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Synthesis and Structure−Activity Relationships of Azamacrocyclic C-X-C Chemokine Receptor 4 Antagonists: Analogues Containing a Single Azamacrocyclic Ring are Potent Inhibitors of T-Cell Tropic (X4) HIV-1 Replication

Gary J. Bridger,*[,†] Renato T. Skerlj,[†,∥] Pedro E. Hernandez-Abad,[‡] David E. Bogucki,[†] Zhongren Wang,[†] Yuanxi Zhou,[†] Susan Nan,[†] Eva M. Boehringer,[†] Trevor Wilson,[†] Jason Crawford,[†] Markus Metz,[†,∥] Sigrid Hatse,[§] Katrien Princen,[§] Erik De Clercq,[§] and Dominique Schols[§]

[†]*AnorMED Inc. now Genzyme Corporation, 500 Kendall Street, Cambridge, Massachusetts 02142,* [‡]*Johnson Matthey Pharmaceutical Research, 1401 King Road, West Chester, Pennsylvania 19380, and* [§]*Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium.* [∥]*Genzyme Corp., 153 Second Avenue, Waltham, Massachusetts 02451.*

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Bis-tetraazamacrocycles such as the bicyclam AMD3100 (**1**) are a class of potent and selective anti-HIV-1 agents that inhibit virus replication by binding to the chemokine receptor CXCR4, the coreceptor for entry of X4 viruses. By sequential replacement and/or deletion of the amino groups within the azamacrocyclic ring systems, we have determined the minimum structural features required for potent antiviral activity in this class of compounds. All eight amino groups are not required for activity, the critical amino groups on a per ring basis are nonidentical, and the overall charge at physiological pH can be reduced without compromising potency. This approach led to the identification of several single ring azamacrocyclic analogues such as AMD3465 (**3d**), **36**, and **40**, which exhibit EC_{50}'s against the cytopathic effects of HIV-1 of 9.0, 1.0, and 4.0 nM, respectively, antiviral potencies that are comparable to **1** (EC_{50} against HIV-1 of 4.0 nM). More importantly, however, the key structural elements of **1** required for antiviral activity may facilitate the design of nonmacrocyclic CXCR4 antagonists suitable for HIV treatment via oral administration.

Introduction

The development of antiviral agents that inhibit alternative targets in the HIV[a]-replicative cycle remains an important goal in order to alleviate the side effects of currently approved agents or to overcome the problem of drug resistance. In this regard, we have focused on the development of compounds that inhibit CXCR4, the coreceptor used by T-tropic (T-cell tropic) viruses for fusion and entry of HIV into target cells of the immune system. The corresponding chemokine receptor CCR5 is used by M-tropic (macrophage tropic) viruses and has been associated with the early stages of infection and replication in HIV-positive patients.[1,2] The transition from M-tropic to T-tropic (or dual/mixed-tropic) virus during the course of HIV infection in approximately 50% of patients is associated with a faster $CD4^+$ T-cell decline and a more rapid disease progression.[3−5]

Recently, we reported the results of clinical trials with our prototype CXCR4 antagonist AMD3100[6−8] (**1**) and an orally bioavailable CXCR4 antagonist, (*S*)-*N*′-(1*H*-benzimidazol-2-ylmethyl)-*N*′-(5,6,7,8-tetrahydroquinolin-8-yl)butane-1,4-diamine (AMD070).[9−11] When administered to HIV positive patients whose virus was confirmed to use CXCR4 for viral entry, both agents were able to suppress the replication of CXCR4 and dual-tropic strains of HIV. Similarly, the CCR5 antagonist Maraviroc suppresses replication of HIV-1 that exclusively uses CCR5 for entry[12] and was recently approved by the FDA for combined antiretroviral therapy in treatment-experienced patients.[13] A combination of CCR5 and CXCR4 antagonists for treatment of dual/mixed-tropic HIV infection is therefore highly desirable.

Beyond its use as a coreceptor for HIV, the CXCR4 chemokine receptor has a more fundamental role in the trafficking of white blood cells, which broadly express CXCR4.[14,15] A member of the superfamily of G-protein coupled receptors, the interaction of CXCR4 and its ligand, stromal cell-derived factor-1 (SDF-1), plays a central role in the homing and retention of cells within the bone marrow microenvironment.[16] Consistent with these observations, administration of **1** to healthy volunteers caused a dose-dependent leukocytosis[6,7] that in subsequent studies was shown to include the mobilization of $CD34^+$ stem and progenitor cells suitable for hematopoietic stem cell transplantation.[17−20] The ability of analogues of **1** to mobilize progenitors correlated with their in vitro capacity to inhibit SDF-1 binding to CXCR4.[21] Because of the need for parenteral administration, **1** was developed in combination with granulocyte colony-stimulating factor (G-CSF) to mobilize hematopoietic stem cells to the peripheral blood for collection and subsequent autologous transplantation in patients with non-Hodgkin's lymphoma (NHL) and multiple myeloma (MM).[22−25] Plerixafor (**1**) was approved by the FDA in December 2008.

We have previously reported the structure−activity relationships of anti-HIV bis-azamacrocycles and their transition

*To whom correspondence should be addressed. Phone: 617-429-7994. Fax: 617-768-9809. E-mail: gary.bridger@genzyme.com. Address: Gary J. Bridger, Genzyme Corporation, 55 Cambridge Parkway, Cambridge MA 02142.

[a]Abbreviations: HIV, Human Immunodeficiency Virus; CXCR4, C-X-C chemokine receptor 4; CCR5, C-C-R chemokine receptor 5.

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metal complexes in detail.[26−28] Because of the common structural features between a doubly protonated cyclam (1,4,8,11-tetraazacyclotetradecane) ring present in **1** (at physiological pH) and a kinetically labile transition metal complex of cyclam with an overall charge of +2, we proposed that both structural motifs may bind to the CXCR4 receptor through interactions with amino acid residues containing carboxylate groups.[29] We have subsequently shown via directed mutagenesis of the aspartate and glutamic acid residues in CXCR4 that binding of **1** and related analogues to the seven transmembrane, G-protein coupled receptor is highly dependent upon the amino acids Asp171 and Asp262, located in transmembrane region (TM)-IV and TM-VI at each end of the main ligand binding crevice of the receptor.[30−35] Mutation of either aspartic acid to aspargine significantly reduced the ability of **1** to inhibit binding of radiolabeled stromal cell derived factor-1α (^{125}I-Met-SDF-1α). More importantly, however, U87 cells stably transfected with CD4 and the mutant coreceptors CXCR4[D171N] and CXCR4[D262N] were less effective at supporting infection of the CXCR4-using HIV-1 strain NL4.3 compared to the wild-type receptor and the double mutant CXCR4[D171N,D262N] completely failed as a coreceptor for HIV infection.[31] Correspondingly, the ability of **1** to inhibit HIV-1 infection via CXCR4[D171N] and CXCR4[D262N] was also diminished, thereby confirming that **1** binds in a region of the receptor that is critical for X4 HIV-1 coreceptor function.

