid: yield 6.56 g, 59%; mp 123−128 °C; TLC R_f 0.33 (EtOAc/MeOH 9:1); ^{13}C NMR δ 12.5, 14.8, 17.6, 22.5, 22.7, 23.3, 24.4, 25.4, 27.5, 28.2, 30.9, 31.5, 32.7, 33.2, 34.5, 35.1, 37.0, 39.2, 41.0, 41.6, 44.7, 45.6, 48.3, 48.6, 52.0, 53.3, 60.2, 63.6, 66.8, 68.1, 72.9, 76.4, 77.0, 77.2, 77.7, 127.7, 128.0, 129.6, 136.1, 156.9, 168.8, 170.6, 171.8, 174.2; MS *m/z* 670 $(M + H)^+$; HPLC (Method B) retention time = 28, 96% (area %). Anal. ($C_{37}H_{55}N_3O_8$) C, H, N.

***N*-[(3*β*,5*β*,7α,12α)-3-[(Aminoacetyl)amino]-7,12-dihydroxy-24-oxocholan-24-yl]glycine Methyl Ester (15d).** To a solution of **17** (21.8 g, 32.5 mmol) in MeOH (100 mL) at room temperature was added 5% Pd/C (2.2 g), and the resulting suspension was stirred under hydrogen atmosphere for 1.5 h. The reaction mixture was filtered, the solvent evaporated, and the crude product purified by flash chromatography (eluent: EtOAc/MeOH 1:1) to afford **15d** as a white solid: yield 15.8 g, 91%; mp 168−172 °C; TLC R_f 0.27 (EtOAc/MeOH 1:1); ^{13}C NMR (CD_3OD) δ 13.4, 18.1, 23.8, 24.5, 25.9, 27.5, 28.9, 30.0, 32.2, 33.2, 34.0, 34.8, 35.7, 36.5, 37.0, 38.7, 41.1, 42.1, 43.1, 45.4, 47.0, 47.7, 48.1, 48.2, 48.5, 48.9, 49.4, 49.9, 50.2, 50.8, 52.9, 69.2, 74.2, 172.1, 174.5, 177.3; MS *m*/*z* 536 $(M + H)^+$; HPLC (Method B) retention time = 3.9, 96% (area %). Anal. ($C_{29}H_{49}N_3O_6$) C, H, N.

(3*β*,5*β*,7α,12α)-3-[[*N*-[*N*-[*N*-[2-[2-[Bis(carboxymethyl)amino]ethyl](carboxymethyl)amino]ethyl]-*N*-(carboxymethyl)glycyl]glycyl]amino]-7,12-dihydroxy-*N*-(carboxymethyl)cholan-24-amide (1d). A solution of H_2O (5.8 mL, 322 mmol) and DMF (60 mL) was added dropwise over 75 min into a suspension of **21**[28] (68.6 g, 0.19 mol) in DMF (1 L) at 80 °C. After 2 h, a solution of **15d** (26.8 g, 50 mmol) in DMF (100 mL) was added dropwise over 30 min. The reaction mixture was cooled to 20 °C, and 2 M NaOH (480 mL) was added dropwise, to afford precipitation of a sticky solid. After 16 h, at room temperature the pH of the mixture was adjusted to 7 with 12 M HCl (31 mL), and the solution was decanted and evaporated to give a crude product which was taken up with H_2O/MeCN (9:1, 100 mL) and 2 M HCl (10 mL) The resulting solution was loaded onto an Amberlite XAD-2 resin column and eluted with MeCN/H_2O gradient to give **1d** as a white solid: yield 21.9 g, 49%; mp 210 °C (dec); ^{13}C NMR (D_2O + KOD) δ 15.8, 19.5, 25.3, 25.8, 26.8, 29.7, 30.1, 30.8, 33.3, 34.3, 34.9, 35.3, 36.3, 37.4, 39.0, 39.3, 42.0, 44.3, 45.0, 46.1. 48.7, 48.8, 53.9, 54.2, 54.4, 54.5, 59.7, 60.9, 61.1, 61.5, 70.9, 75.6, 175.2, 177.5, 179.3, 179.5, 180.8, 181.6; MS *m*/*z* 897 $(M + H)^+$; HPLC (Method A) retention time = 15.1, 100% (area %). Anal. ($C_{42}H_{68}N_6O_{15}$) C, H, N.

(3*β*,5*β*,7α,12α)-3-[[[[(1,1-Dimethylethoxy)carbonyl]amino]acetyl]amino]-7,12-dihydroxycholan-24-oic Acid Methyl Ester (18). Isobutyl chloroformate (11.5 g, 84.0 mmol, 10.9 mL) was dropped over 15 min into a solution of *N*-(*tert*-butoxycarbonyl)glycine (14.7 g, 84.0 mmol) and Et_3N (8.5 g, 84.0 mmol, 11.6 mL) in THF (400 mL) at −5 °C under nitrogen. After 15 min, a solution of **13**[26] (29.5 g, 70.0 mmol) in THF (100 mL) was added dropwise over 1 h into the reaction mixture that was maintained at −4 °C for 30 min and then was allowed to rise to room temperature and stirred overnight. The suspension was filtered, and the solution was evaporated. The residue was taken up with Et_2O and washed with saturated aqueous $NaHCO_3$ and with H_2O. The organic layer was separated, dried (Na_2SO_4), and evaporated to give a crude which was purified by flash chromatography (eluent: EtOAc/MeOH 1:1) to afford **18** as a white solid: yield 25.3 g, 62%; mp 110−114 °C; TLC R_f 0.19 (EtOAc); ^{13}C NMR (CD_3OD) δ 13.1, 17.9, 23.6, 24.3, 25.8, 27.3, 29.0, 29.9, 32.1, 32.2, 32.4, 34.8, 35.7, 36.5, 36.9, 38.7, 41.1, 43.2, 45.0, 47.2, 47.3, 47.7, 48.0, 48.2, 48.4, 48.6, 49.2, 49.7, 50.1, 50.5, 52.3, 69.2, 74.5, 80.9, 159.6, 171.7, 171.8, 176.6; MS *m*/*z* 579 $(M + H)^+$; HPLC (Method A) retention time = 27.7, 97% (area %). Anal. ($C_{32}H_{54}N_2O_7$) H, N; C: calcd, 66.40; found, 65.47.

