Structural Alterations in Desferrioxamine Compatible with Iron Clearance in Animals

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The design, synthesis, and biological evaluation of amideless desferrioxamine analogues are described. The design concept is predicated on the idea that a low molecular weight desferrioxamine analogue would represent a better pharmacophore from which to construct an orally effective or more efficient trihydroxamate than the parent chelator. The study demonstrates that (1) the monohydroxamate units of desferrioxamine must be linked to promote iron clearance, (2) the N-propanoyl-N-pentyl fragments of desferrioxamine can be replaced with smaller, e.g., C-5, methylene units without compromising the analogue's iron-clearing properties, and (3) a delicate balance exists between the molecule's iron-clearing efficiency and its lipophilicity.

Introduction

Although considerable effort has been invested in the development of new therapeutics for managing thalassemia, the treatment has remained essentially unchanged. Subcutaneous infusion of desferrioxamine B, a hexacoordinate hydroxamate iron chelator produced by *Streptomyces pilosus*,¹ is still regarded as the method of choice for handling transfusional iron overload.²⁻⁶ Although the drug's efficacy and long-term tolerability are well documented, it still suffers from a number of shortcomings associated with its poor to moderate efficiency and its marginal oral activity.

It has been demonstrated in situ that desferrioxamine forms a 1:1 hexacoordinate octahedral complex with Fe(III) with a formation constant⁷ of 3×10^{30} M⁻¹. If one assumes that the same stoichiometry applies when desferrioxamine is administered to an animal, 10% or less of the calculated iron excretion is observed.⁸ This situation is further complicated by the fact that desferrioxamine has a very short half-life in the body and must therefore be administered by continuous-infusion therapy over long periods of time. This translates at a clinical level into poor patient compliance.⁹ Although there are several possible solutions to this problem (the development of more efficient

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of iron clearance models. Ann. N.Y. Acad. Sci. 1990, 612, 378-393. (9) Kirking, M. H. Treatment of chronic iron overload. Clin. Pharm. 1991, 10, 775-783. desferrioxamine analogues, the generation of an orally effective desferrioxamine, or some combination thereof), they suffer from the same potential flaw: The target molecules all have increased molecular weight.

An orally effective desferrioxamine could be developed by (1) producing a desferrioxamine prodrug which, once orally absorbed, would collapse in the serum to the free trihydroxamate or (2) fixing desferrioxamine to a vector which would carry it across the gastrointestinal tract. Assuming even 100% bioavailability, an unlikely scenario, we are still looking at only 10% efficiency of the delivered chelator. Because of the therapeutic's increased molecular weight, patients would have to be given even larger quantities of the modified desferrioxamine than Desferal.

The situation would be somewhat different with the development of a more efficient desferrioxamine analogue. If the efficiency of this analogue were, for example, 8 times that of desferrioxamine, but only twice the molecular weight, this would be an acceptable trade-off. Such increased efficiency might be achieved by developing desferrioxamine analogues which have longer residence times in the animals or better access to iron pools.

With either the development of a more efficient desferrioxamine analogue or an orally effective desferrioxamine, the lower the molecular weight of the therapeutic, the more compatible it is likely to be with patient needs. In keeping with the importance of molecular weight in chelator design, we have elected to identify the minimal structural characteristics of desferrioxamine which are still compatible with iron clearance in animals. In spite of its shortcomings, desferrioxamine's clinical record renders it an excellent pharmacophore on which to predicate the design of more effective iron chelators.

Design and Synthesis

Design. When reduced to its simplest structural components, desferrioxamine (DFO, 1) can be viewed as composed of seven fragments (Figure 1): three bidentate hydroxamates insulated by two N-propanoyl-N-pentyl units, a terminal aminopentyl moiety, and a methyl group. While coordination chemistry considerations suggest that the insulating N-propanoyl-N-pentyl units contribute to the entrophy of binding and that the terminal aminopentyl and methyl fragments contribute little if anything to complexation, the role of these fragments in the molecule's iron-clearing properties remains to be defined. In altering

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Figure 1. Desferrioxamine B (DFO 1) and monohydroxamate DFO fragments 2 and 3.

the desferrioxamine backbone in a way which is compatible with the maintenance of its deferration properties in animals while decreasing its molecular weight, three questions must be addressed. (1) Is it necessary for the three hydroxamates of desferrioxamine to be linked? (2) What is the role of the terminal aminopentyl fragment in iron clearance? (3) What lower molecular weight fragments can be employed to replace the two N-propanoyl-N-pentyl units?

The significance of the entropy term in the chelate effect implies that a hexacoordinate ligand in which three bidentate fragments are linked will have a much more favorable free energy of iron binding than the three disconnected bidentate fragments. However, conventional thought also suggests that lower molecular weight molecules are more effectively transported across the gastrointestinal (GI) tract. The corollary to the latter idea is that even though monohydroxamates would not be expected to bind Fe(III) as effectively as the corresponding trihydroxamates, the trade-off might be acceptable if small fragments were absorbed orally. It is clear, however, that the first evaluation of the iron-clearing properties of any bidentate fragments should involve subcutaneous administration. If the fragments are not effective when administered by this route, their potential as oral therapeutics becomes a moot point.