We have also reported that binding of the bis-Zn, Ni, and Cu complexes of **1** were also dependent upon D171 and D262 of the receptor.[36] In a similar manner to **1**, the transition metal complexes were found to be less effective inhibitors of ^{125}I-Met-SDF-1α binding to the mutant receptors CXCR4-[D171N] and CXCR4[D262N] compared to the wild-type receptor. Incorporation of Zn, Ni, or Cu into the cyclam rings of **1** increased the affinity to the wild-type CXCR4 receptor, but the enhancement was selectively eliminated by substitution of Asp262. Supporting physiochemical evidence for the interaction of acetate (carboxylates) with metal complexes of azamacrocycles, including **1**, has been recently reported.[37,38]

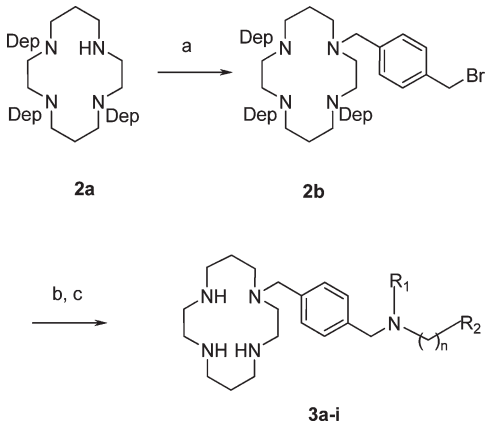
In the current study, we determine the minimum structural features of **1** required for potent antiviral activity, leading to the identification of the single azamacrocyclic ring analogue AMD3465[32,33,39,40] (**3d**) and ultimately the design of nonmacrocyclic, orally biovailable CXCR4 antagonists.[11,41,42] Given the growing body of evidence that the CXCR4/SDF-1 interaction is involved in regulating several human malignancies,[43−45] CXCR4 antagonists may have additional therapeutic applications in addition to HIV treatment.

Chemistry

Analogues containing a single 1,4,8,11-tetraazacyclotetradecane (cyclam) ring were prepared by modifications to previously published routes[26,29] as shown in Scheme 1. Reaction of the selectively protected tris-diethylphosphoramidate (Dep) cyclam ring (**2a**) with α,α-dibromo-*p*-xylene in acetonitrile containing potassium carbonate gave the desired bromomethyl intermediate (**2b**). Reaction of the bromide with an excess of the requisite amine, followed by deprotection of the Dep- groups with a saturated solution of hydrogen bromide in acetic acid at room temperature. gave analogues **3a**−**i** as the corresponding hydrobromide salts.

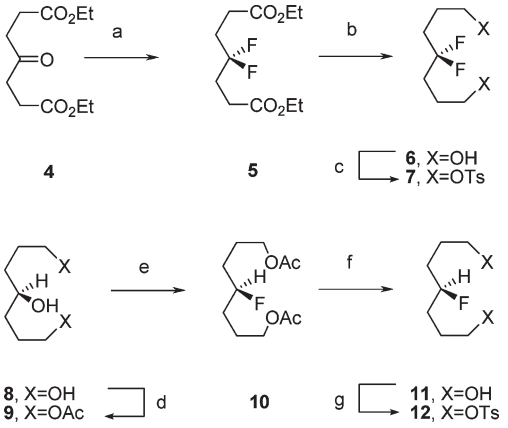
To prepare analogues of **3d** in which the cyclam ring was replaced by a series of 14-membered azamacrocyclic rings, we

Scheme 1[a]



[a] Reagents: (a) α,α′-dibromo-*p*-xylene, K_2CO_3, CH_3CN, reflux; (b) amine, K_2CO_3, CH_3CN, reflux; (c) HBr, acetic acid, room temp.

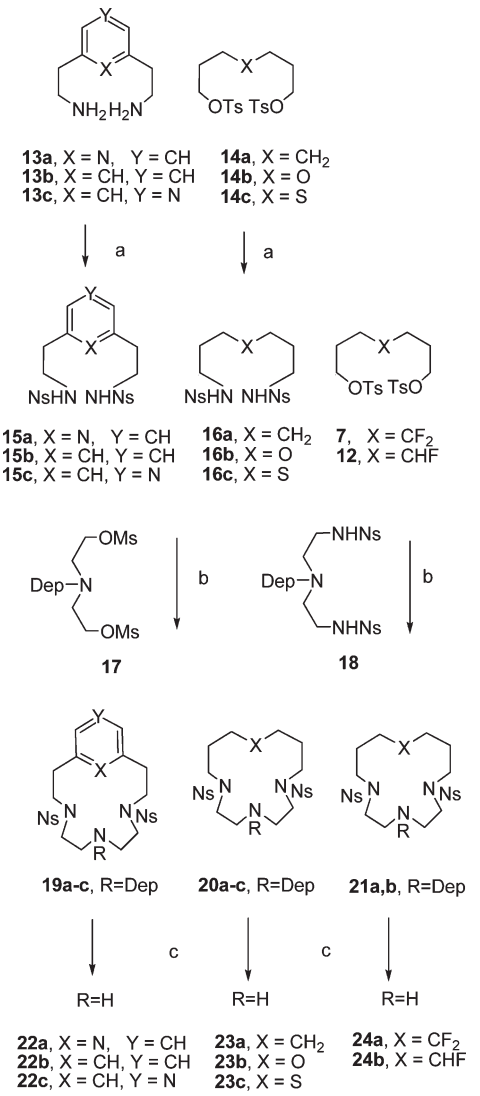
Scheme 2[a]



[a] Reagents: (a) Et_2NSF_3 (neat), room temp; (b) LAH, Et_2O; (c) Ts-Cl, Et_3N, CH_2Cl_2; (d) acetic anhydride, pyridine; (e) Et_2NSF_3, CH_2Cl_2, −78 °C, then room temp; (f) NH_3/MeOH, room temp; (g) Ts-Cl, Et_3N, CH_2Cl_2.

prepared a series of selectively protected macrocyclic ring systems containing a single (unprotected) secondary amine. This approach ensures the regiochemical outcome of the reaction with a benzylic halide during final construction (as shown in Scheme 6). The syntheses of appropriate precursors are shown in Schemes 2−5. To incorporate fluorine groups at the desired position in the macrocyclic ring, suitably fluorinated bis-electrophiles were prepared, starting from 4-oxo-heptanedioic acid diethyl ester (**4**) and heptane-1,4,7-triol (**8**) as depicted in Scheme 2. Reaction of the ketone (**4**) with neat (diethylamino)-sulfur trifluoride[46,47] (DAST) at room temperature for 12 days gave the corresponding difluoro-intermediate (**5**) in 43% yield. Reduction of the ester groups with LAH (to give the diol **6**), followed by derivatization with toluenesulfonyl chloride, gave the bis-electrophile (**7**) required for the impending macrocyclization reaction. The corresponding monofluorinated intermediate was prepared in a similar manner. Protection of the primary alcohols in **8** as the acetyl group using acetic anhydride gave the secondary alcohol **9**, which was rapidly (and virtually quantitatively) converted to the fluorinated intermediate (**10**) with DAST (2.0 equiv) in dichloromethane. Removal of the acetyl protecting groups with saturated ammonia in methanol, followed by reaction of the diol (**11**) with *p*-toluenesulfonyl chloride,

Scheme 3[a]

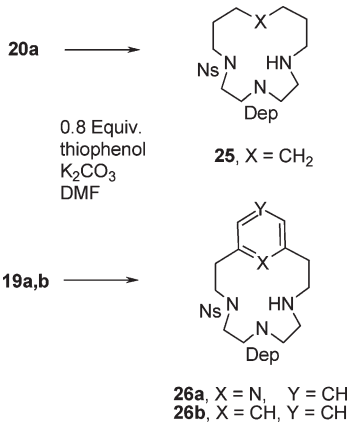


[a] Reagents: (a) Ns-Cl, Et_3N, CH_2Cl_2; (b) Cs_2CO_3, DMF, 80 °C; (c) HBr(g), AcOH, room temp.

