Synthesis and Structure−Activity Relationships of Phenylenebis(methylene)-Linked Bis-azamacrocycles That Inhibit HIV-1 and HIV-2 Replication by Antagonism of the Chemokine Receptor CXCR4

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Bis-tetraazamacrocycles such as the bicyclam AMD3100 are a class of potent and selective anti-HIV-1 and HIV-2 agents that inhibit virus replication by binding to the chemokine receptor CXCR4, the co-receptor for entry of X4 viruses. With the aim of optimizing the anti-HIV-1 and HIV-2 activity of bis-azamacrocycles, a series of analogues were synthesized which contain neutral heteroatom (oxygen, sulfur) or heteroaromatic (of lower pK_a than a secondary amine) replacements for the amino groups of AMD3100. The introduction of one or more heteroatoms such as oxygen or sulfur into the macrocyclic ring of *p*-phenylenebis(methylene)-linked dimers (to give N_3X or N_2X_2 bis-macrocycles) gave analogues with substantially reduced anti-HIV-1 (III_B) and anti-HIV-2 (ROD) potency. In addition, the bis-sulfur analogue was also markedly more cytotoxic to MT-4 cells. However, bis-tetraazamacrocycles featuring a single pyridine group incorporated within the macrocyclic framework exhibited anti-HIV-1 and HIV-2 potency comparable to that of their saturated, aliphatic counterparts. The *p*-phenylenebis(methylene)-linked dimer of the py[14]aneN_4 macrocycle inhibited HIV-1 replication at a 50% effective concentration (EC_{50}) of 0.5 μM while remaining nontoxic to MT-4 cells at concentrations approaching 200 μM. A series of analogues containing macrocyclic heteroaromatic groups of varying pK_a were also synthesized, and their ability to inhibit HIV replication was evaluated. Replacing the pyridine moiety of the py[14]aneN_4 macrocyclic ring with pyrazine or pyridine groups substituted in the 4-position (with electron-withdrawing or -donating groups) either reduced antiviral potency or increased cytotoxicity to MT-4 cells. Finally, we synthesized a series of analogues in which the ring size of the bis-pyridyl macrocycles was varied between 12 and 16 members per ring including the py[*iso*-14]aneN_4 ring system, an isomer of the py-[14]aneN_4 macrocycle. The *p*-phenylenebis(methylene)-linked dimer of the py[*iso*-14]aneN_4 (AMD3329) displayed the highest antiviral activity of the bis-azamacrocyclic analogues reported to date, exhibiting EC_{50}'s against the cytopathic effects of HIV-1 and HIV-2 replication of 0.8 and 1.6 nM, respectively, that is, about 3−5-fold lower than the EC_{50} of AMD3100. AMD3329 also inhibited the binding of a specific CXCR4 mAb and the Ca^{2+} flux induced by SDF-1α, the natural ligand for CXCR4, more potently than AMD3100. Furthermore, AMD3329 also interfered with virus-induced syncytium formation at an EC_{50} of 12 nM.

Introduction

The formally approved chemotherapeutic agents for the treatment of HIV infections such as the reverse transcriptase inhibitors, HIV protease inhibitors, and, more recently, nonnucleoside reverse transcriptase inhibitors interfere with critical steps in the HIV-replicative cycle following release of the viral RNA into the cell.[1] Nevertheless, the identification of potent anti-HIV agents that disrupt early steps in the HIV-replicative cycle preceding the reverse transcription (RT) step remains an important goal to both ameliorate existing therapies and possibly impede the development of drug-resistant strains.

We have previously reported that bis-azamacrocycles such as the bicyclam AMD3100 exhibit potent and selective inhibition of HIV-1 and HIV-2 replication in vitro[2−7] by binding to the chemokine receptor CXCR4,[8−13] the co-receptor utilized by T-tropic (X4) HIV viruses for membrane fusion and subsequent entry of the virus into the cell. Recent reports indicate that antiviral agents targeted at CXCR4 may have several significant therapeutic advantages. For example, CCR5 and CXCR4 were found to be the only physiologically relevant chemokine co-receptors used for viral entry of both laboratory and primary strains of HIV-1 isolated from blood, and progression to X4 using (syncytium-inducing) HIV-1 strains was associated with a more rapid disease course and a faster $CD4^+$ T-cell decline.[14] Furthermore, Verdin et al.[15] and Hesselgesser et al.[16] have observed apoptosis of $CD8^+$ T-cells and neuronal cells, respectively, induced by direct interaction of the HIV-1

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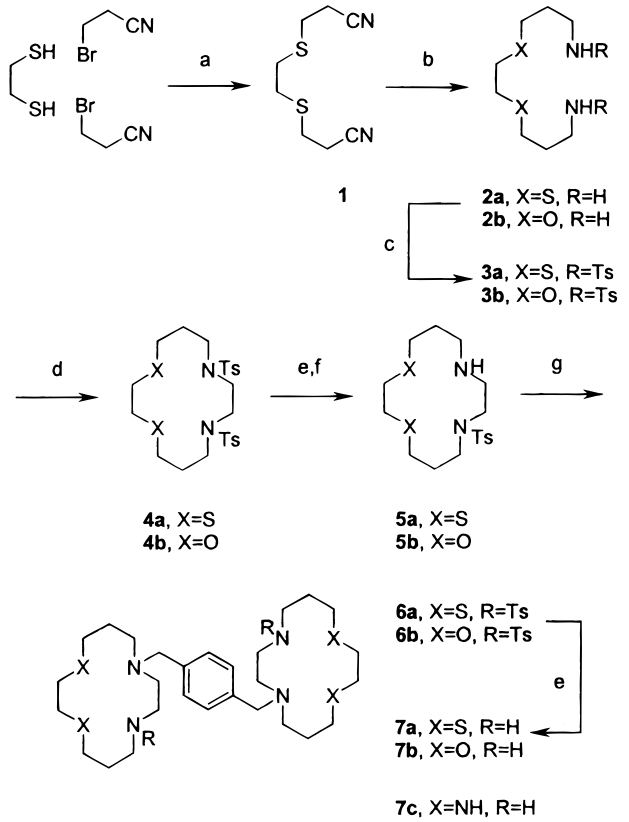
Scheme 1[a]