The idea that smaller fragments of larger chelators, once in the animal, can clear iron is not completely without precedent.¹⁰⁻¹³ For example, although 2,3-dihydroxybenzoic acid (DHB), one of the three dihydroxybenzoyl components of enterobactin, does not bind iron as tightly as the hexacoordinate parent, when DHB is administered to rodents, it does promote iron clearance.^{12,13} Consequently, we were compelled to first synthesize two of the component monohydroxamate fragments, 2 and 3, which make up desferrioxamine (Figure 1) and evaluate their iron-clearing properties.

In an earlier investigation, we developed information to support the idea that the aminopentyl fragment of



6 (C-8)

Figure 2. Amideless DFO analogues 4 (C-5), 5 (C-5/C-10), and 6 (C-8).





^a Reagents: (a) succinic anhydride/pyr; (b) Ac₂O/pyr; (c) H₂/Ra Ni/NH₃/CH₃OH; (d) H₂/10% Pd-C/HCl/CH₃OH.

desferrioxamine was not requisite to its iron-clearing properties. In fact, replacement of the 5-aminopentyl unit with a polyether resulted in an analogue with improved iron-clearing capacity.¹⁴ However, the significance of the central N-propanoyl-N-pentyl units in desferrioxamine's iron-clearing properties remained to be established.

In keeping with the idea of reducing the overall molecular weight of desferrioxamine, the simplest replacement of the N-propanoyl-N-pentyl fragments of desferrioxamine is with a methylene backbone, i.e., creation of an amideless DFO. However, it is important that the distances between the chelating functionalities are not so compressed that the trihydroxamate's optimum binding geometry is compromised. Inspection of molecular models suggests that anything much smaller than C-5 fragments would inhibit the formation of a hexacoordinate octahedral complex. Several amideless desferrioxamine analogues were synthesized (Figure 2), and their iron-clearing properties were evaluated. These include C-5 (4) and C-8 (6) amideless DFO analogues, as well as a C-5 amideless DFO with a nonyl fragment replacing the methyl unit of desferrioxamine (C-5/C-10) (5). The latter analogue should be more lipophilic than either of the simple C-5 (4) or C-8 (6)analogues. This suggests that any chelator-induced iron excretion associated with the latter ligand should be largely of a biliary nature.

Synthesis. Production of both the desferrioxamine fragments (Scheme I) and the amideless DFO analogues (Scheme II) begins with the same synthon, N-(4-cyanobu-

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^a Reagents: (a) Cl(CH₂)₄COCl/1 N NaOH/CH₂Cl₂; (b) PhCH₂-ONHBOC/NaH/DMF; (c) TFA/CH₂Cl₂; (d) Ac₂O/pyr/CH₂Cl₂; (e) CH₃(CH₂)₈COCl/1 N NaOH/CH₂Cl₂; (f) H₂/10 % Pd-C/CH₃OH/HCl.

tyl)-O-benzylhydroxylamine (7). The synthesis and application of this reagent has been described in our earlier papers.¹⁴⁻¹⁶ The aminopentyl half-acid hydroxamate 2 was constructed by first acylating 7 with succinic anhydride in pyridine to produce the half-acid nitrile $8.^{15,16}$ The nitrile was then reduced to the corresponding amino acid 10 utilizing a Raney nickel catalyst, followed by hydrogenolysis of the benzyl group, providing monohydroxamate 2. The acetyl hydroxamate fragment 3 (Scheme I) was also prepared from the nitrile 7 by first reacting it with acetic anhydride in pyridine to produce the nitrile amide $9.^{15}$ The cyano group was then reduced with hydrogen over Raney nickel to generate benzyl-protected hydroxamate $11.^{15}$ Catalytic hydrogenation (1 atm, 10% Pd-C) gave acetyl monohydroxamate 3.

The synthesis of the C-5 amideless desferrioxamine analogues 4 and 5 (Scheme II) also began with nitrile 7. The benzyloxyamine nitrile was acylated with 5-chlorovaleryl chloride under biphasic conditions (1 N NaOH/ CH₂Cl₂), resulting in nitrile halide 12. *N*-(*tert*-Butoxycarbonyl)-O-benzylhydroxylamine¹⁷ was next alkylated (NaH/DMF) with 12 to give the protected dihydroxamate 13. The *N*-(*tert*-butoxycarbonyl) protecting group of 13 was removed by treatment with trifluoroacetic acid (TFA) in CH₂Cl₂, producing benzyloxyamine nitrile 14, and the deprotected nitrogen of 14 was acylated with 5-chlorovaleryl chloride, as above, affording a second ω -chloro nitrile, 15. *N*-(*tert*-Butoxycarbonyl)-O-benzylhydroxylamine was converted to its anion as before and alkylated with primary chloride 15, forming tert-butoxycarbonyl nitrile 16, a versatile precursor to amideless hexacoordinate hydroxamates. This intermediate provides the option of either reducing the nitrile, followed by functionalization of the terminal amine, or removing the tert-butoxycarbonyl protecting group, followed by functionalization of the O-benzylhydroxylamine. The latter choice was applied in the current synthesis. The nitrile 16 was treated with TFA in CH₂Cl₂, collapsing the *tert*-butoxycarbonyl protecting group to produce the O-benzylhydroxylamine nitrile 17. To complete the synthesis of the C-5 amideless DFO, 17 was reacted with acetic anhydride in pyridine. producing chelator precursor 18. The nitrile of 18 was then reduced to the primary amine, and its benzyl groups were cleaved with hydrogen over a palladium on carbon catalyst in methanolic HCl, generating C-5 amideless DFO (4). Alternatively, amine 17 was reacted with decanovl chloride under biphasic conditions to produce hydroxamate precursor 19. Exposure of 19 to hydrogen under mild conditions unmasked the more lipophilic C-5/C-10 amideless trihvdroxamate 5.