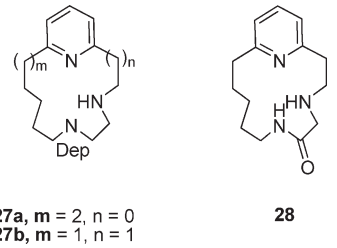
gave the desired bis-electrophile **12** containing a single fluorine group.

The selectively protected azamacrocyclic rings were prepared via directed combinatorial macrocyclization of bis-2-nitrobenzenesulfonamides[48] (Ns) (**15a**−**c**, **16a**−**c**, **18**) with bis-electrophiles (**7**, **12**, **17**) using previously optimized conditions[28] (Scheme 3). To incorporate a phenyl or heterocyclic ring into the macrocycle, the corresponding bis-2-nitrobenzenesulfonamide (**15a**−**c**) was prepared from the bis-aminoethyl intermediates[28] (**13a**−**c**) by reaction with nosyl chloride (Et_3N, CH_2Cl_2). Similarly, **16a**,**b** were obtained by reaction of commercially available intermediates **14a**,**b** with nosyl chloride or in the case of **16c** (X = S) by reduction of 3,3′-thiodipropionitrile with $BH_3 \cdot Me_2S$ and reaction of the intermediate diamine (**14c**) with nosyl chloride to give **16c**. Macrocyclization was accomplished by dropwise addition of a DMF solution of the bis-electrophile to a DMF solution of the bis-2-nitrobenzenesulfonamide containing Cs_2CO_3 maintained at a temperature of 80 °C. Standard workup, followed by purification of the crude product by column chromatography on silica gel, gave the desired macrocycles **19a**−**c**, **20a**−**c**, and **21a**,**b** in yields of 19−55%. Reaction of the

Scheme 4



Scheme 5

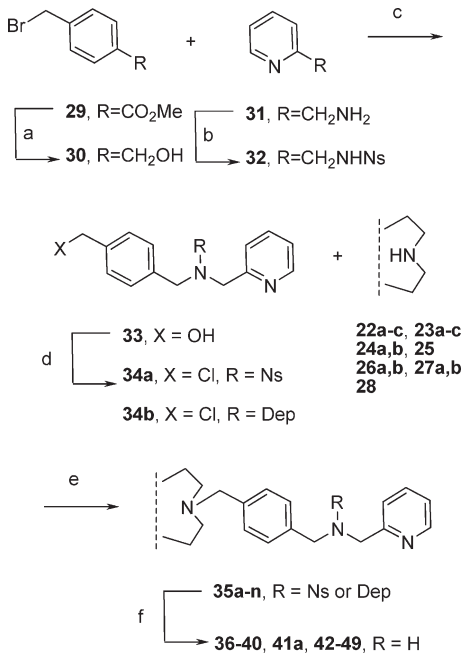


intermediates from above with HBr/acetic acid at room temperature gave **22a**−**c**, **23a**−**c**, and **24a**,**b**, respectively.

Because of synthetic convenience, we also prepared the selectively protected "isomers" of **22a**,**b** and **23a** in which the alternative secondary amine was available for the alkylation reaction. We reasoned that reaction of **19a**,**b** and **20a** with approximately 1 equiv of thiophenol[49] (our reagent of choice for nosyl deprotections) may allow pseudoselective deprotection of a single nosyl group, leaving the Dep group intact. After some optimization, we found that reaction of **19a**,**b** and **20a** with 0.8 equiv of thiophenol and potassium carbonate in DMF (or acetonitrile) gave the precursors **25** and **26a**,**b** in manageable, albeit modest yields (20−50%) following column purification on silica gel (Scheme 4). Finally, the intermediates **27a**,**b** and **28** (Scheme 5) were synthesized as recently described by palladium(0) catalyzed coupling of organozinc iodide reagents with bromopyridines.[50]

Having completed the series of selectively protected azamacrocycles, we proceeded to completion of the desired analogues by straightforward installation of the right-hand portion containing the aminomethyl pyridine moiety. As shown in Scheme 6, this was accomplished in all cases by direct alkylation of the available secondary amine of the macrocycle with the benzylic chlorides **34a**,**b**. Intermediate **34a** was prepared in four steps from 4-bromomethyl benzoic acid methyl ester (**29**) and 2-aminomethylpyridine (**31**): conversion of **31** to the 2-nitrobenzenesulfonamide **32**, followed by alkylation with the benzyl bromide **30** (obtained by reduction of **29** with DIBAL-H) gave the desired alcohol **33**. As previously reported,[28] reaction of benzylic alcohols such as **33** with methanesulfonyl chloride gave the chloride **34a** rather than the corresponding mesylate, presumably via in situ nucleophilic substitution of the initially formed mesylate with chloride. Intermediate **34b** (Scheme 6) containing a Dep-protecting group was prepared by an alternative synthesis

Scheme 6[a]



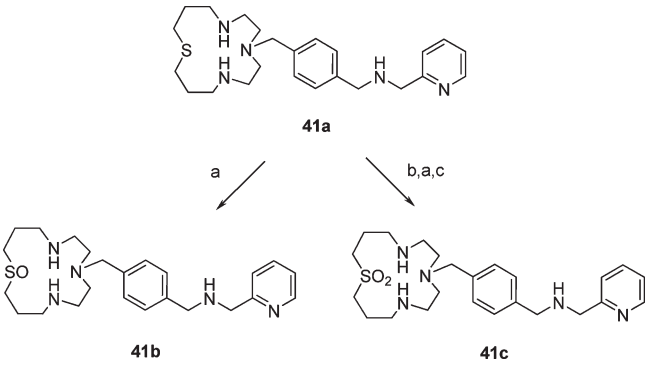
[a] Reagents: (a) DIBAL-H, CH_2Cl_2; (b) Ns-Cl, Et_3N, CH_2Cl_2; (c) K_2CO_3, CH_3CN, 60 °C; (d) Ms-Cl, Et_3N, CH_2Cl_2; (e) K_2CO_3, CH_3CN, 80 °C; (f) R = Ns: thiophenol, K_2CO_3, DMF, or R = Dep: HBr(g), AcOH, room temp.

(procedures in Supporting Information). Alkylation of the available secondary amine of the macrocycles with **34a** (or **34b**) in CH_3CN in the presence of K_2CO_3 gave the penultimate intermediates **35a**−**n**. Deprotection of the nosyl groups with thiophenol and K_2CO_3 in DMF gave the free base of the desired analogues, which in the vast majority of cases were converted to the corresponding hydrobromide salts. For analogues derived from the macrocyclic precursors **25** and **26a**,**b**, the intermediates isolated prior to the deprotection also contained a residual Dep group in addition to nosyl groups. For compound **45**, we found that conversion to the hydrobromide salt using a saturated solution of HBr in acetic acid resulted in concomitant deprotection of the remaining Dep group to obtain compound **45**. For compounds **44** and **46**, the residual Dep group was removed prior to nosyl deprotection and salt formation.