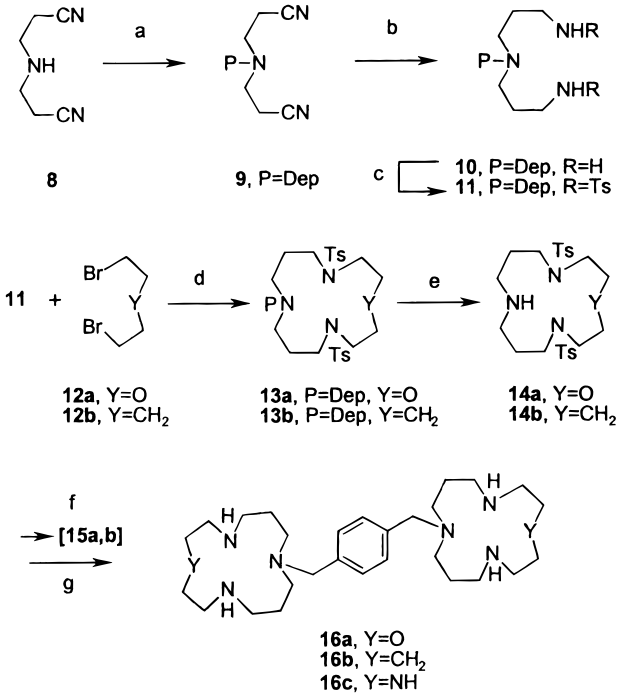
[a] Reagents: (a) Et_3N, CH_2Cl_2, rt; (b) BH_3·THF; (c) Ts-Cl, Et_3N, CH_2Cl_2; (d) ethylene glycol ditosylate, Cs_2CO_3, DMF, 60−70 °C; (e) Na/Hg amalgam; (f) 1.0 equiv Ts-Cl, Et_3N, CH_2Cl_2; (g) 0.5 equiv *p*-dibromoxylene, K_2CO_3, CH_3CN reflux.

envelope glycoprotein gp120 with CXCR4. Thus, CXCR4 antagonists may exhibit potent antiviral activity and concomitant protection of the immune system in vivo.

To demonstrate that inhibition of viral entry into cells is a legitimate target for antiviral chemotherapy of HIV infection, the inhibitory effect of AMD3100 was evaluated in intrathymically HIV-1-infected SCID-hu (Thy/Liv) mice.[17] Once-daily, subcutaneous injections of AMD3100 at nontoxic doses caused a significant reduction in p24 antigen expression, a dose-dependent decrease in viremia, and a protection of the decrease in CD4/CD8 T-cell ratio. Antiviral efficacy was also potentiated by the combined administration of AMD3100 and AZT or ddI. Therefore, AMD3100 exhibits potent antiviral efficacy in vitro and in vivo, clearly establishing inhibition of CXCR4 and virus entry into cells as a target for chemotherapeutic intervention with antiviral agents.

In the present study, we report the results of our continuing efforts to optimize the anti-HIV activity of bis-azamacrocycles. To this end, a series of compounds in which the secondary amine groups of AMD3100 were replaced by neutral heteroatoms or heteroaromatic groups were synthesized and evaluated for their inhibitory effects on HIV-1 and HIV-2 replication in vitro. This approach led to the identification of AMD3329, a bis-tetraazamacrocycle featuring a pyridine group incorporated within the macrocyclic ring, as the most potent inhibitor of HIV-1 and HIV-2 replication among the bis-azamacrocycles reported to date.

Scheme 2[a]



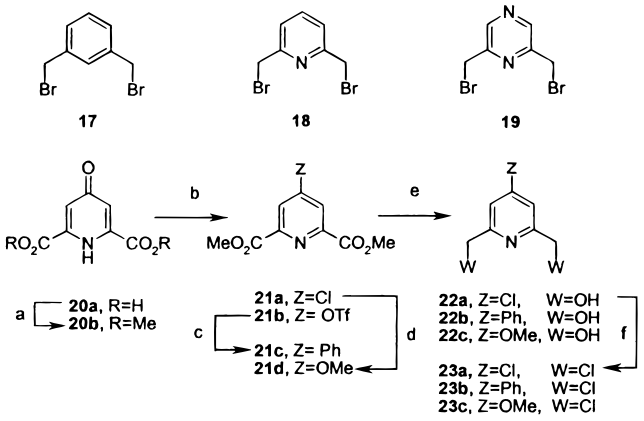
[a] Reagents: (a) $(EtO)_2POCl$, Et_3N, CH_2Cl_2; (b) H_2, Ra Ni, NH_3, MeOH; (c) Ts-Cl, Et_3N, CH_2Cl_2; (d) Cs_2CO_3, DMF, 65−70 °C; (e) HBr, HOAc, rt; (f) 0.5 equiv *p*-dibromoxylene, K_2CO_3, CH_3CN, reflux; (g) 48% aq HBr, HOAc, reflux.

Chemistry

The synthetic route used to prepare phenylenebis-(methylene)-linked [14]aneN_2X_2 dimers is shown in Scheme 1. The left-hand portion of the dithia ring system was prepared by reaction of ethanedithiol and bromopropionitrile in CH_2Cl_2 containing Et_3N to give the dithia-dinitrile **1** in 88% yield. A straightforward reduction of the dinitrile with BH_3·THF and subsequent derivatization with *p*-toluenesulfonyl chloride under standard conditions gave the ditosylate **3a**. Macrocyclization of **3a** was accomplished using our previously established conditions.[4,18] Dropwise addition of a DMF solution of ethyleneglycol ditosylate into a solution of **3a** in DMF containing Cs_2CO_3 gave the fully protected [14]aneN_2S_2 macrocycle **4a** in 42% yield following purification by column chromatography on silica gel. To complete the synthesis of the symmetrical dimer **7a**, the ditosylated macrocycle **4a** was converted to the monotosylated intermediate **5a** by a two-step procedure involving deprotection of the tosyl groups with 3% sodium amalgam followed by monoamine protection. Dimerization of the available secondary amine in **5a** with α,α′-dibromo-*p*-xylene in refluxing CH_3CN in the presence of K_2CO_3, as previously described,[4,5] gave the ditosyl-protected dimer **6a** which was deprotected with 1% sodium amalgam to give the free base of **7a**. Inexplicably, deprotection of **6a** with 3% sodium amalgam led to decomposition. The free base was finally converted to the tetrahydrochloride salt giving **7a**. The corresponding [14]aneN_2O_2 bis-azamacrocycle **7b** was prepared in a manner similar to that for **7a** starting from the commercially available dioxo-diamine **2b**.