(3*β*,5*β*,7α,12α)-3-[[[[(1,1-Dimethylethoxy)carbonyl]amino]acetyl]amino]-7,12-dihydroxycholan-24-oic Acid (19). To a solution of **18** (24.5 g, 42.3 mmol) in MeOH/H_2O (2:1, 160 mL) at room temperature was added 1 M NaOH (49.7 mL) dropwise over 2 h. After 48 h, the reaction mixture was filtered and evaporated. The glassy residue was taken up with EtOAc (160 mL), and the solution was washed with 1 M HCl. After separation, the aqueous phase was saturated with NaCl and extracted with EtOAc. The organic layers were combined, dried (Na_2SO_4), and evaporated to give **19** as a white solid: yield 22.2 g, 93%; mp 150−155 °C; TLC R_f 0.47 (EtOAc/*i*-PrOH/AcOH 90:15:1); ^{13}C NMR (CD_3OD) δ 13.1, 17.3, 23.8, 24.4, 25.8, 27.5, 28.9, 29.9, 32.2, 32.5, 34.8, 35.5, 36.5, 37.0, 38.7, 41.1, 43.2, 45.0, 47.2, 47.3, 47.7, 48.0, 48.2, 48.4, 48.8, 49.2, 49.7, 50.1, 50.5, 69.3, 74.2, 81.0, 159.5, 171.7, 178.3; MS *m*/*z* 565 $(M + H)^+$; HPLC (Method A) retention time = 24.1, 96% (area %). Anal. ($C_{31}H_{52}N_2O_7$) C, N: H: calcd, 9.28; found, 9.98.

2-[[(3*β*,5*β*,7α,12α)-3-[(Aminoacetyl)amino]-7,12-dihydroxy-24-oxocholan-24-yl]amino]ethanesulfonic Acid (15e). Taurine (5.38 g, 43.1 mmol) and Et_3N (5.16 g, 51.0 mmol, 7.07 mL) were added to a solution of **19** (22.1 g, 39.1 mmol) and 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) (12.6 g, 51.0 mmol) in DMF (100 mL) under nitrogen. The resulting suspension was heated to 90 °C for 70 min, obtaining a clear solution which was then cooled to 25 °C. After 30 min, the reaction mixture was slowly poured into cold Et_2O (0 °C, 900 mL) to give the precipitation of a resinous product. The suspension was kept at 4 °C overnight. The mixture was decanted, and the resinous residue was dried under vacuum. The resulting white solid was added to a stirred solution of HCl in MeOH (0.85 M, 190 mL) at room temperature. The initially milky reaction mixture slowly became thicker and after 24 h was filtered. The solid was washed thoroughly with Et_2O/MeOH (1:1) and dried to give **15e** as a white solid: yield 14.3 g, 64%; mp 200 °C (dec); TLC R_f 0.67 (MeOH/AcOH 95:5); ^{13}C NMR (D_2O + KOD) δ 15.5, 19.7, 25.3, 25.9, 26.8, 28.7, 30.2, 31.0, 33.5, 34.3, 35.1, 35.5, 36.5, 37.5, 38.0, 38.4, 39.3, 42.2, 44.2, 46.7, 48.6, 48.8, 48.9, 52.6, 71.0, 75.7; MS *m*/*z* 572 $(M + H)^+$; HPLC (Method A) retention time = 14.3, 94% (area %). Anal. ($C_{28}H_{49}N_3O_7S$) H, N, S; C: calcd, 52.17; found, 51.70.

(3*β*,5*β*,7α,12α)-3-[[*N*-[*N*-[*N*-[2-[2-[bis(carboxymethyl)amino]ethyl] (carboxymethyl)amino]ethyl]-*N*-(carboxymethyl)glycyl]glycyl]amino]-7,12-dihydroxy-*N*-(2-sulfoethyl)cholan-24-amide (1e). A solution of H_2O (3.6 mL, 227 mmol) and DMF (150 mL) was added dropwise over 30 min into a suspension of **21**[28] (54.3 g, 152 mmol) in DMF (700 mL) at 80 °C. After 2 h, a solution of **15e** (21.7 g, 37.9 mmol) and Et_3N (3.85 g, 38 mmol, 5.27 mL) in DMF (300 mL) was added dropwise over 30 min. The reaction mixture was cooled to 20 °C, and 2 M NaOH (380 mL) was added dropwise, to afford the precipitation of a sticky solid. After 16 h, at room temperature the pH of the mixture was adjusted to 7 with 12 M HCl (25 mL), and the solution was decanted and evaporated to give a crude product which was taken up with H_2O (300 mL) and 12 M HCl (35 mL). The resulting solution was loaded onto an Amberlite XAD-2 resin column and eluted with MeCN/H_2O gradient to give **1e** as a white solid: yield 20.4 g, 57%; mp 174−176 °C; ^{13}C NMR (D_2O + KOD) δ 15.1, 19.6, 25.4, 25.9, 26.7, 28.8, 32.2, 32.9, 33.4, 34.4, 35.1, 35.4, 36.6, 37.5, 38.0, 39.0, 39.3, 42.0, 44.3, 45.1, 49.9, 52.6, 53.2, 53.5, 54.4, 54.8, 58.6, 60.7, 61.0, 61.3, 70.9, 75.6, 173.0, 176.6, 177.3, 179.5, 179.6, 191.5; MS *m*/*z* 947 $(M + H)^+$; HPLC (Method A) retention time = 16.6, 94% (area %); Anal. ($C_{42}H_{70}N_6O_{16}S$) C, H, N, S.