The sequence of reactions in Scheme II was also performed with 8-bromooctanoyl chloride¹⁸ in place of 5-chlorovaleryl chloride to generate the corresponding C-8 amideless desferrioxamine analogue (6). N-(4-Cvanobutyl)-O-benzylhydroxylamine (7) was acylated with the longer acid chloride under biphasic conditions, furnishing ω -bromonitrile 20. Alkylation of N-(tert-butoxycarbonyl)-O-benzylhydroxylamine with 20 gave tetracoordinate precursor 21, which on exposure to TFA under mild conditions collapsed to benzyloxyamine 22. Acylation of 22 with 8-bromooctanoyl chloride generated bromide 23. which was utilized to alkylate N-(tert-butoxycarbonyl)-O-benzylhydroxylamine, yielding hexacoordinate reagent 24. After treatment of 24 with TFA, the resulting amine 25 was reacted with acetyl chloride under biphasic conditions, providing nitrile 26. Simultaneous reduction of the cyano group and cleavage of the O-benzyl protecting groups (H₂/Pd-C) provided C-8 amideless desferrioxamine analogue (6).

Biological Evaluation

Iron Clearance Model. Biological evaluation of the hydroxamates was performed using a non-iron-overloaded bile duct-cannulated rat model. The drug-promoted iron excretion was monitored in both the bile and the urine and the results compared to a standard desferrioxamine dose given subcutaneously (sc). The effectiveness of various ligands was compared on an equivalent iron binding basis, 150 μ mol/kg for trihydroxamates and 450 μ mol/kg for monohydroxamates. The iron excretion data for the drugs evaluated in this study are reported in Table I and Figures 3 and 4. Table I includes the total "induced" iron excreted per kilogram of rat weight, over 24 h, the percentage of iron excreted in the bile, the percentage of iron excreted in the urine, and the efficiencies of the six ligands. In evaluating the efficiencies of the chelators ([observed chelator-promoted iron excretion/calculated chelator-promoted iron excretion] $\times 100\%$), the assumption was that the stoichiometry of ligand/metal complex is 1:1 under physiological conditions. Figure 3 indicates

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Table I. Effect of Iron-Chelating Drugs on 24-h Cumulative Iron Excretion in the Urine and the Bile of Non-Iron-Overloaded Bile Duct-Cannulated Rats

	iron excretion											
	$DFO^a (n = 6)$		$2^{b} (n = 2)$		$3^{b} (n = 2)$		$4^{a} (n = 5)$		$5^a (n = 5)$		$6^a (n = 5)$	
measurement	mg/kg	%	mg/kg	%	mg/kg	%	mg/kg	%	mg/kg	%	mg/kg	%
theor total	8.37	100	8.37	100	8.37	100	8.37	100	8.37	100	8.37	100
exptl total	0.232 ± 0.042	2.8	-0.018 ± 0.001	-2.1	-0.001 ± 0.000	-1.7	0.228 ± 0.030	2.7	0.184 ± 0.015	2.2	0.098 ± 0.009	1.2
in urine	0.059 ± 0.023	25.4	-0.003 ± 0.000	0	-0.001 ± 0.000	0	0.021 ± 0.004	9.4	0.017 ± 0.004	9.2	0.000 ± 0.000	0
in bile	0.173 ± 0.035	74.6	-0.015 ± 0.001	0	-0.002 ± 0.000	0	0.207 ± 0.030	90.6	0.167 ± 0.015	90.8	0.098 ± 0.010	100

^a Concentration, 150 µmol/kg sc. ^b Concentration, 450 µmol/kg sc.



Figure 3. Response of the non-iron-overloaded bile ductcannulated rats to DFO and amideless DFO analogues 4-6 administered at 150 μ mol/kg sc.