Bis-azamacrocycles featuring a single heteroatom substitution per macrocyclic ring (N_3X macrocycles) were synthesized as illustrated in Scheme 2. Due to the

Scheme 3[a]

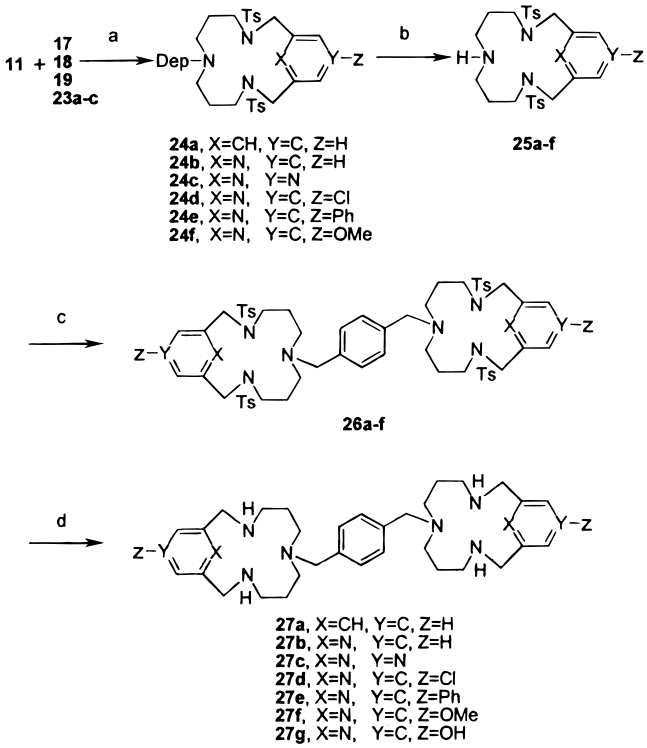


[a] Reagents: (a) MeOH, cat. H_2SO_4; (b) when R = H: PCl_5, $CHCl_3$, reflux then MeOH; or R = Me: Tf_2O, pyridine; (c) $PhB(OH)_2$, K_2PO_3, KBr, $Pd(PPh_3)_4$, dioxane, reflux; (d) NaOMe, MeOH, reflux; (e) $NaBH_4$, EtOH, reflux; (f) Ms-Cl, Et_3N, CH_2Cl_2.

difficulties associated with the synthesis of "unsymmetrical" N_3X macrocycles (for example, construction of the corresponding cyclam ring in which a single aza group has been replaced with a heteroatom), a short series of compounds based on the [*iso*-14]aneN_4 ring system were prepared using a combination of diethoxyphosphoryl (Dep) and *p*-toluenesulfonyl groups.[4] For the synthesis of the bis-[*iso*-14]aneN_3O macrocycle **16a**, iminodipropionitrile (**8**) was chosen as a convenient starting material. Protection of the amino group of **8** with diethyl chlorophosphate in CH_2Cl_2 in the presence of Et_3N gave the Dep-protected dinitrile **9** in 64% yield. Reduction of the nitrile groups by hydrogenation over Raney Ni in a saturated solution of NH_3 in MeOH gave the diamine **10** followed by derivatization of the corresponding amines with *p*-toluenesulfonyl chloride under standard conditions provided the required precursor **11**. Using our standard macrocyclization conditions,[4,18] cyclization of the ditosylate **11** with 2-bromoethyl ether (**12a**) was highly efficient, giving the protected N_3O macrocycle **13a** in a 79% isolated yield following silica gel column purification. To liberate a single secondary amine of known regiochemistry for the impending dimerization reaction, the Dep group in **13a** was subsequently deprotected under conditions in which the tosyl group is stable. Thus, reaction of **13a** with HBr/acetic acid at room temperature for 2−3 h gave the bistosyl macrocycle **14a**. The synthesis of the ditosyl [14]aneN_3 macrocycle **14b** was accomplished in a manner similar to that described for **14a** by replacing 2-bromoethyl ether with 1,5-dibromopentane (**12b**) in the macrocyclization reaction with **11**. The dimerization and final deprotection reactions were performed as illustrated in Scheme 2.

For construction of azamacrocyclic rings containing an aromatic or heteroaromatic group (pyridine or pyrazine) within the macrocyclic framework, the Dep-protected ditosylate **11** was macrocyclized with a series of bis-electrophiles **17**−**19** and **23a**−**c** as shown in Schemes 3 and 4. Compounds **18** and **19** were prepared as previously described,[4,5] and compounds **23a**−**c**, which feature a 4-pyridine substituent, were synthesized from a common starting material, chelidamic acid (**20a**) (Scheme 3). To introduce the 4-chloro substituent, **20a** was reacted with PCl_5 in chloroform at reflux and

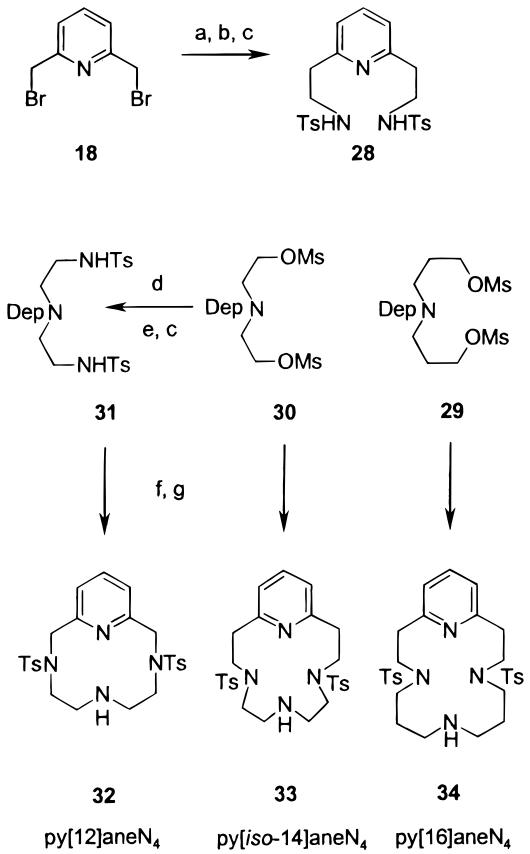
Scheme 4[a]