The thioether analogue **41a** was also used to prepare the corresponding sulfoxide and sulfone analogues for antiviral evaluation as shown in Scheme 7. Initially, we globally protected the amino groups of **41a** with Boc and subjected this intermediate to oxidation with oxone in MeOH[51] at −10 °C to give a mixture of the sulfoxide and sulfone that were separated by column chromatography on silica gel. However, while deprotection of the Boc groups with simultaneous conversion to the hydrobromide salt proceeded without incident for the sulfone (to give **41c**), we found that deprotection of the corresponding sulfoxide led to substantial reduction and hence recovery of the starting analogue **41a**. To overcome this problem, the sulfoxide was synthesized by direct oxidation of **41a** with 1 equiv of oxone in MeOH to give **41b** in a 21% isolated yield and was subsequently tested as the free base in antiviral assays.

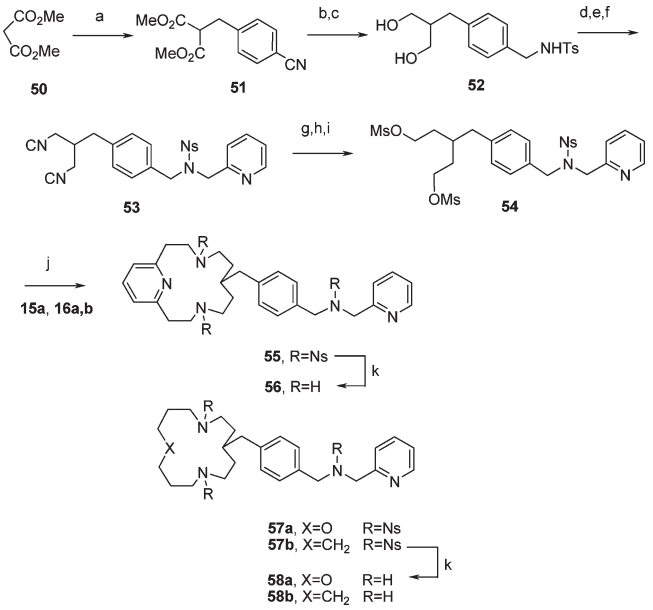
Finally, we prepared a short series of analogues containing a carbon atom in place of a tertiary nitrogen group at the ring junction. To economize on the number of synthetic steps, we

Scheme 7[a]



[a] Reagents: (a) oxone, MeOH, −10 °C; (b) $(Boc)_2O$, THF; (c) HBr(g), AcOH, room temp.

Scheme 8[a]



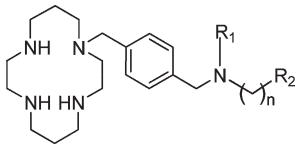
[a] Reagents: (a) NaH, α-bromo-tolunitrile, THF; (b) $LiAlH_4$, THF; (c) Ns-Cl, Et_3N, CH_2Cl_2; (d) 2-picolyl chloride, Et_3N, K_2CO_3, KBr, CH_3CN, reflux; (e) Ms-Cl, Et_3N, CH_2Cl_2; (f) cetyltrimethyammonium bromide, NaCN, benzene, H_2O, reflux; (g) conc HCl/AcOH (4:1), reflux; (h) $BH_3.Me_2S$, THF; (i) Ms-Cl, Et_3N, CH_2Cl_2; (j) Cs_2CO_3, DMF, 80 °C; (k) thiophenol, K_2CO_3, CH_3CN (or DMF), 40 °C.

elected to synthesize the dimesylate **54** (Scheme 8), an intermediate that could be commonly used for the synthesis of multiple analogues via macrocylization with the bis-2-nitrobenzenesulfonamide precursors already in our possession (namely **15a**, **16a**,**b** from Scheme 3). Intermediate **54** was prepared from the commercially available starting material bromo-*p*-tolunitrile via a double one-carbon homologation of the malonate **51**, followed by derivatization to gave the requisite bis-methanesulfonate **54**. Macrocyclizations of **54** with bis-sulfonamides **15a** and **16a**,**b** were performed as described above. Deprotection of the nosyl groups followed by conversion to the corresponding hydrobromide salts gave analogues **56** and **58a**,**b**.

Discussion

Having previously established the optimum ring size and distance between the amines of both aliphatic and

Table 1. Antiviral Activity of Single Ring Azamacrocycles



	n	R_1	R_2	HIV-1(III_B) EC_{50} (μM)	MT-4 cells CC_{50} (μM)
3a	1	H	Ph	0.491	160
3b	1	H	2-amino-Ph	1.825	24
3c	1	H	4-amino-Ph	0.717	227
3d	1	H	2-pyridine	0.009	>112
3e	1	H	3-pyridine	8.470	37
3f	1	H	4-pyridine	9.977	>279
3g	1	Me	2-pyridine	0.416	38
3h	2	H	2-pyridine	49.135	>110
3I	1	H	5-Me-pyrazine	1.895	78
1				0.004	>421

pyridine-fused bis-tetraazamacrocycles required for potent X4 anti-HIV activity, we designed a series of compounds to address the question of structural redundancy. The prototype bis-macrocycle **1** has a center of symmetry and contains eight amino groups, of which four are positively charged at physiological pH. In the current study, we aimed to answer two specific questions: (1) Are all four positive charges required for potent anti-HIV activity? (2) On a per ring basis, what are the minimum structural requirements for activity?

Assuming that the structural requirements are not identical for both rings of **1**, we reasoned that the simplest replacement for a single tetraaza-macrocyclic ring would be a pseudo diamine-segment, representing the first two amino groups of the macrocyclic ring from the point of attachment at the benzylic position. A judicious choice of "diamine" would also reduce the overall charge to +1. Having previously established that the optimum distance between the first two amino groups was a two-carbon unit, we prepared a series of aminomethyl-substituted analogues in which the second amino group was a substituent upon an aromatic ring or part of a heterocyclic ring. In either case, the second pK_a would be sufficiently low to prevent a second protonation at physiological pH. The compounds were tested for their ability to inhibit replication of HIV-1 III_B in MT-4 cells, a strain of HIV-1 that uses exclusively CXCR4 for fusion and viral entry into target cells. The results are shown in Table 1.

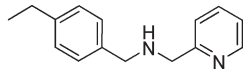
Compared to **1**, the introduction of a benzylamine group (**3a**) in place of the azamacrocyclic ring substantially reduced anti-HIV potency, although the compound remained active at submicromolar concentrations. The concentration of **3a** required to inhibit HIV-1 replication by 50% (the EC_{50}) was 0.49 μM, which was approximately 100-fold higher than the 50% inhibitory concentration of **1**. Aromatic amino groups at the 2-position (**3b**) or 4-position (**3c**) did not affect antiviral potency. Both **3b**,**c** exhibited comparable EC_{50}'s to the unsubstituted benzyl group (**3a**). However, we observed a substantial increase in anti-HIV potency when the benzyl group was replaced by a pyridyl group (**3d**). Compound **3d** exhibited a 50% inhibitory concentration of 0.009 μM, which was only ca. 2-fold higher than the EC_{50} of **1**. Furthermore, the 50% cytotoxic concentration (CC_{50}) of compound **3d** in MT-4 cells was greater than 112 μM. Thus **3d** exhibits a selectivity index of greater than 12000.