Figure 4. Ferrokinetics of chelator-induced biliary iron clearance in rodents given 4-6 at 150 μ mol/kg sc.

the micrograms of chelator-induced iron excreted in the bile and urine per kilogram of rat weight over a period of 24 h. Because of the differences in water solubility among the various drugs, it was necessary to employ a vehicle in which all of the compounds could be administered. We utilized a mixture of Cremophor RH-40 and water (40: 60). Furthermore, as dietary iron can reduce or in some cases enhance the amount of biliary iron excreted, all animals were fasted for 24 h before the administration of the drug, and the fast was maintained throughout the course of the experiment. Although fasting reduces the bile flow, data interpretation is facilitated. Dietary iron can complex with the ligand, reducing its bioavailability for absorption, or alternatively, the iron/chelator complex itself can be absorbed and then excreted in the bile, thus confusing data interpretation.

Biological Results. When the dihydroxamate fragments 2 and 3 (Figure 1) were administered subcutaneously to the rodents at a dose of $450 \,\mu \text{mol/kg}$, total iron excretion remained at the base level in all of the test animals. These results strongly suggest that the bidentate hydroxamate fragments of desferrioxamine must be linked for effective iron chelation in animals. The idea of monohydroxamates as orally effective chelators is now questionable. All three trihydroxamate analogues (Figure 2) cleared iron in the rodent model, although there were obvious differences in their efficiencies (Figure 3 and Table I). The lowest molecular weight trihydroxamate, the C-5 analogue (4), was the most effective at overall iron clearance, with an efficiency of $2.7 \pm 0.9\%$. With 9% of the induced iron cleared in the urine and 91% in the bile, this compound, although as efficient as DFO itself, had a larger fraction of the total induced iron in the bile. The C-8 analogue (6), although less efficient than DFO, still maintained a higher fraction of iron clearance in the bile. Interestingly, when the terminal methyl fragment of the C-5 analogue (4) was replaced with a nonyl unit, the iron clearance efficiency of the resulting trihydroxamate (5) was reduced to nearly half that of DFO. All of the iron cleared by 5. the most lipophilic of the three desferrioxamine analogues, was in the bile. A consideration of Figure 4 reveals rather striking differences in the kinetics of biliary iron clearance. Biliary iron clearance, induced by DFO or C-5 analogue 4, is back to baseline in the bile in 15 h. However, the more lipophilic analogues, C-8 (6) and C-5/C-10 (5), demonstrated a far more protracted clearance of iron in the bile.

Conclusion

The idea that a low molecular weight desferrioxamine analogue represents a better pharmacophore from which to construct an orally effective or more efficient trihydroxamate now seems tenable. The synthetic methodologies developed in this paper have made it possible to access such compounds. We have been able to identify some of the minimal structural requirements of desferrioxamine which are still compatible with iron clearance in an animal model. It is now clear that (1) a monohydroxamate pharmacophore is not viable, (2) the terminal aminopentyl fragment of desferrioxamine is not critical to its biological activity,¹⁴ and (3) the N-propanoyl-Npentyl fragments of DFO can be replaced with smaller methylene-containing fragments; i.e., the amides are not significant. On the basis of these observations, it should now be possible to develop either an orally effective desferrioxamine analogue or a more efficient desferrioxamine analogue which is realistic in terms of dosing levels.

Experimental Section

All reagents were purchased from Aldrich Chemical Co. and were used without further purification. Fisher Optima grade solvents were routinely used, and organic extracts were dried with sodium sulfate. Distilled solvents and acid-washed glassware (3 N HCl for 15 min) were employed for transformations involving hydroxamates 2-6. Silica gel 60 (70-230 mesh) obtained from EM Science (Darmstadt, Germany) or Sephadex LH-20 from Sigma Chemical Co. was used for column chromatography. Proton NMR spectra were recorded at 90 or 300 MHz in CDCl₃

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(unless otherwise indicated) with chemical shifts given in parts per million downfield from an internal tetramethylsilane or sodium 3-(trimethylsilyl)propanesulfonate (D_2O) standard; coupling constants (J) are in hertz. Elemental analyses were performed by Atlantic Microlabs, Norcross, GA. Sprague–Dawley rats were purchased from Charles River, Wilmington, MA. Bile and urine samples were analyzed on a Perkin-Elmer 5100 PC atomic absorption spectrophotometer fitted with a Model AS-51 autosampler using a quartz sampling probe as previously described.⁸ Cremophor RH-40 was obtained from BASF, Parsippany, NJ.

N-(4-Cyanobutyl)-O-benzylhydroxylamine (7) was previously synthesized in these laboratories^{15,16} in 84% yield.

N-(Benzyloxy)-N-(4-cyanobutyl)succinamic acid (8) was prepared by our method in 98% yield.^{15,16}

N-(4-Cyanobutyl)-N-(benzyloxy)acetamide (9) was synthesized in these laboratories¹⁵ in quantitative yield.