[a] Reagents: (a) Cs_2CO_3, DMF, 65 °C; (b) HBr, HOAc, rt; (c) 0.5 equiv *p*-dibromoxylene, K_2CO_3, CH_3CN, reflux; (d) 48% aq HBr, HOAc, reflux or concd H_2SO_4, 110 °C.

quenched with methanol to give the corresponding 4-chloropyridine-2,6-dicarboxylate dimethyl ester (**21a**). Reduction of the diester with $NaBH_4$ in refluxing ethanol gave the diol **22a** in 65% yield which was subsequently reacted with methanesulfonyl chloride in CH_2Cl_2 in the presence of Et_3N. Contrary to our expectations, we obtained the bis-chloromethyl intermediate **23a** rather than the desired bis-mesylate under these conditions (by in situ nucleophilic substitution of the initially formed mesylate by chloride), even though the reaction time was relatively short (30 min at 0 °C). Since a bis(chloromethyl)pyridine intermediate is an equally suitable precursor for the macrocyclization reaction, subsequent conversions of diols to bis-electrophiles using this procedure were allowed a minimum reaction time of 30 min to complete the conversion to the respective dichlorides (**23b**,**c**). To prepare the 4-methoxypyridine precursor, the 4-chloropyridine intermediate **21a** was reacted with freshly prepared sodium methoxide in refluxing MeOH to give **21d**. In a similar two-step reduction and chlorination procedure utilized for the preparation of **23a**, the methoxy compound **21d** gave **23c**. Finally, the 4-phenylpyridine intermediate **23b** was also prepared from chelidamic acid (**20a**). Esterification of **20a** with MeOH and catalytic concentrated H_2SO_4 gave the dimethyl ester **20b**. The 4-hydroxy substituent on the pyridine ring was then converted to the corresponding triflate by treatment with Tf_2O in pyridine to give **21b**. Palladium-catalyzed cross-coupling of the triflate **21b** with phenylboronic acid in the presence of K_3PO_4 and KBr introduced the 4-phenyl substituent to give **21c**, which was subsequently converted to the desired bis(chloromethyl) precursor **23b** as previously described.

Scheme 5[a]



[a] Reagents: (a) NaCN, cetyltrimethylammonium bromide, benzene, water, reflux; (b) H_2/Ra Ni, NH_3, MeOH; (c) Ts-Cl, Et_3N, CH_2Cl_2; (d) NaN_3, DMF, 80 °C, 18 h; (e) H_2/Pd/C, EtOAc; (f) Cs_2CO_3, **18** or **28**, DMF, 65 °C; (g) HBr, HOAc, rt.

Macrocycles were constructed by cyclization of **11** with the appropriate bis-electrophile under established conditions affording **24a**−**f** (Scheme 4). Following removal of the Dep group post-macrocyclization (to give **25a**−**f**) and the penultimate step of dimerization with α,α′-dibromo-*p*-xylene, the tosyl-protecting groups of **26a**−**f** were subjected to hydrolytic deprotection to give the desired bis-azamacrocycles **27a**−**g**. However, the 4-methoxypyridine precursor **26f** provided two final products, **27f**,**g**, depending on the choice of conditions used for the final deprotection step. Treatment of **26f** with 48% aqueous HBr and acetic acid at reflux simultaneously cleaved the tosyl and methyl ether groups giving the 4-hydroxypyridine analogue **27g**, whereas reaction of **26f** with concentrated H_2SO_4 at 100−110 °C for 2 h selectively deprotected the tosyl groups in the presence of the methyl ether giving the 4-methoxypyridine analogue **27f**.

Finally, a series of analogues were prepared in which the ring size of the pyridine-containing tetraazamacrocycle was varied between 12 and 16 members per ring, including the py[*iso*-14]aneN$_4$ ring system, an isomer of the py[14]aneN$_4$ macrocycle featured in compound **27b** [compare structure **33** (Scheme 5) to **27b** (Scheme 4)]. This synthetic goal was accomplished by straightforward manipulation of our collection of macrocyclic precursors as illustrated in Scheme 5. For assembly of the protected py[*iso*-14]aneN$_4$ (**33**) and py[16]aneN$_4$ (**34**) rings, the *N*,*N*′-bis-tosyl derivative of 2,6-(2-aminoethyl)-pyridine (**28**) was macrocyclized with the appropriate bis-electrophiles **29** and **30**,[4] respectively, which contain a Dep group targeted for the selective deprotection reaction. The key intermediate **28** was prepared in three steps from 2,6-bis(bromomethyl)pyridine (**18**). Alternatively, the dimesylate **30** can be converted to the corresponding bis-tosylamide **31** and macrocyclized with **18** to give the corresponding py[12]aneN$_4$ macrocyclic ring, which provided **32** on selective removal of the Dep group. The aforementioned macrocyclic intermediates **32**−**34** were used to prepare analogues **35a**,**b**, **36**, and **37** (Table 2, isolated as their octahydrobromide salts) using the appropriately substituted α,α′-dibromo-*p*-xylene intermediate and the procedures shown in Schemes 1, 2, and 4.