(3*β*,5*β*,7α,12α)-7,12-Dihydroxy-3-[[1-oxo-6-[[(phenylmethoxy)carbonyl]amino]hexyl]amino]cholan-24-oic Acid Methyl Ester (20). Isobutyl chloroformate (1.78 g, 13 mmol, 1.69 mL) was dropped over 5 min into a solution of 6-[[(phenylmethoxy)carbonyl]amino]hexanoic acid (3.45 g, 13.0 mmol) and Et_3N (1.31 g, 13.0 mmol, 1.8 mL) in THF (70 mL) at −5 °C under nitrogen. After 15 min, a solution of **13**[26] (5 g, 11.9 mmol) in THF (30 mL) was added dropwise over 1 h into the reaction mixture that was maintained at −4 °C for 30 min and then was allowed to rise to room temperature and stirred overnight. The suspension was filtered, and the solution was evaporated. The residue was taken up with CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$ and with H_2O. The organic layer was separated, dried (Na_2SO_4), and evaporated. Crystallization of the crude product from EtOAc gave **20** as a white solid: yield 5.60 g, 70%; mp 145−150 °C; TLC R_f 0.11 (EtOAc), R_f 0.41 (EtOAc/*i*-PrOH 95:5); ^{13}C NMR δ 13.3, 17.9, 23.8, 24.5, 25.9, 27.1, 27.6, 28.9, 30.0, 30.9, 32.1, 32.2, 32.4, 34.9, 35.7, 36.5, 36.9, 37.1, 38.5, 41.1, 41.9, 43.1, 47.2, 47.7, 48.0, 48.2, 48.4, 48.9, 49.3, 49.7, 50.1, 50.6, 52.3, 67.5, 69.3, 74.2, 128.9, 129.2, 129.7, 139.7, 159.0, 175.7, 175.9, 176.6; MS *m*/*z* 669 $(M + H)^+$; HPLC (Method A) retention time = 28.4, 98.5% (area %). Anal. ($C_{39}H_{60}N_2O_7$) C, H, N.

(3β,5β,7α,12α)-3-[(6-Amino-1-oxohexyl)amino]-7,12-dihydroxycholan-24-oic Acid Methyl Ester (15f). To a solution of **20** (4.0 g; 5.99 mmol) in EtOH (50 mL) at room temperature 5% Pd/C (0.8 g) was added, and the resulting suspension was stirred under hydrogen atmosphere for 6 h. The reaction mixture was filtered, and the solvent was evaporated to afford **15f** as a white solid: yield 2.70 g, 84%; mp 70−75 °C; TLC R_f 0.33 (MeOH/Et_3N 95:5), ^{13}C NMR δ 12.4, 17.2, 23.0, 23.2, 24.5, 25.5, 25.8, 26.3, 27.4, 28.4, 30.8, 31.0, 31.2, 32.7, 33.4, 34.4, 35.1, 35.2, 36.7, 37.4, 39.3, 41.6, 45.2, 43.4, 47.1, 51.3, 67.9, 72.8, 76.4, 77.0, 77.2, 77.6, 172.4, 174.7; MS *m*/*z* 535 $(M + H)^+$; HPLC (Method A) retention time = 18, 95% (area %). Anal. ($C_{31}H_{54}N_2O_5$) C,N; H: calcd, 10.18; found, 9.56.

18-[[(3β,5β,7α,12α)-23-Carboxy-7,12-dihydroxy-24-norcholan-3-yl]amino]-3,6,9-tris(carboxymethyl)-11,18-dioxo-3,6,9,12-tetraazaoctadecanoic Acid (1f). A solution of H_2O (2.34 mL, 146 mmol) and DMF (190 mL) was added over 30 min into a suspension of **21**[28] (34.7 g, 97.2 mmol) in DMF (500 mL) at 80 °C. After 2 h, a solution of **15f** (13 g, 24.3 mmol) in DMF (195 mL) was added over 30 min. The reaction mixture was cooled to 20 °C, and 2 M NaOH (245 mL) was dropped, to afford the precipitation of a sticky solid. After 24 h at room temperature, the pH of the mixture was adjusted to 7 with 12 M HCl (18 mL), and the solution was decanted and evaporated to give a crude product which was taken up with H_2O/MeCN (9:1, 150 mL) and 12 M HCl (42 mL). The resulting solution was loaded onto an Amberlite XAD-2 resin column and eluted with MeCN/H_2O gradient to give **1f** as a white solid: yield 15 g, 69%; mp 172−174 °C; ^{13}C NMR δ 15.0, 19.7, 25.5, 26.0, 26.5, 29.1, 29.5, 30.2, 31.1, 33.5, 35.0, 35.3, 36.4, 37.1, 37.5, 38.5, 39.4, 41.6, 42.2, 44.2, 48.9, 53.4, 53.5, 54.2, 54.4, 58.6, 60.5, 61.2, 61.6, 70.5, 75.5, 176.3, 177.5, 178.7, 180.0, 181.5, 186.6; MS *m*/*z* 896 $(M + H)^+$; HPLC (Method A) retention time = 18, 93% (area %). Anal. ($C_{45}H_{75}N_5O_{14}$) C, H, N.

(3β(S),5β,7α,12α)-3-[4-Carboxy-4-[bis[2-[bis(carboxymethyl)amino]ethyl]amino]-1-oxobutyl]amino]-7,12-dihydroxycholan-24-oic Acid (1 g). This product was synthesized according to the procedure described in ref 29.