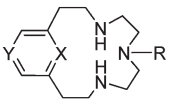
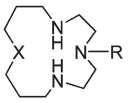
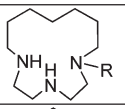
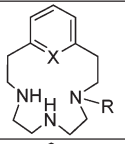
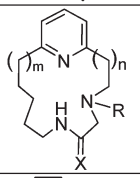
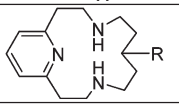
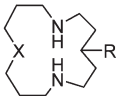
The positional specificity of the pyridine-*N* in **3d** was also examined. Replacement of the 2-pyridyl group with the 3-pyridyl (**3e**) or 4-pyridyl (**3f**) group had a detrimental effect on anti-HIV potency. For example, the EC_{50}'s of analogues **3e**,**f** were approximately 3 orders of magnitude higher than the concentration of **3d** required to inhibit HIV-1 replication by 50% (the EC_{50}'s of **3e** and **3f** were 8.470 and 9.977 μM, respectively). Methylation of the amine in **3d** (to give **3g**) or extension of the connectivity to an aminoethyl pyridine group (to give **3h**) also adversely affected the anti-HIV potency. Finally, we replaced the pyridine moiety with a comparable heterocycle of lower pK_a than pyridine, namely the pyrazine group (**3i**). Perhaps not surprisingly, the antiviral potency of analogue **3i** was approximately comparable to the benzyl analogue **3a**, which did not contain a vicinal heterocycle nitrogen atom.

With the optimized "right-hand" replacement for the azamacrocycle ring of **1** fixed as the 2-aminomethyl pyridine group, we then turned our attention to the "left-hand" ring. Needless to say, the mandatory synthesis of the symmetrical analogue in which both rings were replaced by a 2-aminomethyl pyridine group turned out to be a predictably fruitless exercise (EC_{50} was >250 μM, data not shown). We therefore focused on systematically replacing individual amine groups of the left ring. As shown in Table 2, we first prepared an analogue in which the [14]aneN_4 (cyclam) ring had been replaced by the optimized and equally suitable, py[*iso*-14]-aneN_4 ring (to give compound **36**). Consistent with the structure−activity relationship of py[*iso*-14]aneN_4 bis-azamacrocycles, compound **36** proved to be a potent inhibitor of HIV-1 replication, exhibiting an EC_{50} of 0.001 μM, that is, around 9-fold and 4-fold lower, respectively, than the concentration of **3d** or **1** required to inhibit viral replication by 50%. Although the pyridine-*N* of the macrocyclic ring in **36** was previously found to be critical for high antiviral potency, we reasoned that a precise determination of the pyridine-*N* contribution to potency could help redesign a less basic mimic. Compounds **37** and **38** were then prepared to answer this question. Both analogues **37**, containing a phenyl replacement and **38**, containing an "exocyclic" pyridine fused group, retained reasonable anti-HIV potency (the EC_{50}'s of **37** and **38** were 0.040 and 0.104 μM, respectively) but were at least 40- to 100-fold less potent than analogue **36**. So what role does the pyridine group play?

At physiological pH, the overall charge of the py[*iso*-14]-aneN_4 ring in **36** is also +2 (in a similar manner to cyclam[52]) and the likely protonation sequence is indicated in Figure 1A, based on the sequence reported by Delgado et al.[53] for similar 14-membered tetraazamacrocyclic rings containing pyridine. Presumably, the secondary amino groups are predominantly protonated and the overall structure is stabilized by intramolecular hydrogen bond interactions from the adjacent hydrogen-bond acceptors, the pyridine and tertiary benzylic amine groups (while minimizing the electrostatic repulsion of two positive charges in a confined macrocyclic ring). This is confirmed by a conformational analysis of **36** on B3LYP/6-31G* level followed by single point energy calculations. In the energetically most stable ring conformation (LMP2/6-311+G* + ZPE), the pyridine nitrogen forms two six-membered intramolecular hydrogen bond interactions with the two adjacent protonated nitrogens as shown in Figure 2. Potential five-membered intramolecular hydrogen bond interactions are formed with the tertiary amine.

Table 2. Antiviral Activity of Single Ring Azamacrocycles

R = 

Compound#	Ring Structure	X	Y	HIV-1(III_B) EC_{50} (μM)	MT-4 cells CC_{50} (μM)
36		N	CH	0.001	36
37		CH	CH	0.040	58
38		CH	N	0.104	>209
39		CH_2		0.043	>116
40		O		0.004	>122
41a		S		0.013	>122
41b		SO		0.485	79
41c		SO_2		11.878	69
42		CF_2		0.920	56
43		CHF		1.239	143
44		CH_2		11.131	76
45		N		0.063	>103
46		CH		14.106	91
47		H_2	n=0 m=2	4.561	84
48		H_2	n=1 m=1	1.245	41
49		O	n=1 m=1	126.401	>126
56				0.217	48
58a		O		0.426	>125
58b		CH_2		2.185	>164

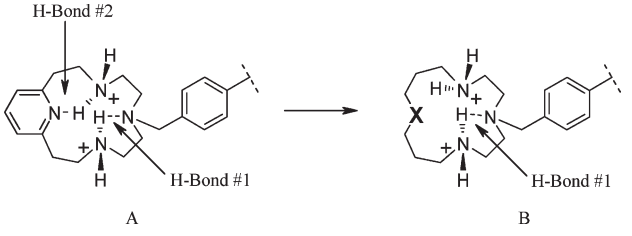


Figure 1. Proposed hydrogen-bond structure of protonated azamacrocycles.

The stabilization provided by this "shared" protonated structure could account for the high basicity of azamacrocyclic rings, as suggested by Kimura et al.[54] It did not seem unreasonable, therefore, that a potential role of the pyridine group is the contribution of a single intramolecular hydrogen-bond, which locks the conformation of the protonated azamacrocyclic ring in manner that is beneficial to antiviral potency. To test this hypothesis, we prepared a series of analogues (depicted in Figure 1B, data in Table 2) in which the fused aromatic group had been removed and replaced by an aliphatic group, in some cases containing a hydrogen-bond acceptor at the key position "x," the position occupied by the pyridine nitrogen in compound **36**.

Consistent with the hydrogen-bonding hypothesis, the alkyl analogue **39** exhibited an anti-HIV potency that was comparable to the phenyl and exocyclic pyridine analogues **37** and **38** (the EC_{50}'s of **37** and **39**, were 0.040 and 0.043 μM, respectively). This result categorically rules out the possibility that the conformational restrictions imposed by the fused aromatic groups in compounds **37**, **38** were even partially responsible for the high potency of **36**. However, incorporation of a hydrogen-bond acceptor at position x (Figure 1B) in some cases restored activity comparable to **36**. For example, the oxygen analogue **40** exhibited an EC_{50} that was only 4-fold higher than the concentration of **36** required to inhibit HIV-1 replication by 50% (the EC_{50} of **40** was 0.004 μM). The corresponding thioether analogue **41a** exhibited an EC_{50} of 0.013 μM, which is approximately 3-fold higher than compound **40**. Although the antiviral potency of the thioether analogue **41a** compared to the ether analogue **41** is greater than one would predict from the strength of the hydrogen-bond acceptor acceptor capabilities (thioether groups are considerably weaker H-bond acceptors than the oxygen in