Table 1. Anti-HIV Activity Data for Bis-azamacrocycles

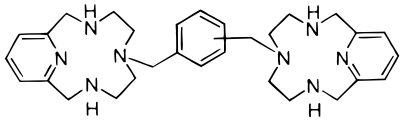
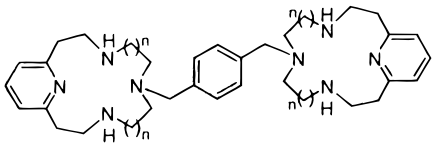
		EC_{50} (μM)[b]		
compd	formula[a]	HIV-1 (III_B)	HIV-2 (ROD)	CC_{50}[c] (μM)
7a	$C_{28}H_{50}N_4S_4 \cdot 4HCl$	>6.8357	>6.8357	6.8
7b	$C_{28}H_{50}N_4O_4 \cdot 4HCl \cdot 2 \cdot 5H_2O$	>363	>363	>363
7c	$C_{28}H_{54}N_8 \cdot 8HCl \cdot 2H_2O$ (AMD3100)	0.0042[d]	0.0059	>421
16a	$C_{28}H_{52}N_6O_2 \cdot 6HBr \cdot H_2O \cdot$ HOAc	4.4919	NT[e]	>244
16b	$C_{30}H_{56}N_6 \cdot 6HBr \cdot 5H_2O$	9.6078	NT	133
16c	$C_{28}H_{54}N_8 \cdot 8HBr \cdot 2H_2O$	0.0337[d]	0.0422	>421
27a	$C_{36}H_{52}N_6 \cdot 6HBr \cdot 2H_2O$	7.9605	NT	133
27b	$C_{34}H_{50}N_8 \cdot 6HBr \cdot 5H_2O$	0.5342	NT	199
27c	$C_{32}H_{48}N_{10} \cdot 7HBr \cdot HOAc$	30.942	20.741	209
27d	$C_{34}H_{48}N_8Cl_2 \cdot 6HBr \cdot HOAc$	0.7509	0.7593	17.8
27e	$C_{46}H_{58}N_8 \cdot 8HBr \cdot HOAc$	1.7547	NT	19.3
27f	$C_{36}H_{54}N_8O_2 \cdot 6HBr \cdot 2H_2O$	3.0979	8.4605	216
27g	$C_{34}H_{50}N_8O_2 \cdot 8HBr$	174.37	199.97	200

[a] Microanalyses were within ±0.4 of theoretical values. [b] 50% antiviral effective concentration. [c] 50% cytotoxic concentration. The greater than symbol (>) is used to indicate the highest concentrations at which the compounds were tested. [d] Anti-HIV-1 (III_B) and anti-HIV-2 (ROD) data from ref 4. [e] NT, not tested.

Biological Results and Discussion

The bis-azamacrocycles reported in Tables 1 and 2 were tested for their inhibitory effects on HIV-1 (III_B) and HIV-2 (ROD) replication in MT-4 cells according to published procedures. These assays are considered representative of inhibition via the co-receptor CXCR4 due to the following observations. First, whereas HIV-1 (III_B) predominantly uses CXCR4 for entry, HIV-2 (ROD) is multitropic and can use CCR3 and CCR5 in addition to CXCR4 to enter cells.[13] However, MT-4 cells are $CD4^+$ and $CXCR4^+$ but do not express detectable levels of CCR3 or CCR5 (D. Schols, unpublished data), and both viral strains are inhibited by SDF-1α, the natural ligand for CXCR4, in MT-4 cells at comparable EC_{50}'s (20−100 ng/mL). Second, while AMD3100 dose-dependently inhibited HIV-1 (III_B) and HIV-2 (ROD) infection of U373MG-CD4 cells stably transfected with the chemokine receptor CXCR4, AMD3100 failed to inhibit infection of cells stably transfected with the chemokine receptors CCR5, CCR3, BOB, and Bonzo and cells transiently expressing US28.[13] These receptors are members of a growing family of chemokine receptors reported to mediate infection by diverse HIV and SIV strains. Finally, we recently reported a mechanism-of-action study on a diverse series of bicyclam analogues,[11] which indicated a close correlation between antiviral potency against X4 strains of HIV-1, inhibition of

Table 2. Anti-HIV Activity Data for Pyridine-Containing Bis-azamacrocycles

compd[a]	structure	EC_{50} (μM)[b] HIV-1 (III_B)	EC_{50} (μM)[b] HIV-2 (ROD)	CC_{50}[c] (μM)
35a, *para*		0.5238	0.1273	8.5
35b, *meta*		0.0971	0.0025	30
36, $n = 1$		0.0008	0.0016	194
37, $n = 2$		0.4213	1.2068	142

[a] All compounds were tested as their octahydrobromide salts (see Experimental Section). Microanalyses were within ±0.4 of theoretical values. [b] 50% antiviral effective concentration. [c] 50% cytotoxic concentration.

binding of the CXCR4-specific monoclonal antibody 12G5 to CXCR4, and inhibition of the intracellular Ca^{2+} signal induced by the natural ligand SDF-1α (stromal cell-derived factor 1α). Thus, it can be concluded that MT-4 cells do not express functional co-receptors that are resistant to inhibition by AMD3100 and that assays which measure viral replication of HIV-1 (III_B) and HIV-2 (ROD) strains in MT-4 cells are representative of inhibition via antagonism of CXCR4.

Compared to the corresponding bis-tetraazamacrocycle **7c** (AMD3100), replacing two aza groups of the macrocyclic ring with heteroatoms such as sulfur or oxygen gave analogues of substantially reduced antiviral potency. For example, both the [14]aneN_2S_2 (**7a**) and [14]aneN_2O_2 (**7b**) bis-macrocycles exhibited 50% effective concentrations (EC_{50}'s) against HIV-1 that were similar to their respective 50% cytotoxic concentrations (CC_{50}'s) in MT-4 cells, thereby negating selectivity for inhibition of virus replication. Furthermore, the introduction of sulfur rather than oxygen heteroatoms significantly affected cytotoxicity: **7a** displayed a greater than 50-fold lower CC_{50} than **7b** (or **7c**).

A second short series of analogues were prepared in which a single amine group of the [*iso*-14]aneN_4 ring system (featured in **16c**, Scheme 2) was replaced by oxygen or a methylene group (to give the [14]aneN_3 bis-macrocycle **16b**). The results are also shown in Table 1. Although the [*iso*-14]aneN_3O bis-macrocycle **16a** exhibited a modest selectivity for inhibition of HIV-1 replication in MT-4 cells [EC_{50} against HIV-1 of 4.4919 μM, CC_{50} of >244 μM, selectivity index (SI) of >54] compared to the [14]aneN_2X_2 bis-macrocycles **7a,b**, its EC_{50} value was only 2-fold lower than that of the methylene analogue **16b**. Moreover, both **16a**,**b** were still significantly less potent than the parent tetraazamacrocyclic dimer **16c**, exhibiting EC_{50}'s against HIV-1 replication that were 2 orders of magnitude higher than the concentration of **16c** required to inhibit HIV-1 replication by 50%. These data clearly indicate that all four amine groups of the tetraazamacrocyclic rings of **7c** or **16c** are required to achieve high antiviral potency.