(3β(S),5β,7α,12α)-3-[[4-[Bis[2-[bis[2-(1,1-dimethylethoxy)-2-oxoethyl]amino]ethyl]amino]-5-(1,1-dimethylethoxy)-1,5-dioxopentyl]amino]-7,12-dihydroxycholan-24-oic Acid phenylmethyl Ester (25). (**CAUTION:** see General Methods). This compound was synthesized according to the procedure described for **7** using **22**[29] (37 g, 49.6 mmol), **24**[26] (27.4 g, 55 mmol), and DEPC (8.97 g, 55 mmol, 8.35 mL) in DMF (750 mL) and Et_3N (5.3 g, 52.5 mmol, 7.3 mL). The solid residue was purified by flash chromatography (eluent: *n*-hexane/EtOAc 8:2) to give **25** as a white solid: yield 36 g, 59%; mp 54−56 °C; TLC R_f 0.3 (*n*-hexane/EtOAc 8:2); ^{13}C NMR δ 12.3, 17.1, 22.8, 23.2, 24.6, 25.4, 25.7, 27.4, 28.0, 29.1, 30.9, 31.2, 32.8, 33.5, 34.6, 35.1, 37.0, 39.2, 41.5, 45.1, 46.4, 47.0, 49.4, 53.1, 55.7, 63.2, 65.9, 68.2, 72.9, 76.5, 77.1, 77.8, 80.6, 80.7, 170.5, 172.2, 172.3, 190.1; MS *m*/*z* 1226 $(M + H)^+$; Anal. ($C_{68}H_{112}N_4O_{15}$) C, H, N.

(3β(S),5β,7α,12α)-3-[[4-[Bis[2-[bis[2-(1,1-dimethylethoxy)-2-oxoethyl]amino]ethyl]amino]-5-(1,1-dimethylethoxy)-1,5-dioxopentyl]amino]-7,12-dihydroxycholan-24-oic Acid triethylammonium salt (26). To a solution of **25** (36 g, 29.4 mmol) in EtOH (1.5 L) at room temperature was added 5% Pd/C (3.6 g), and the resulting suspension was stirred under hydrogen atmosphere for 3 h. The reaction mixture was filtered, the solvent evaporated, and the oily residue purified by flash chromatography (eluent CH_2Cl_2/MeOH/Et_3N, 95:5:1) to give **26** as a white solid: yield 22 g, 61%; mp 57−59 °C; TLC R_f 0.33 (CH_2Cl_2/MeOH/Et_3N 95:5:1); ^{13}C NMR δ 9.0, 12.2, 17.2, 22.9, 23.2, 24.5, 25.4, 25.8, 27.5, 27.9, 28.0, 28.3, 31.0, 31.6, 32.8, 33.4, 34.5, 35.0, 35.6, 37.1, 39.2, 41.6, 44.8, 45.1, 46.3, 46.5, 48.7, 49.4, 49.9, 53.1, 55.7, 63.2, 67.6, 72.6, 76.5, 77.1, 77.8, 90.6, 90.7, 170.5, 172.2, 172.3, 190.1; MS *m*/*z* 1135 $(M\text{-}Et_3NH^+ + H^+)^+$; HPLC (Method C) retention time = 28.8, 95% (area %). Anal. ($C_{67}H_{121}N_5O_{15}S$) C, H, N.

N^2,N^2-Bis[2-[bis(carboxymethyl)amino]ethyl]-*N*-[(3β,5β,7α,12α)-7,12-dihydroxy-24-oxo-24-[(2-sulfoethyl)amino]cholan-3-yl]-L-glutamine (1h). (**CAUTION**: see General Methods). Triethylamine (1.36 g; 13.4 mmol, 1.86 mL) was added dropwise to a solution of **26** (12.5 g, 10.1 mmol), taurine (1.5 g, 12 mmol), and DEPC (2.1 g, 12.8 mmol, 1.82 mL) in DMF (50 mL) at 0 °C under nitrogen. After 3 h, the solvent was evaporated, the residue was dissolved in dioxane (250 mL), and 0.5 M aq H_2SO_4 (250 mL, 125 mmol) was added dropwise. The resulting mixture was heated at 90 °C for 2 h. After the solution was cooled to room temperature, the pH was adjusted to neutrality with 2 N NaOH (30 mL) and the solvent evaporated. The crude was purified by flash chromatography (eluent CH_2Cl_2/MeOH/25% NH_4OH, 5:4:1) and desalted by elution through an Amberlite XAD-16.00 resin column with a MeCN/H_2O gradient and further purified by reverse-phase preparative HPLC (Method B) to give **1h**: yield 1.5 g, 14%; mp >200 °C; TLC R_f 0.3 (CH_2Cl_2/MeOH/25% NH_4OH 5:4:1); ^{13}C NMR δ 15.0, 19.6, 25.2, 25.8, 26.7, 28.5, 28.8, 30.2, 30.9, 33.3, 34.3, 35.1, 35.9, 36.3, 37.4, 37.5, 37.9, 39.2, 41.9, 44.3, 48.6, 50.4, 52.6, 55.1, 60.6, 68.7, 70.9, 75.8, 177.6, 179.6, 180.4; MS *m*/*z* 962 $(M + H)^+$; HPLC (Method D) retention time = 5.61, 95% (area %). Anal. ($C_{43}H_{71}N_5O_{17}S$) C, H, N, S.

10-[2-[[2-[[(3β,5β,7α,12α)-7,12-Dihydroxy-24-oxo-24-[(2-sulfoethyl)amino]cholan-3-yl]amino]-2-oxoethyl]-amino]-2-oxoethyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic Acid (1i). *N*,*N*-Diisopropylethylamine (7.5 g, 58 mmol, 9.9 mL) was added dropwise to a solution of **27**[30] (13.3 g, 20.3 mmol), **15e** (12.1 g, 21.2 mmol), and benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (9.4 g, 21.2 mmol) in DMF (250 mL) at room temperature under nitrogen. After 3 h, the reaction mixture was evaporated and the oily residue was treated twice with H_2O and filtered. The resulting solid was dissolved in H_2O/AcOH/*n*-PrOH (2:1:1, 200 mL) and refluxed for 8 h. The solvent was evaporated, and the resulting viscous oil was dissolved in 0.1 M HCl (50 mL), loaded onto Amberlite XAD-16.00, and eluted with a EtOH/H_2O gradient to afford **1i** as a white solid: yield 4.7 g, 24%; mp 200 °C (dec); TLC R_f 0.4 (CH_2Cl_2/MeOH/25% NH_4OH 6:3:1); ^{13}C NMR (D_2O) δ 15.1, 19.8, 25.7, 26.6, 28.5, 30.2, 30.9, 33.6, 34.2, 35.0, 35.4, 35.5, 37.5, 37.8, 38.0, 39.9, 42.1, 44.2, 45.5, 48.5, 48.6, 48.9, 52.6, 53.2, 59.7, 62.3, 70.9, 75.4, 175.4, 176.4, 179.4, 182.3, 182.4; MS *m*/*z* 958 $(M + H)^+$; HPLC (Method A) retention time = 14.1, 98% (area %). Anal. ($C_{44}H_{75}N_7O_{14}S$) H, N; C: calcd, 55.15; found, 55.60.