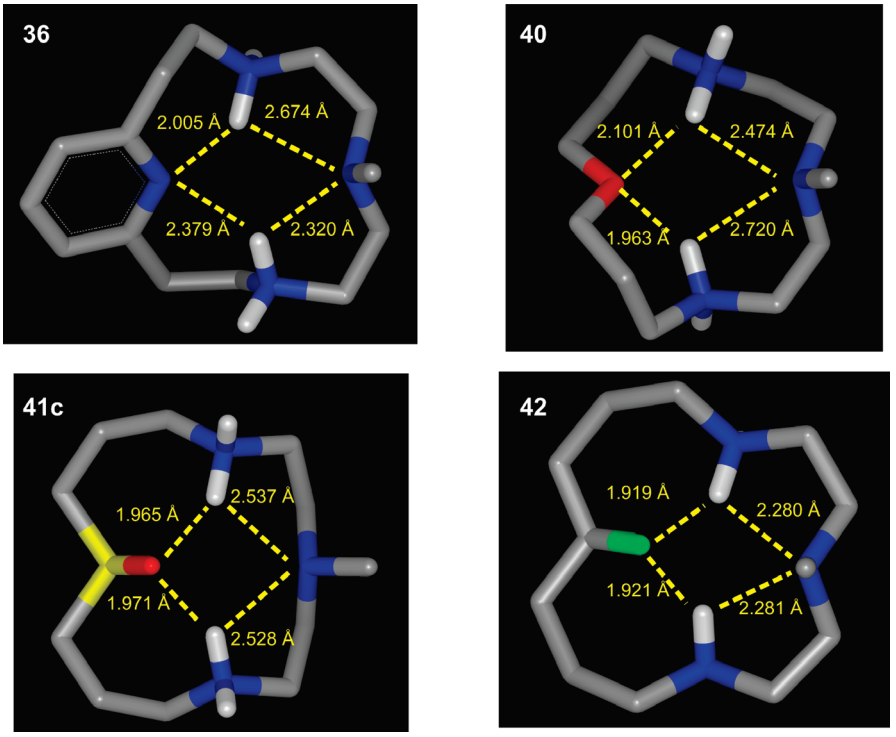


Figure 2. Lowest energy conformations of compounds **36**, **40**, **41c**, and **42**. View from top on a plane defined by three nitrogens and X (see Figure 1). Dashed lines indicate hydrogen bond interactions: the hydrogen bond acceptors in **36** and **40** are in one plane with the three nitrogens. This is not the case for **41c** and **42**. Bond angles: **36**: ∠(N···H−N+) = 140.5°, 122.4°, 102.1°, 108.4°. **40**: ∠(O···H−N+) = 135.1°, 141.5°; ∠(N···H−N+) = 104.6°, 102.8°. **41c**: ∠(O···H−N+) = 112.8°, 112.8°; ∠(N···H−N+) = 108.2°, 108.0°. **42**: ∠(F···H−N+) = 142.2°, 142.2°; ∠(N···H−N+) = 114.7°, 114.7°.

40), this result can be reconciled by considering the nature of the H-bond required; a six-membered intramolecular H-bond constrained by the macrocyclic ring (Figure 2).

With the thioether compound **41a** in hand, we also prepared the sulfoxide (**41b**) and sulfone (**41c**) analogues by direct oxidation of **41a**. We reasoned that the oxygen atoms of the sulfoxide and sulfone are stronger H-bond acceptors than the sulfur atom of **41a** and may consequently improve the anti-HIV potency. However, both **41b** and **41c** were considerably weaker antiviral agents, exhibiting 50% effective concentrations for inhibition of HIV-1 replication that were at least 79-fold higher than the EC_{50} of **41a** (the EC_{50}'s of **41b** and **41c** were 0.485 and 11.878 μM, respectively). The precise reason for the poor antiviral activity exhibited by analogues **41b**,**c** was unclear; although the sulfoxide and sulfone are more sterically demanding than the thioether and could induce a ring conformation that is detrimental to antiviral activity, we could not rule out the possibility that the H-bond acceptor oxygen is now "one-bond" outside of the ring, and the intramolecular H-bond itself induces an unfavorable conformation (a seven-membered ring H-bond in **41b**,**c** (Figure 2) compared to a six-membered in **41a**). To complete this series of compounds therefore, we decided to introduce the fluoro and difluoro substituents at position x (Figure 1B). Several reports have demonstrated that the fluoro group can participate as an acceptor for intramolecular H-bonds, particularly within highly constrained ring structures.[55−57] This is also confirmed by our calculations, as shown in Figure 2. The fluoro (**43**) and difluoro (**42**) analogues were also attractive substituents for two other reasons: (1) the substituents would be situated at the fourth carbon from the adjacent amine group, thereby minimizing the affect on pK_a; (2) in a similar manner to the sulfoxide and sulfone, the H-bond acceptor would be one-bond outside of the macrocyclic ring. However in this case, because the fluorine atom in C−F groups is isostructural with hydrogen, a negative effect of the fluoro substituents on antiviral activity can only be attributed to an inappropriately positioned H-bond rather than steric requirements (that is, in the absence of an H-bond, we would expect the fluoro or difluoro analogues to exhibit an EC_{50} comparable to the methylene analogue **39**). In antiviral testing, the fluoro (**43**) and difluoro (**42**) analogues displayed EC_{50}'s that were greater than 20-fold higher than the methylene analogue **39** (the EC_{50}'s of **39**, **42**, and **43** were 0.043, 0.920, and 1.239 μM, respectively), confirming the negative consequences of an incorrectly positioned hydrogen-bond (Figure 2).

Next, we focused on the sequence of aliphatic amine groups in the macrocyclic ring required for potent antiviral activity. By straightforward synthetic manipulation of our collection of ring systems, we prepared the structural isomers of analogues **36**, **37**, and **39** in which the side-chain (R, in Table 2) was connected to the alternative secondary amine group to give compounds **44**, **45**, and **46**. In antiviral testing, analogue **44** was substantially less potent than its corresponding regioisomer **39**: the EC_{50} of **44** was 11.131 μM, which was approximately 260-fold higher than the EC_{50} of **39**. A similar loss of antiviral potency was observed with the phenyl analogue **46** and its isomer **37** (the EC_{50}'s of **46** and **37** were 14.106 and 0.040 μM, respectively). Interestingly, the loss of antiviral potency with the pyridine-fused isomer **45** compared to **36** was significant but not as substantial; the EC_{50} of **45** was 0.063 μM, around 60-fold higher than the concentration of **36** required to inhibit HIV-1 replication by 50%. There was a possibility, therefore, that while the "tri-aza" ring configuration required for potent antiviral activity is clearly represented

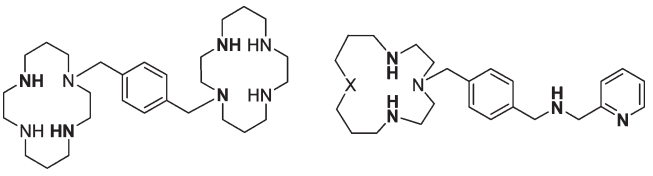
Table 3. Protonation Constants of Selected Azamacrocycles

compd	pK_{a1}	pK_{a2}	pK_{a3}
37	8.89 ± 0.02	7.73 ± 0.02	6.90 ± 0.02
39	9.66 ± 0.02	8.60 ± 0.02	7.53 ± 0.02
41a	9.59 ± 0.02	8.15 ± 0.02	7.34 ± 0.02

by analogues **36**−**43**, selective replacement of a single secondary amine (in **39**) with a fused pyridine group might provide an analogue that displays comparable antiviral potency to **45** (and **39**). This hypothesis was tested via the synthesis of analogues **47**−**49** with mixed results. Compounds **47** and **48** (isomeric 14-membered triaza rings) inhibited replication of HIV-1 but were approximately 70-fold and 20-fold less potent, respectively, than analogue **45**. Consistent with the ring configuration of **45** (a three carbon unit connecting the tertiary amine and pyridine-*N* groups), the optimum configuration was a 4,7,17-triazabicyclo system (**48**, structurally related to **45**) rather than a 3,6,17-triazabicyclo ring (analogue **47**, structurally related to **39**). Compound **48** inhibited HIV-1 replication with an EC_{50} that was 3-fold lower than **47**. Because of synthetic convenience, an analogue of **48** containing the nonbasic amide group (**49**) was also completed for antiviral testing. As expected, removing the positively charged secondary amine group was highly detrimental to antiviral potency (the EC_{50} of **49** was 126.4 μM).