N-(Benzyloxy)-N-(5-aminopentyl)succinamic Acid (10). Raney nickel (W-2 form, 1.05 g) and concentrated NH₄OH (1.6 mL) were added to a solution of 8 (0.60 g, 1.97 mmol) in CH₃OH (8 mL) in a 250-mL Parr bottle. The reaction mixture was cooled to 0 °C. A slow stream of NH₃ was bubbled through the cold mixture for 10 min, and hydrogenation using a Parr shaker was carried out for 3 h at 50 psi. Catalyst was filtered using Celite and the filtrate concentrated in vacuo. Silica gel column chromatography, eluting with 0.6% concentrated NH₄OH/CH₃-OH gave 0.55 g (90%) of 10 as a white amorphous solid: NMR (D₂O) δ 1.05-1.84 (m, 6 H), 2.20-2.78 (m, 4 H), 2.91 (t, 2 H, J = 7), 3.68 (t, 2 H, J = 6), 4.91 (s, 2 H), 7.45 (s, 5 H). Anal. (C₁₆H₂₄N₂O₄) C, H, N.

N-(5-Aminopentyl)-N-(benzyloxy)acetamide (11) was synthesized according to our published procedure¹⁵ in 88% yield.

N-Hydroxy-*N***-(5-aminopentyl)succinamic Acid Hydrochloride (2).** Compound 10 (0.71 g, 2.30 mmol) was dissolved in CH₃OH (90 mL), followed by addition of 0.1 N HCl (from concentrated HCl and methanol, 24 mL, 2.4 mmol) and 10% Pd-C (0.24 g). The mixture was stirred under H₂ (1 atm) for 3 h, the catalyst was filtered, and the solvent was removed in vacuo. The residue was purified by Sephadex LH-20 column chromatography, eluting with 5–30% EtOH/benzene, giving 133 mg (23%) of 2 as a glass: NMR (D₂O) δ 1.14–1.87 (m, 6 H), 2.47–3.10 (m, 6 H), 3.60 (t, 2 H, J = 6). Anal. (C₉H₁₉ClN₂O₄) C, H, N.

N-Hydroxy-*N*-(5-aminopentyl)acetamide Hydrochloride (3). Compound 11 (3.66 g, 14.6 mmol) was dissolved in CH₃OH (150 mL), and 10% Pd–C (0.6 g) was added. The mixture was stirred under H₂(1 atm) at room temperature for 4 h. The catalyst was filtered, an equivalent of 0.1 N HCl added, and the solvent was removed, providing 2.2 g (77%) of 3 as a white solid: NMR (D₂O) δ 1.2–1.8 (m, 6 H), 2.12 (s, 3 H), 2.98 (t, 2 H, *J* = 7), 3.60 (t, 2 H, *J* = 7). Anal. (C₇H₁₇ClN₂O₂) C, H, N.

N-(Benzyloxy)-N-(4-cyanobutyl)-5-chloropentanamide (12). A solution of 5-chlorovaleryl chloride (3.66 g, 23.6 mmol) in CH₂Cl₂ (50 mL) was slowly dripped into a mixture of amine 7 (4.08 g, 20.0 mmol) in CH₂Cl₂ (100 mL) and 1 N NaOH (200 mL), which had been cooled to 0 °C. The mixture was stirred at 0 °C for 30 min and at room temperature overnight. The layers were separated, and the aqueous layer was further extracted with CH₂Cl₂ (2 × 150 mL). The combined organic extracts were washed with saturated sodium chloride, and solvent was removed in vacuo. The crude oil was passed through a short silica gel column, eluting with EtOAc, to give 6.0 g (93%) of 12 as an oil: NMR δ 1.57–1.97 (m, 8 H), 2.35 (t, 2 H, J = 6), 2.40 (t, 2 H, J = 6), 3.50 (t, 2 H, J = 6), 3.67 (t, 2 H, J = 6), 4.80 (s, 2 H), 7.37 (s, 5 H). Anal. (C₁₇H₂₃ClN₂O₂) C, H, N.

12-(tert-Butoxycarbonyl)-6,12-bis(benzyloxy)-7-oxo-6,12diazadodecanenitrile (13). N-(tert-Butoxycarbonyl)-O-benzylhydroxylamine¹⁷ (4.2 g, 18.8 mmol) was dissolved in DMF (120 mL) and cooled to 0 °C. Sodium hydride (80%, 0.564 g, 18.8 mmol) was added, and the mixture was stirred at 0 °C for 30 min. A solution of 12 (6.0 g, 18.6 mmol) in DMF (30 mL) was slowly added to the cold solution, which was then allowed to stir at room temperature for 20 min and at 80 °C overnight. The DMF was removed under high vacuum, and the residue was quenched with H₂O (50 mL) and extracted with CH₂Cl₂ (3×70 mL). Organic extracts were washed with saturated sodium chloride, and the solvent was removed by rotary evaporation. Silica gel column chromatography, eluting with 2:1 hexane/EtOAc produced 7.29 g (77%) of 13 as an oil: NMR δ 1.50 (s, 9 H), 1.40–1.90 (m, 8 H), 2.30 (t, 2 H, J = 6), 2.40 (t, 2 H, J = 6), 3.40 (t, 2 H, J = 6), 3.63 (t, 2 H, J = 6), 4.77 (s, 2 H), 4.80 (s, 2 H), 7.33 (s, 10 H). Anal. (C₂₉H₃₉N₃O₅) C, H, N.