(3α,5β,7α,12α)-7,12-Dihydroxy-3-(oxyranylmethoxy)-cholan-24-oic Acid 1,1-Dimethylethyl Ester (29). A solution of **28**[31] (45.5 g, 97.8 mmol) in CH_2Cl_2 (300 mL) was added dropwise over 30 min to a vigorously stirred mixture of 1-chloro-2,3-epoxypropane (154 g, 1.66 mol, 130 mL) (**CAUTION**: cancer suspected agent), 50% aq NaOH (220 mL) and Bu_4NHSO_4 (6.79 g, 20 mmol), keeping the temperature at 20 °C. After 24 h, the reaction mixture was diluted with H_2O (100 mL), and the organic phase was separated, dried (Na_2SO_4), and evaporated. The oily residue was purified by flash chromatography (eluent EtOAc/*n*-hexane, 1:1) to afford **29** as a white solid: yield 23.6 g, 46%; mp 65−67 °C; TLC R_f 0.35 (EtOAc/*n*-hexane 6:4); ^{13}C NMR δ 12.2, 17.2, 22.4, 23.1, 26.3, 26.8, 27.0, 27.3, 28.0, 28.1, 30.8, 32.4, 34.7, 34.9, 35.0, 36.0, 36.2, 39.4, 41.4, 41.6, 44.5, 46.4, 47.0, 51.1, 68.1, 68.3, 68.6, 72.6, 76.7, 77.0, 77.7, 79.7, 79.8, 173.6,; MS *m*/*z* 521$(M + H)^+$; Anal. ($C_{31}H_{52}O_6$) H; C: calcd, 71.50; found, 70.66.

10-[3-[[(3α,5β,7α,12α)-24-(1,1-Dimethylethoxy)-7,12-dihydroxy-24-oxocholan-3-yl]oxy]-2-hydroxypropyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic Acid Tris(1,1-dimethylethyl) Ester Adduct with NaCl (30). A solution of **29** (10.4 g, 20 mmol) in absol EtOH (60 mL) was added dropwise over 20 min to a refluxing solution of DO3A *t*Bu ester[32] (10.3 g, 20 mmol) in absol EtOH (100 mL). After 3 h of reflux, the solvent was evaporated, and the residue was dissolved in EtOAc (200 mL) and washed with brine. The organic phase was separated, dried (Na_2SO_4), and evaporated. The crude was purified by flash chromatography (eluent CH_2Cl_2/MeOH 15:1) to give **30** as a white solid: yield 13 g, 60%; mp 117−119 °C; TLC R_f 0.36 (CH_2Cl_2/MeOH 15:1); ^{13}C NMR δ 12.3, 17.1, 22.4, 23.0, 26.4, 26.7, 27.1, 27.3, 27.7, 27.9,

28.0, 28.1, 30.8, 32.4, 34.5, 34.8, 34.9, 35.8, 35.9, 39.3, 41.3, 41.6, 46.2, 46.9, 48.3, 49.2, 49.8, 50.4, 52.0, 52.3, 55.4, 55.8, 56.1, 66.6, 67.8, 70.1, 70.3, 72.7, 76.5, 77.1, 77.8, 79.6, 79.9, 81.8, 81.9, 82.1, 171.7, 172.2, 172.6, 173.5; MS *m/z* 1036 $(M + H)^+$; Anal. ($C_{57}H_{102}ClN_4NaO_{12}$) H, Cl, N, Na; C: calcd, 62.59; found, 60.28.

10-[3-[[(3α,5*β*,7α,12α)-23-Carboxy-7,12-dihydroxy-24-norcholan-3-yl]oxy]-2-hydroxypropyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic Acid (1j). A suspension of **30** (35.1 g, 32.1 mmol) in H_2O (130 mL) and 0.5 M H_2SO_4 (130 mL) was heated to 90 °C. After 2.5 h the homogeneous reaction mixture was evaporated. The solid residue was purified by flash chromatography (eluent CH_2Cl_2/MeOH/25% NH_4OH, 6:3:1). The product was dissolved in H_2O (160 mL) and 6 N HCl (40 mL), the solution was loaded onto an Amberlite XAD-16 resin column and eluted with a MeCN/H_2O gradient. Ligand **1j** was recovered as a white solid: yield 16 g, 61%, mp 220 °C (dec); TLC R_f 0.5 (CH_2Cl_2/MeOH/25% NH_4OH 6:3:1); ^{13}C NMR (D_2O+KOD) δ 15.2, 19.7, 25.4, 25.9, 28.0, 30.3, 35.0, 36.1, 37.6, 39.6, 42.4, 44.1, 44.3, 48.5, 53.6, 60.8, 62.3, 69.7, 70.3, 74.1, 75.3, 83.2, 182.0, 182.4, 188.3; MS *m/z* 811 $(M + H)^+$; HPLC (Method E) retention time = 11.2, 99% (area %). Anal. ($C_{41}H_{70}N_4O_{12}$) C, H, N.