In view of these results, we reasoned that bis-tetraazamacrocycles featuring a heteroaromatic replacement for a secondary amine of **16c** may also provide analogues that are doubly protonated at physiological pH [in a manner similar to the cyclam rings[5] of **7c** (AMD3100)] but contain a more powerful hydrogen-bond acceptor than the ether oxygen of **16a**. To this end, we prepared a phenylenebis(methylene)-linked dimer of a tetraazamacrocyclic ring containing a pyridine group incorporated within the macrocyclic framework (bis-py-[14]aneN_4, **27b**). Compound **27b** was found to inhibit HIV-1 replication at submicromolar concentrations (EC_{50}: 0.5342 μM) while remaining nontoxic to MT-4 cells at a CC_{50} approaching 200 μM. The corresponding phenyl analogue **27a** was ca. 15-fold less potent against HIV-1 replication than **27b**, thereby confirming the beneficial effect of the pyridine-N in the macrocyclic rings of **27b** on antiviral activity. We further prepared a series of compounds in which the basicity of the pyridine-N was systematically varied by incorporation of substituents in the 4-position of the pyridine ring. Analogue **27d**, containing an electron-withdrawing chloro substituent (that lowers the pK_a and therefore basicity of the pyridine-N), displayed comparable antiviral potency to the unsubstituted pyridine analogue **27b** (exhibiting an EC_{50} against HIV-1 replication of 0.7509 μM) but was ca. 11-fold more cytotoxic to MT-4 cells. However, electron-donating substituents (which increase the basicity of the pyridine-N) had a detrimental effect on antiviral activity. The 4-phenyl and 4-methoxy analogues **27e**,**f** exhibited EC_{50}'s against HIV-1 replication that were approximately 3- and 6-fold higher, respectively, than those of compounds **27b**,**g**. The latter was essentially inactive, exhibiting a EC_{50} against HIV-1 replication that was comparable to its CC_{50} in MT-4 cells. Although we are unable to fully explain these results at the present time, the most likely explanation for the variation in antiviral activity with pyridine basicity is that the pyridine pK_a controls the

Table 3. Data for Inhibition of CXCR4-Specific mAb (12G5) Binding to CXCR4 by Selected Compounds from Tables 1 and 2

compd	12G5 mAb inhibition IC_{50} (μM)[a]	compd	12G5 mAb inhibition IC_{50} (μM)[a]
7a	>35	**27c**	>35
7b	>35	**27d**	5.737
7c[b]	0.007	**27g**	>35
16b	>35	**35a**	4.363
16c	0.067	**36**[c]	0.001

[a] 50% inhibitory concentration. The greater than symbol (>) is used to indicate the highest concentrations at which the compounds were tested. [b] AMD3100. [c] Tested as the octahydrochloride salt.