6,12-Bis(benzyloxy)-7-oxo-6,12-diazadodecanenitrile (14). Trifluoroacetic acid (25 mL) was slowly dripped into a solution of 13 (7.10 g, 13.93 mmol) in CH₂Cl₂ (150 mL), which had been cooled to 0 °C. The solution was stirred at 0 °C for 30 min and at room temperature for 15 min. Solvent and excess TFA were removed by rotary evaporation, and the concentrate was cooled to 0 °C, treated with saturated NaHCO₃ (150 mL), and extracted with CH₂Cl₂ (4 × 80 mL). After solvent removal, the crude oil was purified by silica gel column chromatography with 1:1 hexane/EtOAc as the eluant, generating 4.6 g (81%) of 14 as an oil: NMR δ 1.40–1.90 (m, 8 H), 2.30 (t, 2 H, J = 6), 2.40 (t, 2 H, J = 6), 2.90 (t, 2 H, J = 6), 3.63 (t, 2 H, J = 6), 4.63 (s, 2 H), 4.75 (s, 2 H), 4.93 (br s, 1 H), 7.27 (s, 5 H), 7.33 (s, 5 H). Anal. (C₂₄H₃₁N₃O₃) C, H, N.

17-Chloro-6,12-bis(benzyloxy)-7,13-dioxo-6,12-diazaheptadecanenitrile (15). Compound 14 (2.44 g, 5.96 mmol) was reacted with 5-chlorovaleryl chloride (1.13 g, 7.29 mmol) in CH₂-Cl₂ (150 mL) and 1 N NaOH (200 mL) by the procedure used for 12. Silica gel column chromatography, eluting with 3% EtOH/ CHCl₃, gave 15 in quantitative yield as an oil: NMR δ 1.50–1.87 (m, 12 H), 2.23–2.53 (m, 6 H), 3.47 (t, 2 H, J = 6), 3.60 (t, 4 H, J = 6), 4.77 (s, 4 H), 7.33 (s, 10 H). Anal. (C₂₉H₃₈ClN₃O₄) C, H, N.

18-(tert-Butoxycarbonyl)-6,12,18-tris(benzyloxy)-7,13-dioxo-6,12,18-triazaoctadecanenitrile (16). N-(tert-Butoxycarbonyl)-O-benzylhydroxylamine (1.43 g, 6.40 mmol) was alkylated with 15 (3.00 g, 5.68 mmol) using NaH (80%, 0.19 g, 6.33 mmol) in DMF (100 mL), following the procedure used for 13. Column chromatography on silica gel with 4:1 hexane/EtOAc yielded 3.0 g (74%) of 16 as an oil: NMR δ 1.50 (s, 9 H), 1.40–1.85 (m, 12 H), 2.20–2.50 (m, 6 H), 3.27–3.70 (m, 6 H), 4.75 (s, 6 H), 7.33 (s, 15 H). Anal. (C₄₁H₅₄N₄O₇) C, H, N.

6,12,18-Tris(benzyloxy)-7,13-dioxo-6,12,18-triazaoctadecanenitrile (17). Compound 16 (2.85 g, 3.99 mmol) in CH₂Cl₂ was treated with excess TFA following the procedure used for 14. Silica gel column chromatography, eluting with 3:2 EtOAc/ hexane gave 1.4 g (57%) of 17 as an oil: NMR δ 1.43–1.83 (m, 12 H), 2.20–2.50 (m, 6 H), 2.90 (t, 2 H, J = 6), 3.60 (t, 4 H, J = 6), 4.63 (s, 2 H), 4.75 (s, 4 H), 7.28 (s, 5 H), 7.33 (s, 10 H). Anal. (C₃₆H₄₆N₄O₅) C, H, N.

6,12,18-Tris(benzyloxy)-7,13,19-trioxo-6,12,18-triazaeicosanenitrile (18). Acetic anhydride (8 mL) was slowly added to a solution of 17 (1.25 g, 2.03 mmol) in pyridine (50 mL) and CH₂Cl₂ (30 mL), which had been cooled to 0 °C. Stirring was continued at 0 °C for 30 min and then at room temperature overnight. Solvents were removed under high vacuum, and the residue was dissolved in CHCl₃ (120 ml), followed by washing with 0.5 N HCl (2 × 40 mL) and saturated NaHCO₃ (2 × 40 mL). After solvent removal, the crude oil was purified by silica gel column chromatography, eluting with 3:1 EtOAc/hexane, to furnish 0.8 g (60%) of 18 as an oil: NMR δ 1.40–1.80 (m, 12 H), 2.05 (s, 3 H), 2.20–2.50 (m, 6 H), 3.40–3.73 (m, 6 H), 4.75 (s, 6 H), 7.32 (s, 15 H). Anal. (C₃₈H₄₈N₄O₆) C, H, N.