***N*⁶-[(Phenylmethoxy)carbonyl]-*N*²-[(3α,5*β*,7α,12α)-3,7,12-trihydroxy-24-oxocholan-24-yl]-L-lysine Methyl Ester (31).** Isobutyl chloroformate (6.56 g, 48 mmol, 6.22 mL) was dropped over 10 min into a suspension of cholic acid (16.3 g, 39.9 mmol) and Et_3N (4.86 g, 48 mmol, 6.65 mL) in THF (350 mL) at 0 °C under nitrogen. After 15 min, a solution of N^6-(phenylmethoxy)carbonyl-L-lysine (11.2 g; 40 mmol) in 0.67 M NaOH (60 mL) was added dropwise to the reaction mixture that was then maintained at 0 °C for 1h and at room temperature for 5 h. After addition of 2 N HCl (30 mL) to the mixture, the organic solvent was evaporated. The aqueous phase was diluted with brine and extracted with EtOAc. The organic layer was separated, dried (Na_2SO_4), and evaporated. The residue was dissolved in MeOH (600 mL) and *p*-toluenesulfonic acid monohydrate (1.56 g, 8.2 mmol) was added. After 20 h Et_3N (0.83 g, 8.2 mmol, 1.14 mL) was added, the solvent was evaporated and the crude was purified by flash chromatography (eluent: i-PrOH/EtOAc, 8−20%) to give **31** as a white solid: yield 24.1 g, 88%; mp 80−83 °C; TLC R_f 0.22 (EtOAc/*i*-PrOH 9:1); ^{13}C NMR (CD_3OD) δ 13.3, 18.0, 23.5, 24.3, 24.5, 25.5, 28.1, 29.0, 29.5, 30.6, 31.4, 32.3, 33.4, 33.9, 36.1, 36.7, 37.0, 40.7, 41.2, 41.7, 43.2, 43.4, 47.7, 48.0, 48.3, 48.4, 48.8, 49.3, 49.4, 49.7, 50.1, 50.5, 52.5, 53.5, 64.9, 67.5, 69.2, 73.2, 74.2, 128.9, 129.1, 129.7, 139.6, 159.1, 174.5, 177.1; MS *m/z* 707 $(M + Na)^+$; HPLC (Method A) retention time = 27.8, 99.5% (area %). Anal. ($C_{39}H_{60}N_2O_8$) H,N; C: calcd, 68.39; found, 67.75.

***N*²-[(3α,5*β*,7α,12α)-3,7,12-Trihydroxy-24-oxocholan-24-yl]-L-lysine Methyl Ester Monohydrochloride (32)** To a solution of **31** (15 g, 21.9 mmol) in MeOH (150 mL) at room temperature 5% Pd/C (1.5 g) was added, and the resulting suspension was stirred under hydrogen atmosphere for 1.5 h. After filtration, the solution was cooled to 0 °C and HCl in MeOH (1.1 M, 20.5 mL) was added. Evaporation of the solvent gave **32** as a white solid: yield 12.4 g, 96%, mp 108−110 °C; TLC R_f 0.33 (MeOH/Et_3N 95:5); ^{13}C NMR δ 13.3, 18.1, 23.5, 24.2, 28.2, 28.3, 32.2, 33.4, 36.2, 37.2, 40.8, 41.3, 43.3, 43.4, 48.3, 49.1, 49.3, 49.5, 53.1, 53.8, 69.3, 73.1, 74.3, 174.3, 177.3; MS *m/z* 551 $(M + H)^+$; HPLC (Method A) retention time = 17.1, 92% (area %). Anal. ($C_{31}H_{55}ClN_2O_6$) C,H,N,Cl.

***N*⁶-[*N*-[2-[[2-[Bis(carboxymethyl)amino]ethyl](carboxymethyl)amino]ethyl]-*N*-(carboxymethyl)-*O*-(phenylmethyl)-DL-seryl]-*N*²-[(3α,5*β*,7α,12α)-3,7,12-trihydroxy-24-oxocholan-24-yl]-L-lysine (1k).** *N*,*N*-Diisopropylethylamine (6.48 g, 50.1 mmol, 8.52 mL) was added dropwise to a solution of **32** (9.35 g, 15.9 mmol), **10** (9.06 g, 15.9 mmol), and BOP (6.3 g, 15.9 mmol) in DMF (140 mL) at room temperature under nitrogen. After 6 h, the solvent was evaporated, and the residue was dissolved in EtOAc and washed with saturated aqueous NH_4Cl then with H_2O. The organic phase was separated, dried (Na_2SO_4), and evaporated. The crude was purified by flash chromatography (eluent: MeOH/CH_2Cl_2 5−10%) to obtain a yellow-brown solid that was dissolved in MeOH/H_2O (2:1, 100 mL), and the solution was basified to pH 12 with 1 M NaOH. The reaction mixture was stirred for 21 h at room temperature, maintaining pH 12 by addition of 1 M NaOH (35.5 mL) through a pH-stat apparatus. The resulting solution was neutralized by addition of 1 M HCl and evaporated. The yellow oily residue was dissolved in 1 M HCl/MeOH (7:3, 200 mL), loaded onto an Amberlite XAD-16.00 resin column, and eluted with a MeOH/H_2O gradient. Ligand **1k** was recovered as a white solid: yield 4.5 g, 27%; mp 158−160 °C; TLC R_f 0.25 ($CHCl_3$/MeOH/H_2O/Et_3N 80:30:5:5); ^{13}C NMR (CD_3OD) δ 13.2, 19.4, 23.3, 24.4, 24.5, 29.2, 29.6, 29.8, 30.4, 30.6, 31.4, 33.5, 34.5, 36.0, 36.7, 37.2, 40.2, 40.3, 40.6, 41.2, 43.2, 43.4, 47.7, 47.9, 48.2, 48.6, 48.8, 49.2, 49.7, 50.1, 50.5, 53.1, 53.7, 55.6, 56.4, 58.5, 59.1, 59.7, 67.6, 69.0, 69.2, 69.5, 71.0, 73.0, 74.1, 74.5, 129.0, 129.1, 129.4, 129.7, 129.8, 139.6, 139.7, 173.3, 174.1, 174.7, 175.3, 176.1, 176.2, 177.5, 179.4, 179.5; MS *m/z* 1031 $(M-H)^-$ HPLC (Method A) retention time = 19.8, 97% (area %). Anal. ($C_{52}H_{81}N_5O_{16}$) C,H,N.