Finally, we prepared a short series of analogues in which the tertiary amine group in analogue **36** (and analogues **39**, **40** in Table 1) connecting the side-chain R to the macrocyclic ring, has been replaced by a carbon (CH) group. Using **56** as an example, one would predict that the loss of a hydrogen-bond acceptor provided by (in this case) the tertiary amine group (H-bond no. 1 in Figure 1A) would lead to a similar reduction in antiviral potency compared to the replacement of the pyridine group in **36** with a phenyl group (to give **37**). Consistent with this analogy, the antiviral activity of **56** was comparable to **37** (the EC_{50}'s were 0.217 and 0.040 μM, respectively) and both compounds were at least 40-fold less potent inhibitors of HIV-1 replication than **36** (EC_{50} = 0.001 μM). Interestingly, replacement of the tertiary amine group in **39** or **40** (to give **58a** and **58b**) led to a substantially greater reduction in antiviral potency: the EC_{50}'s of **58a** and **58b** were ca. 40- to 50-fold higher than the concentration of **39** or **40** required to inhibit viral replication by 50%. Significantly, however, the simple diaza-macrocycle **58b** remained active, exhibiting, albeit, modest antiviral potency (EC_{50} = 2.185 μM). These combined results clearly supported our original pharmacophore hypothesis that (1) the minimum macrocyclic requirements for potent activity are the protonated secondary amine groups in a 14-membered ring and (2) the activity is improved by hydrogen-bond acceptors which presumably lock the ring in a favorable conformation for antiviral activity.

To complete the study and our intitial goals, several analogues were selected for pK_a determinations in an attempt to confirm the overall charge. The results are shown in Table 3. As expected, the compounds in general exhibit two high pK_a's, consistent with the azamacrocyclic literature and therefore most likely due to double protonation of the azamacrocyclic ring. The third pK_a is closer to physiological pH, precluding the absolute assignment of protonation status on the aminomethylpyridine moiety during the HIV inhibitory step. Nevertheless, we can estimate the overal charge of these analogues to be in the range +2 to +3, which compares favorably with **1** (+4).



(**1**)

(Overall Charge +4) (Overall Charge +2 to +3)

Figure 3. Key nitrogen atoms (bold) per ring of **1**, required for potent antiviral activity.

In summary, we have determined the key structural features of **1** required for potent antiviral activity and, in the process, identified several single azamacrocyclic ring structures with comparable or improved antiviral inhibitory potency. As shown in Figure 3, there is considerable structural redundency in **1**: all eight amino groups are not required for activity, the critical amino groups on a per ring basis are nonidentical, and the overall charge at physiological pH can be reduced without compromising potency. These features have been used to design nonmacrocyclic analogues that will be reported in a subsequent manuscript.[41]

Experimental Section

Compound **1** (Mozobil (plerixafor)) is 1,1′-[1,4-phenylenebis-(methylene]-bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride, dihydrate (formula weight = 830.51).

General experimental procedures are provided in refs 26−28. ^1H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively, on a Bruker Avance 300 spectrometer. Electrospray mass spectral analysis was performed on a Bruker Esquire spectrometer. Fast atom bombardment mass spectral analysis was carried out by M-Scan (West Chester, PA). Microanalyses for C, H, N, and halogen were performed by Atlantic Microlabs (Norcross, GA) and were within ±0.4% of theoretical values. Purity was determined by reversed phase HPLC and was ≥95% for all compounds tested.

Preparation of Cyclam Analogues. To a stirred solution of 4,8,11-tris(diethoxyphosphoryl)-1,4,8,11-tetra-azacyclotetradecane (**2a**[26,27]) (6.1 g, 0.01 mol) and K_2CO_3 (1.89 g, 0.013 mol) in CH_3CN (150 mL) was added α,α′-dibromo-*p*-xylene (13.2 g, 0.05 mol) and the reaction mixture stirred at 70 °C for 1 h. The solution was cooled to room temperature and the solvent removed under reduced pressure. The residue was partitioned between brine (50 mL) and CH_2Cl_2 (100 mL). The organic phase was separated, dried (Na_2SO_4), and concentrated to a minimum volume. The solid was filtered off and the solvent evaporated under reduced pressure to give the crude product. Purification by column chromatography on silica gel (25:1 CH_2Cl_2/CH_3OH) gave 1-[1-methylene-4-(bromo-methylene)phenylene]-4,8,11-tris-(diethoxyphosphoryl-1,4,8,11-tetraazacyclotetra-decane (4.7 g, 59%) (**2b**) as a pale-yellow oil. ^1H NMR ($CDCl_3$) δ 1.21−1.37 (m, 18H), 1.66−1.74 (m, 2H), 1.82−1.91 (m, 2H), 2.30−2.35 (m, 2H), 2.58−2.63 (m, 2H), 2.99−3.16 (m, 12H), 3.48 (s, 2H), 3.95−4.07 (m, 12H), 4.48 (s, 2H), 7.21−7.35 (4H).

To a solution of the appropriate amine (5.0 equiv) in dry CH_3CN (5 mL) containing a suspension of K_2CO_3 (1.5 equiv) at 80 °C was added dropwise with stirring a solution of 1-[1-methylene-4-(bromomethylene)phenylene]-4,8,11-tris(diethoxyphosphoryl-1,4,8,11-tetraazacyclotetradecane (**2b**) (0.6 mmol) in CH_3CN (10 mL) over 15−20 min. After stirring for a further 1 h at 80 °C, the solution was concentrated to dryness and the residue was partitioned between CH_2Cl_2 and water. The organic layer was separated and washed with water (3×) and then dried ($MgSO_4$) and evaporated. The crude residue was purified by

column chromatography on silica gel eluting with 5−15% $MeOH/CH_2Cl_2$ to afford a viscous oil.

To a stirred solution of the protected cyclam derivative from above (0.1−0.5 mmol) in acetic acid (3 mL) was added a saturated solution of HBr(g) in acetic acid (5 mL) and the solution was stirred at room temperature for 14 h. The resulting precipitate was collected by filtration and washed with acetic acid then Et_2O. The solid was then dissolved in H_2O (3 mL) and treated with charcoal (100 mg) and the mixture was heated to 80 °C for 30 min. The hot solution was filtered through celite and the filtrate was concentrated to approximately 1 mL, after which acetic acid was added, resulting in the immediate formation of a white precipitate. The white solid was collected by filtration and dried in vacuo.

Compounds **3a**−**i** were prepared by these methods.