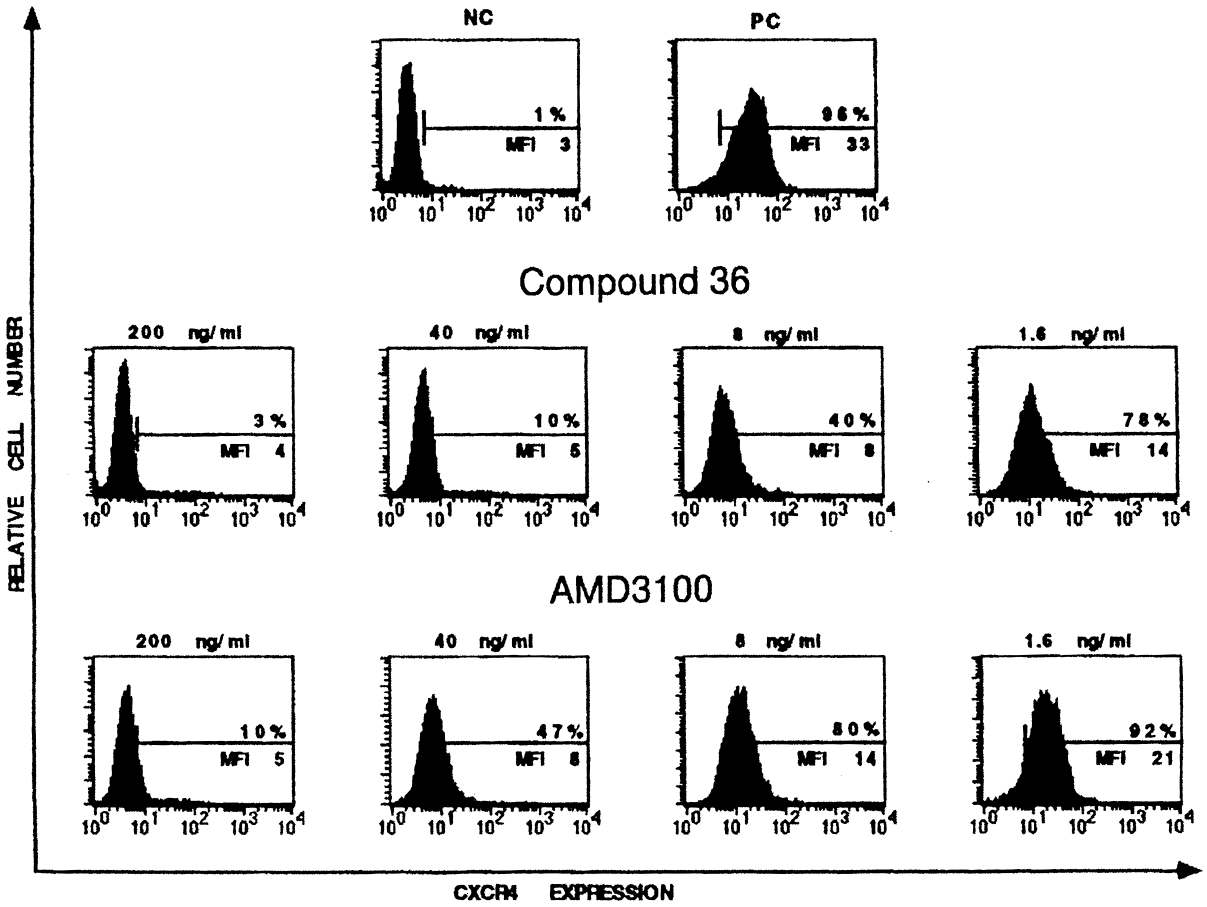


Figure 1. Inhibition of the binding of an anti-CXCR4 mAb (12G5) to SUP-T1 cells in the presence of compound **36** or AMD3100 at different concentrations (200, 40, 8, and 1.6 ng/mL). NC is the isotype control mAb, and PC is the specific staining obtained with 12G5 in the absence of test compound. The percentage of positive cells and the MFI values are indicated in each histogram.

position and degree of protonation on the azamacrocyclic ring.[19] However, the substantial reduction in antiviral activity observed with the hydroxyl analogue **27g** is probably due to dominance of the pyridone (rather than pyridine) tautomeric form under the pH conditions of the assay. Since pyridine analogues of lower basicity (such as the chloro analogue **27d**) exhibited comparable activity to the unsubstituted parent compound **27b**, we decided to incorporate an aromatic heterocycle that is substantially less basic than pyridine into the macrocyclic framework. For this purpose we chose to prepare the pyrazine analogue **27c** (pK_a of pyridine is 5.25 and pK_{a1} of pyrazine is 0.65[5]). Although we expected that **27c** would exhibit (at the very least) an HIV-1 activity profile that was similar to that of analogues **27a**,**b**, we were disappointed to find that the incorporation of a pyrazine group reduced antiviral potency: the EC_{50} of **27c** against HIV-1 was 30.942 μM, a 3- and 58-fold increase in the concentrations of **27a**,**b**, respectively, required to inhibit HIV-1 replication by 50%.

Having established that a pyridine group incorporated into the azamacrocyclic ring has a favorable effect on antiviral activity, we prepared a series of compounds in which the macrocyclic ring contained 12 and 16 ring members (py[12]aneN_4 and py[16]aneN_4). Unlike the structure−activity relationship observed in the series of aliphatic tetraazamacrocyclic compounds,[4] the *p*-phenylenebis(methylene)-linked dimers of the py[12]-aneN_4 (**35a**), py[14]aneN_4 (**27b**), and py[16]aneN_4 (**37**) ring systems exhibited comparable EC_{50}'s for inhibition of HIV-1 (and HIV-2) replication: EC_{50}'s for **35a**, **27b**, and **37** were 0.5238, 0.5342, and 0.4213 μM, respectively. However, compounds **27b** and **37** were significantly less cytotoxic to MT-4 cells than **35a**: the CC_{50} of **35a** (8.5 μM) was approximately 23- and 17-fold lower than that of analogues **27b** and **37**, to give a selectivity index for **35a** against HIV-1 replication of only >16. In contrast, the corresponding *m*-phenylenebis(methylene)-linked dimer of the py[12]aneN_4 macrocycle (**35b**) displayed higher antiviral potency for inhibition of HIV-1 and HIV-2 replication than the corresponding *para*-analogue **35a** (and the activity against HIV-2 was greater than against HIV-1), consistent with the structure−activity relationship of the aliphatic tetraazamacrocycles previously reported.[4] Compound **35b** exhibited EC_{50}'s against HIV-1 (0.0971 μM) and HIV-2 (0.0025 μM) replication that were ca. 5- and 50-fold lower than the concentration of **35a** required to inhibit HIV-1 and HIV-2 replication by 50%. Finally, we synthesized the *p*-phenylenebis(methylene)-linked dimer of the py[*iso*-14]aneN_4 macrocycle (**36**), an isomer of the macrocyclic ring in analogue **27b**. To our surprise, compound **36** exhibited an EC_{50} against HIV-1 replication that was 3 orders of magnitude lower than the EC_{50} of **27b**: EC_{50}'s of **36** against HIV-1 and HIV-2 replication were 0.8 and 1.6 nM; the CC_{50} in MT-4 cells was 199 μM, which gives a selectivity index for **36** against HIV-1 of greater than 2.4×10^5. In addition, **36** inhibited HIV-1 and HIV-2 replication at a 3−5-fold lower concentration than the concentration of AMD3100 (**7c**) required to inhibit viral

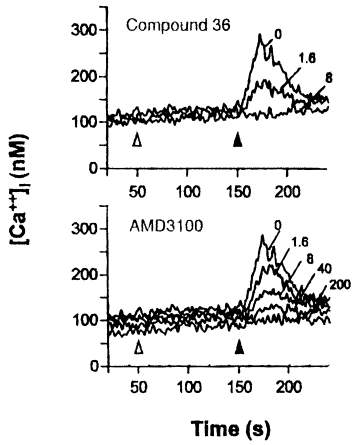


Figure 2. Inhibition of SDF-1α-induced Ca^{2+} flux in SUP-T1 cells by compound **36** (8 and 1.6 ng/mL) and AMD3100 (200, 40, 8, and 1.6 ng/mL). Test compounds or buffer was added at 50 s (△), and SDF-1α was given as a second stimulus at a concentration of 30 ng/mL (▲), 100 s after addition of the compound.

replication by 50%. We also compared **36** and **7c** for their ability to interfere with virus-induced syncytium formation between persistently HIV-1 (III_B)-infected HUT-78 cells and uninfected MOLT-4 cells. In these assays, the concentration of **36** required to inhibit syncytium formation by 50% was 90-fold lower than that of **7c** (AMD3100) (EC_{50}'s for **36** and **7c** were 0.012 and 1.130 μM,[3] respectively).