***N*⁶-[*N*-[2-[[2-[Bis(carboxymethyl)amino]ethyl](carboxymethyl)amino]ethyl]-*N*-(carboxymethyl)glycyl]-*N*²-[(3α,5*β*,7α,12α)-3,7,12-trihydroxy-24-oxocholan-24-yl]-L-lysine (1l).** A solution of H_2O (3.76 mL, 240 mmol) and DMF (155 mL) was added dropwise over 75 min into a suspension of **21**[28] (57.2 g, 160 mmol) in DMF (700 mL) at 80 °C. After 3 h, a solution of **32** (23.5 g; 40 mmol) and Et_3N (4.05 g, 40 mmol, 5.54 mL,) in DMF (330 mL) was added dropwise over 30 min. The reaction mixture was cooled to 20 °C, and 2 M NaOH (400 mL) was added dropwise, to afford the precipitation of a sticky solid. After 24 h at room temperature, the pH of the mixture was adjusted to 7 with 12 M HCl (25 mL), and the solution was decanted and evaporated to give a crude product which was taken up with H_2O/MeCN (85:15, 500 mL) and 6 M HCl (25 mL). The resulting solution was loaded onto an Amberlite XAD-2 resin column and eluted with MeCN/H_2O gradient to give **1l** as a white solid: yield 20 g, 55%; ^{13}C NMR δ 15.1, 19.5, 25.2, 25.5, 29.1, 30.1, 31.0, 32.0, 34.1, 34.6, 35.0, 36.6, 37.2, 39.2, 41.1, 41.6, 42.3, 44.1, 48.6, 48.9, 54.1, 54.2, 54.6, 57.6, 60.5, 61.2, 61.3, 61.8, 70.6, 74.1, 75.4, 176.7, 179.2, 180.9, 181.7, 181.9, 182.0; MS *m/z* 912 $(M + H)^+$; HPLC (Method A) retention time = 15, 98.5% (area %). Anal. ($C_{44}H_{73}N_5O_{15}$) H,N; C: calcd, 57.94; found, 58.52.

General Procedures for the Preparation of Gd(III) Complexes 2a−l. Procedure A. To a solution of the ligand (10 mmol) and base [NaOH or 1-deoxy-1-(methylamino)-D-glucitol (meglumine); 1 or 2 mol equiv according to the stoichiometry of the complex] in H_2O (300 mL) was added Gd_2O_3 (5 mmol). The resulting suspension was heated at 50 °C for 5 h. The reaction mixture was filtered and the solvent evaporated to give the complex as a solid. All yields obtained with this procedure are at least 90%.

Procedure B. The ligand (12 mmol) was suspended in H_2O (300 mL), and base (NaOH or meglumine; 3 mol equiv) was added until complete solution. A solution of $GdCl_3 \cdot H_2O$ (12 mmol) in H_2O (50 mL) was added dropwise to the reaction mixture, maintaining pH 7 by addition of base (NaOH or meglumine; 1 or 2 mol equiv according to the stoichiometry of the complex). Desalting was performed by nanofiltration or by preparative HPLC (Method B) or by elution through an Amberlite XAD-16 resin column with a MeCN/H_2O gradient. Evaporation of the solvents gave the complex as a solid. All yields obtained with this procedure are at least 70%.

2a: procedure B with meglumine; mp 178−180 °C; MS *m/z* 564 $(M - 2Meglu^+)^{2-}$; Anal. ($C_{62}H_{104}GdN_7O_{26}$) C,H,Gd,N.

2b: procedure B with meglumine; mp > 250 °C; MS *m/z* 1099 $(M - Meglu^+)^-$; Anal. ($C_{55}H_{89}GdN_6O_{19}$) C,H,Gd,N.

2c: procedure A with NaOH; mp > 250 °C; MS *m/z* 496 $(M - 2Na^+)^{2-}$; Anal. ($C_{40}H_{60}GdN_5Na_2O_{14}$) C,H,Gd,N,Na.

2d: procedure A with NaOH; mp > 250 °C; MS *m/z* 525 $(M - 2Na^+)^{2-}$; Anal. ($C_{42}H_{63}GdN_6Na_2O_{15}$) C,H,Gd,N,Na.

2e: procedure A with NaOH; mp > 250 °C; MS *m/z* 549 $(M - 2Na^+)^{2-}$; Anal. ($C_{42}H_{65}GdN_6Na_2O_{16}S$) C, H,Gd,N,Na,S.

2f: procedure B with NaOH; mp >250 °C; MS *m/z* 532 $(M - 2Na^+)^{2-}$; Anal. ($C_{44}H_{68}GdN_5Na_2O_{14}$) C,H,Gd,N,Na.

2g: procedure A with NaOH; mp > 250 °C; MS *m*/*z* 335 ($M - 3Na^+)^{3-}$; Anal. ($C_{41}H_{60}GdN_4Na_3O_{15}$) C,H,Gd,N,Na.

2h: procedure A with NaOH; mp > 250 °C; MS *m*/*z* 371 ($M - 3Na^+)^{3-}$; Anal. ($C_{43}H_{65}GdN_5Na_3O_{17}S$) H,Gd,N,Na,S; C: calcd, 43.78; found, 43.10.

2i: procedure A with NaOH; mp >250 °C; MS *m*/*z* 1111 ($M - Na^+)^-$; Anal. ($C_{44}H_{71}GdN_7NaO_{14}S$) H,Gd,N,Na,S; C: calcd, 46.59; found, 47.09.

2j: procedure A with NaOH; mp > 250 °C; MS *m*/*z* 984 ($M - Na^+)^-$; Anal. ($C_{41}H_{66}GdN_4NaO_{12}$) H,Gd,N,Na; C: calcd, 49.88; found 50.34.

2k: procedure A with meglumine; mp 171−174 °C; MS *m*/*z* 592 ($M - 2Meglu^+)^{2-}$; Anal. ($C_{66}H_{112}GdN_7O_{26}$) H,Gd,N; C: calcd, 50.27; found, 50.86.