***N*-[1,4,8,11-Tetraazacyclotetradecanyl-1,4-phenylenebis(methylene)]-2-(amino-methyl)pyridine hexahydrobromide (3d).** White solid: mp 200−205 °C (dec). ^{1}H NMR (D_2O) δ 2.04 (m, 4H), 3.20−3.40 (m, 8H), 3.40−3.60 (m, 8H), 4.34 (s, 2H), 4.38 (s, 2H), 4.51 (s, 2H), 7.50 (m, 4H), 7.75 (t, 1H, J = 6.6 Hz), 7.82 (d, 1H, J = 7.9 Hz), 8.26 (t, 1H, J = 7.9 Hz), 8.63 (d, 1H, J = 5.3 Hz). ^{13}C NMR (D_2O) δ 18.30, 18.96, 37.04, 37.28, 37.40, 40.92, 41.13, 41.49, 44.26, 47.61, 48.01, 51.29, 58.88, 127.46, 127.75, 130.40, 131.05, 131.23, 131.47, 132.10, 132.44, 144.95, 145.81, 146.01. FAB MS m/z 493 (M + H^{81}Br, 7), 491 (M + H^{79}Br, 7), 411 (M + H, 100). Anal. ($C_{24}H_{38}N_6 \cdot 6HBr$) C, H, N, Br.

General Procedure A: Macrocyclization. To a stirred solution of the requisite bis-nitrobenzenesulfonamide and anhydrous Cs_2CO_3 (2.5 equiv) in DMF (50 mL of DMF per mmol of bis-nitrobenzenesulfonamide) maintained at 80 °C under N_2 was added a solution of the bis-electrophile (1.0−1.5 equiv) in DMF (5 mL of DMF per mmol of bis-electrophile), dropwise over 10 h. The reaction mixture was allowed to stir at 80 °C for a further 30 h and then cooled to room temperature and concentrated in vacuo. The residue was partitioned between EtOAc and water, and the organic layer was separated, washed with satd $NaHCO_3$ and then brine and dried over $MgSO_4$ or Na_2SO_4. Evaporation of the solvent and purification of the residue by column chromatography on silica gel (conditions indicated) gave the desired Dep-protected macrocycle.

General Procedure B: Deprotection of the Diethoxyphosphoryl (Dep) group. To a stirred solution of the Dep-protected macrocycle in acetic acid (ca. 2.5 mL of acetic acid per mmol of Dep-macrocycle) was added a freshly prepared solution of saturated HBr(g) in acetic acid (10 mL per mmol of Dep-macrocycle), and the resulting homogeneous solution was stirred at room temperature for a further 22 h. Addition of diethyl ether (125 mL per mmol of macrocycle) to the reaction mixture gave a precipitate that was allowed to settle to the bottom of the flask, and the supernatant solution was decanted. The precipitate was washed with ether by decantation (repeated 3×), and the residue was then partitioned between CH_2Cl_2 and 1N aq NaOH. The separated aqueous layer was extracted with CH_2Cl_2 (2×), and the combined organic extracts were washed with brine and then dried ($MgSO_4$ or Na_2SO_4) and concentrated in vacuo. The macrocycle was purified by column chromatography on silica gel or used directly without further purification in the next step.

General Procedure C: Alkylation of the Macrocycle with *N*-[1-Methylene-4-(chloromethylene)phenylene]-*N*-(2-nitrobenzenesulfonyl)-2-(aminomethyl) pyridine. To a stirred solution of the macrocycle and anhydrous K_2CO_3 (5.0 equiv) in anhydrous CH_3CN (10−15 mL per mmol of macrocycle) under N_2 was added *N*-[1-methylene-4-(chloromethylene)phenylene]-*N*-(2-nitrobenzenesulfonyl)-2-(aminomethyl)pyridine (**34a**) (1.0−3.0 equiv), and the reaction mixture was allowed to stir at 80 °C for 18 h and then concentrated in vacuo. The residue was partitioned between EtOAc and water, and the organic layer was separated, washed with satd $NaHCO_3$ and then brine and dried over $MgSO_4$ or Na_2SO_4. Evaporation of the solvent and purification of the residue by column chromatography on silica gel gave the fully Ns-protected product.

General Procedure D: Deprotection of the 2-Nitrobenzenesulfonyl (Ns) Groups. To a stirred solution of the intermediate from the above procedure and anhydrous K_2CO_3 (3.0−4.0 equiv per Ns group) in anhydrous DMF (12 mL per mmol of intermediate) under N_2 was added dropwise, thiophenol (1.0−2.5 equiv per Ns group). The reaction mixture was allowed to stir at room temperature for a further 4 h and then concentrated in vacuo. The residue was partitioned between EtOAc and water, and the organic layer was separated, washed with satd $NaHCO_3$ and then brine and dried over $MgSO_4$ or Na_2SO_4. Evaporation of the solvent and purification of the residue by column chromatography on silica gel or alumina gave the desired product as the free base.

General Procedure E: Conversion to the Hydrobromide Salt. The free base was dissolved in MeOH (15 mL per mmol of free base), and a freshly prepared solution of saturated HBr(g) in MeOH (35 mL per mmol of free base) was added giving a precipitate. The mixture was stirred for 5 min, and diethyl ether was added (50 mL per mmol of free base). The solid was allowed to settle to the bottom of the flask, and the supernatant solution decanted. The solid was washed by decantation with MeOH (5×) and then ether (10×), and the last traces of ether were removed by evaporation in vacuo followed by drying in vacuo at 40−50 °C overnight to give the desired product as the hydrobromide salt.

Anti-HIV Activity Assays. Inhibition of HIV-1 (III_B) replication assays were performed as previously described.[26−28] Anti-HIV activity and cytotoxicity measurements were carried out in parallel. They were based on the viability of MT-4 cells that had been infected with HIV in the presence of various concentrations of the test compounds. After the MT-4 cells were allowed to proliferate for 5 days, the number of viable cells was quantified by a tetrazolium-based colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyletetrazolium bromide (MTT) procedure in 96-well microtrays. In all of these assays, viral input (viral multiplicity of infection, MOI) was 0.01, or 100 times the 50% cell culture infective dose ($CCID_{50}$). The EC_{50} was defined as the concentration required to protect 50% of the virus-infected cells against viral cytopathicity. The 50% cytotoxic concentration (CC_{50}) was defined as the compound concentration required to reduce the viability of mock-infected cells by 50%. The greater than symbol (>) is used to indicate the highest concentrations at which the compounds were tested and still found to be non-cytotoxic. Average EC_{50} and CC_{50} values for several separate experiments are presented as defined above. As a rule, the individual values did not deviate by more than 2-fold up or down from the EC_{50} and CC_{50} values indicated in Tables 1 and 2.

Potentiometric Titrations. Aza-macrocylic pK_a determinations were obtained by potentiometric titration in aqueous solution (I = 0.16, NaCl) under an argon atmosphere at 25 °C in the pH range 2.5−11.0. Error limits in Table 3 were estimated from multiple independent titrations.

Computational Details. Three-dimensional conformations for all compounds were obtained with Macromodel 9.7 within Maestro 9.0 using the OPLS 2005 force field.[58] Standard options have been used for all other parameters of the conformational search panel. These geometries were further optimized on B3LYP/6-31G* level of theory. All conformations are local energy minima with 0 imaginary frequencies as identified by B3LYP/6-31G* frequency calculations. The energies of conformations were determined on LMP2/6-311+G* level including zero-point correction energies (ZPE) from frequency calculations. Elimination of redundant low energy conformations provided mostly conformations displaying intramolecular hydrogen bonds resembling the respective lowest energy conformation.[59] Therefore we limited our discussion to this energy conformation. Three-dimensional representations have been generated with Vida 4.0.0.[60]

Supporting Information Available: Experimental procedures and characterization data for the synthesis of intermediate **34b** and compounds **30** through **58b**. Characterization data for compounds **3a**−**i**. This material is available free of charge via the Internet at http://pubs.acs.org.

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