6,12,18-Trihydroxy-7,13,19-trioxo-6,12,18-triazaeicosanamine Hydrochloride (4). Compound 18 (1.000 g, 1.522 mmol) was dissolved in CH₃OH (210 mL), followed by addition of 0.1 N HCl (from concentrated HCl and methanol, 16.75 mL, 1.675 mmol) and 10% Pd-C (0.6 g). The mixture was stirred under H₂ (1 atm) at room temperature overnight. The catalyst was filtered, and the solvent was removed. The residue was purified by Sephadex LH-20 column chromatography, eluting with 15% MeOH/toluene, to generate 560 mg (86%) of 4 as a colorless solid: NMR (CD₃OD) δ 1.30–1.97 (m, 14 H), 2.17 (s, 3 H), 2.60 (t, 4 H, J = 6), 3.00 (t, 2 H, J = 6), 3.70 (t, 6 H, J = 6). Anal. (C₁₇H₃₅ClN₄O₆) C, H, N.

6,12,18-Tris(benzyloxy)-7,13,19-trioxo-6,12,18-triazaoctacosanenitrile (19). Compound 17 (1.20g, 1.95 mmol) was reacted with decanoyl chloride (0.565 g, 2.96 mmol) in CH₂Cl₂ and 1 N NaOH following the procedure used for 12. Purification by silica gel column chromatography with 2:1 EtOAc/hexane as the eluant provided 1.20 g (80%) of 19 as an oil: NMR δ 0.90 (t, 3 H, J = 6), 1.13–1.40 (m, 14 H), 1.47–1.90 (m, 12 H), 2.23–2.53 (m, 8 H), 3.50–3.77 (m, 6 H), 4.77 (s, 6 H), 7.33 (s, 15 H). Anal. ($C_{46}H_{64}N_4O_6$) C, H, N.

6,12,18-Trihydroxy-7,13,19-trioxo-6,12,18-triazaoctacosanamine Hydrochloride (5). Compound 19 (1.13 g, 1.47 mmol) was hydrogenated in CH₃OH (150 mL) and 0.1 N HCl (16.2 mL, 1.62 mmol) with 10% Pd-C (0.6 g) by the procedure used for 4. Purification by Sephadex LH-20 column chromatography with 20% EtOH/toluene as the eluant produced 0.508 g (64%) of 5 as a colorless solid: NMR (CD₃OD) δ 0.90 (t, 3 H, J = 6), 1.17-1.87 (m, 28 H), 2.33-2.63 (m, 6 H), 2.92 (t, 2 H, J = 6), 3.60 (t, 6 H, J = 6). Anal. (C₂₅H₅₁ClN₄O₆) C, H, N.

14-Bromo-6-(benzyloxy)-7-oxo-6-azatetradecanenitrile (20). Compound 7 (3.2 g, 15.7 mmol) was treated with 8-bromooctanoyl chloride¹⁸ (4.55 g, 18.8 mmol) in CH₂Cl₂ and 1 N NaOH following the procedure used for 12. Silica gel column chromatography with 2:1 hexane/EtOAc afforded 3.8 g (59%) of 20 as an oil: NMR δ 1.17-2.00 (m, 14 H), 2.23-2.50 (m, 4 H), 3.37 (t, 2 H, J = 6), 3.63 (t, 2 H, J = 6), 4.77 (s, 2 H), 7.33 (s, 5 H). Anal. (C₂₀H₂₉BrN₂O₂) C, H, N.

15-(tert-Butoxycarbonyl)-6,15-bis(benzyloxy)-7-oxo-6,15diazapentadecanenitrile (21). N-(tert-Butoxycarbonyl)-Obenzylhydroxylamine (0.991 g, 4.44 mmol) was alkylated with 20 (2.0 g, 4.89 mmol) by employment of the procedure used for 13. Purification of the crude oil by silica gel column chromatography with 3:2 hexane/EtOAc gave 1.98 g (81%) of 21 as an oil: NMR δ 1.13-1.90 (m, 14 H), 1.50 (s, 9 H), 2.23-2.47 (m, 4 H), 3.37 (t, 2 H, J = 6), 3.63 (t, 2 H, J = 6), 4.75 (s, 2 H), 4.77 (s, 2 H), 7.33 (s, 10 H). Anal. (C₃₂H₄₅N₃O₅) C, H, N.

6,15-Bis(benzyloxy)-7-oxo-6,15-diazapentadecanenitrile (22). Excess TFA was added to 21 (2.00 g, 3.63 mmol) in CH₂Cl₂ at 0 °C. The solution was stirred at 0 °C for 30 min and at room temperature for 15 min, and then the reaction was worked up by the method used for 14. Silica gel column chromatography with 2% MeOH/CHCl₃ provided 1.51 g (92%) of 22 as an oil: NMR δ 1.17–1.90 (m, 14 H), 2.23–2.50 (m, 4 H), 2.90 (t, 2 H, J = 6), 3.40 (s, 1 H), 3.63 (t, 2 H, J = 6), 4.67 (s, 2 H), 4.77 (s, 2 H), 7.30 (s, 5 H), 7.33 (s, 5 H). Anal. (C₂₇H₃₇N₃O₃) C, H, N.