2l: procedure B with NaOH; MS *m*/*z* 532 ($M - 2Na^+)^{2-}$ Anal. ($C_{44}H_{68}GdN_5Na_2O_{15}$) C, H, N,Gd; C: calcd, 47.60; found, 48.25.

Toxicity. Crl-CD-1(ICR)BR mice of both sexes (five males and five females per dose group) were used in the body weight range 18−25 g at treatment. Animals were housed under controlled environmental conditions according to EEC guidelines. For intravenous administration, aqueous solutions of the compounds were injected via lateral tail vein at 1 mL/min injection speed. For intracerebral administration, aqueous solutions of the compounds were injected in the left cerebral ventricle according to the method of Haley and Mc Cormick.[40] Animals were treated by single administration at escalating doses and mortality and onset of clinical signs were recorded throughout the following 7- or 14-day observation period. Terminal sacrifice was then performed, followed by gross necropsy. The median lethal dose (LD_{50}) and its 95% confidence limits (95% CL) were calculated by probit analysis using specialized software.

Cardiovascular Safety Assessment. Cardiovascular safety assessment on conjugates **2c** and **2g** was performed in NZW rabbits ($n = 3$) in the body weight range 2 to 3 kg. Each rabbit (fasted overnight) was anaesthetized with about 30 mg/kg iv of sodium pentobarbital. The dose of anaesthetic was supplemented when appropriate to maintain a surgical plan of anaesthesia. Animals were then placed on an operating table for surgical preparation. Tracheostomy was then performed, followed by intubation to allow artificial ventilation when the chest was opened. Artificial respiration was achieved using a respiratory pump. A 21G needle was inserted into the marginal ear vein for compounds administration; afterward, a 2F Millar transducer (Millar, Texas), with two probes connected to Gould pressure amplifiers (Gould, Ohio), was introduced into the left cardiac ventricle by the right carotid artery to measure blood pressure in both ascending aorta and left ventricle. Sternotomy was then performed. The pericardium was opened and segment of ascending aorta was dissected free from the other tissues. Electromagnetic flowmeter was placed around the aorta for aortic flow determination (flowmeter Biotronex Laboratory Inc., Kensington). Another electromagnetic probe was placed around the left carotid artery for determination of carotid flow. Then, subcutaneouos needle electrodes were inserted into the legs and connected to an ECG amplifier (Gould) to record the ECG (normally II lead) and to monitor the heart rate. Compounds **2c** and **2g** were administered as 0.25 M aqueous solutions at a dose of 1 mmol/kg (4 mL/kg) with an injection rate of about 0.1 mL/s. The cardiovascular parameters were then monitored for 30 min after the injection and recorded at the following times: end administration, 1, 5, 10, 15, and 30 min after the administration. The following parameters were examined: heart rate (HR) in beats/min, mean arterial blood pressure (mABP) in mmHg, systolic left ventricular pressure (sLVP) in mmHg, cardiac output (CO) in mL/min, stroke volume (SV) in mL/beat, dP/dt in mmHg/s^2, systemic vascular resistance (SVR) in 10^3 dyne·s cm^{-5}. All the cardiovascular parameters were recorded using a Gould polygraph and stored magnetically on an on-line data acquisition system (Morra Ing., Cuneo, Italy). The same software elaborated the data in order to give values relative to the examined cardiovascular parameters. Each animal was sacrificed at the end of the experiment by injecting an overdose of sodium pentobarbital.

Mutagenicity. In in vitro assessment of the mutagenic potential, histidine-dependent auxotrophic mutants of *Salmonella typhimurium* (strains TA1535, TA1537, TA98, and TA100) and a tryptophan-dependent mutant of *Escherichia coli* (strain CM891) were exposed to the test substance diluted in water, which was also used as a negative control. Two independent mutation tests were performed in the presence and absence of liver preparations from Aroclor 1254-induced rats (S9 mix). The first was a standard plate incorporation assay, and the second involved a preincubation stage. Dose levels of up to 5000 μg/plate were tested in the mutation tests. This is the standard limit dose recommended in the regulatory guidelines this assay follows. Other dose levels used were a series of ca. half-log dilutions of the highest concentration.

Biliary and Urinary Excretion in Normal Rats. Surgical Procedures. Animals were anesthetized by intraperitoneal injection of sodium pentobarbital at 30 mg kg^{-1}. Laparatomy was performed, and the common bile duct and the urinary bladder were cannulated with a PE 50 polyethylene catheter. The abdominal cavity was closed with sutures and the animal placed on a surgical table warmed to 37°C to keep the body temperature within physiological limits. The right femoral vein was exposed for the contrast medium injection.

Dosing and Sampling Procedures. The compounds (as aqueous solutions: 0.1 M for **2a**, **b**, **d**, **i**; 0.25 M for **2f**−**h**, **k**, **l**; 0.5 M for **2c**, **e**, **j**) were injected at a dose level of 0.25 mmol kg^{-1} into a femoral vein at a rate of 6 mL min^{-1}. Bile and urine were collected for 30 min before test article administration and every 30 min starting from 0 up to 480 min.

Assay of Gadolinium in Bile and Urine. Bile and urine contents of the compounds were calculated in terms of gadolinium concentration. Gadolinium was assayed in bile and urine by X-ray fluorescence, XRF. Quantitation was performed by interpolation from standard curves.

Sodium Cholyltaurine (CT) Competition. Surgical procedures were the same as for the biliary and urinary excretion experiments. Moreover, jugular vein was isolated and cannulated for CT solution infusion.

Dosing and Sampling Procedures. Bolus injection (7.5 μmol kg^{-1}) of the CT solution (3% w/v in physiological saline) was followed by infusion at a rate of 7.5 μmol kg^{-1} via jugular vein (total time of infusion 120 min). After 30 min from the start of the infusion, the compounds were injected (0.25 mmol kg^{-1}) into a femoral vein. Bile and urine were collected every 30 min starting from the end of test article administration up to 90 min after injection. Assay of gadolinium in bile and urine was performed as reported above.

References

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JM0310683