23-Bromo-6,15-bis(benzyloxy)-7,16-dioxo-6,15-diazatricosanenitrile (23). Compound 22 (1.50 g, 3.32 mmol) was combined with 8-bromooctanoyl chloride (0.963 g, 3.99 mmol) following the conditions used for 12. The crude oil was purified by silica gel column chromatography with 1:1 hexane/EtOAc as the eluant, giving 2.06 g (95%) of 23 as an oil: NMR δ 1.17-2.00 (m, 24 H), 2.23-2.50 (m, 6 H), 3.36 (t, 2 H, J = 6), 3.60 (t, 2 H, J = 6), 3.63 (t, 2 H, J = 6), 4.77 (s, 4 H), 7.33 (s, 10 H). Anal. (C₃₅H₅₀BrN₃O₄) C, H, N.

24-(tert-Butoxycarbonyl)-6,15,24-tris(benzyloxy)-7,16-dioxo-6,15,24-triazatetracosanenitrile (24). N-(tert-Butoxycarbonyl)-O-benzylhydroxylamine (0.445 g, 1.99 mmol) was alkylated by 23 (1.37 g, 2.09 mmol), utilizing the procedure followed for 13. Silica gel column chromatography with 1:1 hexane/EtOAc gave 1.50 g (94%) of 24 as an oil: NMR δ 1.50 (s, 9 H), 1.17–1.90 (m, 24 H), 2.27–2.50 (m, 6 H), 3.40 (t, 2 H, J = 6), 3.60 (t, 2 H, J = 6), 3.63 (t, 2 H, J = 6), 4.77 (s, 4 H), 4.80 (s, 2 H), 7.33 (s, 15 H). Anal. ($C_{47}H_{66}N_4O_7$) C, H, N.

6,15,24-Tris (ben zyloxy)-7,16-dioxo-6,15,24-triazatetracosanenitrile (25). Excess TFA was added to a solution of 24 (1.32 g, 1.65 mmol) in CH₂Cl₂ at 0 °C following the procedure used for 14. The crude oil was purified by silica gel column chromatography using 3:2 EtOAc/hexane to generate 0.96g (83%) of 25 as an oil: NMR δ 1.17-1.97 (m, 24 H), 2.23-2.50 (m, 6 H), 2.90 (t, 2 H, J = 6), 3.57 (t, 2 H, J = 6), 3.63 (t, 2 H, J = 6), 4.67 (s, 2 H), 4.77 (s, 4 H), 7.30 (s, 5 H), 7.33 (s, 10 H). Anal. (C₄₂H₅₈N₄O₅) C, H, N.

6,15,24-Tris(benzyloxy)-7,16,25-trioxo-6,15,24-triazahexacosanenitrile (26). Compound **25** (0.93 g, 1.33 mmol) was treated with acetyl chloride (0.160 g, 2.04 mmol) by the procedure used for **12**. Silica gel column chromatography (3:1 EtOAc/hexane) produced 0.95 g (96%) of **26** as an oil: NMR δ 1.17–1.87 (m, 24 H), 2.07 (s, 3 H), 2.27–2.47 (m, 6 H), 3.47–3.73 (m, 6 H), 4.77 (s, 6 H), 7.33 (s, 15 H). Anal. (C₄₄H₆₀N₄O₆) C, H, N.

6,15,24-Trihydroxy-7,16,25-trioxo-6,15,24-triazahexacosanamine Hydrochloride (6). Compound 26 (1.20 g, 1.62 mmol) was hydrogenated in CH₃OH and 0.1 N HCl with 10% Pd-C by the procedure used for 4. Purification by Sephadex LH-20 column chromatography with 15% EtOH/toluene as the eluant produced 0.386 g (47%) of 6 as a colorless solid: NMR (CD₃OD) δ 1.17-1.87 (m, 26 H), 2.07 (s, 3 H), 2.30-2.55 (m, 4 H), 2.90 (t, 2 H, J = 6), 3.43-3.70 (m, 6 H). Anal. (C₂₃H₄₇ClN₄O₆) C, H, N.

Bile Duct Cannulation. Male Sprague-Dawley rats averaging 400 g were housed in Nalgene plastic metabolic cages during the experimental period and were given free access to water. The animals were anesthetized using sodium pentobarbital (50 mg/ kg), given intraperitoneally (ip). The bile duct was cannulated, using 22-gauge polyethylene tubing (Intramedic), about 1 cm from the duodenum. The cannula was inserted about 2 cm into the duct, and once bile flow was established, the cannula was tied snugly in place. A skin-tunneling needle was inserted from the shoulder area around to the abdominal incision. The cannula was threaded through the needle until it emerged from the shoulder opening.

The cannula was passed from the rat to the swivel inside a metal torque-transmitting tether, which was attached to a rodent jacket around the animal's chest. The cannula was directed from the rat to a Gilson microfraction collector by a fluid swivel mounted above the metabolic cage. This system allowed the animal to move freely in the cage while continuous bile samples were being collected. Bile samples were collected at 3-h intervals for 48 h. Urine samples were taken every 24 h.

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