The effects of the compounds described herein were studied in more detail for their interactions with CXCR4, the main co-receptor for entry of T-tropic HIV strains. In this regard, a group of compounds that exhibited varying EC_{50}'s for HIV inhibition were selected and tested for their ability to inhibit binding of a CXCR4-specific mAb (12G5) to CXCR4 on SUP-T1 cells (Table 3). A strong correlation between inhibition of HIV-1 replication and inhibition of 12G5 binding to CXCR4 was observed. For example, compounds **7c**, **16c** (Table 1), and **36** (Table 2) exhibited EC_{50}'s for inhibition of HIV-1 replication that were comparable to their IC_{50}'s for inhibition of 12G5 binding, whereas compounds that were ineffective antiviral agents did not inhibit 12G5 binding at concentrations exceeding 35 μM. As shown in Figure 1, both **7c** (AMD3100) and **36** (for these experiments, isolated as the octahydrochloride salt) dose-dependently inhibited binding of 12G5 to CXCR4. Compound **36** at 200, 40, 8, and 1.6 ng/mL inhibited 12G5 binding by 97%, 93%, 83%, and 63%, respectively, as calculated from the MFI values, whereas **7c** at 200, 40, 8, and 1.6 ng/mL inhibited 12G5 binding by 94%, 83%, 63%, and 40%, respectively. From these data, the calculated IC_{50} value for **36** was found to be 7-fold lower than the concentration of **7c** required to inhibit 12G5 binding by 50% (IC_{50}'s for **36** and **7c** were 0.001 and 0.007 μM, respectively).

Their effects on SDF-1α-induced Ca^{2+} flux ($[Ca^{2+}]_i$) in SUP-T1 cells were also investigated (Figure 2). Compound **36** completely blocked $[Ca^{2+}]_i$ increases induced by SDF-1α at 8 ng/mL, whereas at this concentration, **7c** partially inhibited (42%) the $[Ca^{2+}]_i$ increase. AMD3100 (**7c**) completely blocked the response at 100 ng/mL[9] (Figure 2). These combined assays clearly demonstrate the more potent interaction of **36** (AMD3329) with CXCR4, consistent with its higher antiviral activity.

In summary, we have prepared a series of bis-azamacrocycles in which selected secondary amines of the bis-tetraazamacrocycle AMD3100 (**7c**) have been replaced by neutral heteroatoms or heteroaromatic groups. This approach to optimizing the anti-HIV activity has led to the identification of AMD3329 (**36**), a *p*-phenylenebis(methylene)-linked dimer of the py[*iso*-14]aneN_4 macrocycle, as the most potent inhibitor of HIV-1 and HIV-2 replication among the bis-azamacrocycles reported to date.

Experimental Section

AMD3100 is 1,1′-[1,4-phenylenebis(methylene]bis(1,4,8,11-tetraazacyclo tetradecane)octahydrochloride, dihydrate[3,4] (formula weight = 830.51).

General experimental procedures are provided in ref 4. ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively, on a Bruker AC300 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.

2,2′-(Ethylenedithio)bis[2-propionitrile] (1). To a solution of 1,2-ethanedithiol (3.8 g, 40.3 mmol) and bromopropionitrile (10.8 g, 80.7 mmol) in 100 mL of CH_2Cl_2 was added triethylamine (12.23 g, 121 mmol) and the resulting white slurry was stirred at room temperature for 4 h. The solution was diluted with CH_2Cl_2 (100 mL), washed with H_2O (2 × 50 mL) and brine (50 mL), dried ($MgSO_4$), and concentrated to give **1** (7.1 g, 88%) as a white solid: ^{1}H NMR ($CDCl_3$) δ 2.62−2.70 (m, 4H), 2.79−2.90 (m, 8H).

1,12-Bis(*p*-toluenesulfonyl)-5,8-dithia-1,12-diazadodecane (3a). To a solution of **1** (7.1 g, 35.5 mmol) in anhydrous THF (100 mL) was added BH_3·THF (1.0 M solution in THF, 180 mL) and the mixture was heated to reflux for 24 h. After cooling, the excess borane was destroyed by addition of MeOH (50 mL) and evaporation (repeated three times) affording a viscous oil (**2a**) which was used without purification in the subsequent step: ^{1}H NMR ($CDCl_3$) δ 1.27 (br s, 4H), 1.72 (quintet, 4H, J = 7.1 Hz), 2.61 (m, 4H), 2.70−2.85 (m, 8H).

To a vigorously stirred solution of the crude diamine **2a** and NaOH (3.55 g, 88.25 mmol) in H_2O (150 mL) was added dropwise a solution of *p*-toluenesulfonyl chloride (13.5 g, 71.0 mmol) in Et_2O (100 mL) and the mixture was stirred for 20 h at room temperature. The solution was diluted with CH_2Cl_2 (300 mL), washed twice with brine (100 mL), dried ($MgSO_4$), and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (EtOAc/hexane, 1:1) to give **3a** (7.44 g, 36%) as a white solid: ^{1}H NMR ($CDCl_3$) δ 1.79 (quintet, 4H, J = 7.1 Hz), 2.42 (s, 6H), 2.61 (t, 4H, J = 7.1 Hz), 2.70 (s, 4H), 3.00−3.09 (m, 4H), 5.19 (t, 2H, J = 6.1 Hz), 7.30 (d, 4H, J = 8.3 Hz), 7.74 (d, 4H, J = 8.3 Hz).

General Procedure A: Macrocyclization. 8,11-Bis(*p*-toluenesulfonyl)-1,4-dithia-8,11-diazacyclotetradecane (4a). To a stirred solution of **3a** (7.44 g, 14.42 mmol) in anhydrous DMF (400 mL) containing cesium carbonate (14.1 g, 43.25 mmol) maintained at 65 °C was added a solution of ethylene glycol bis-*p*-toluenesulfonate (5.33 g, 14.42 mmol) in DMF (100 mL) dropwise over a period of 14−16 h. The reaction mixture was stirred at 65 °C for a total of 24 h and then allowed to cool to room temperature and evaporated in vacuo. The residue was partitioned between CH_2Cl_2 (500 mL) and brine (300 mL) and the aqueous layer was separated and extracted with CH_2Cl_2 (2 × 75 mL). The combined organic phases were dried ($MgSO_4$) and evaporated in vacuo to give the crude product as an orange oil. Purification by column chromatography on silica gel (EtOAc/hexane, 3:7) gave **4a** (3.3 g, 42%) as a white solid: ^{1}H NMR ($CDCl_3$) δ 1.89 (quintet,

4H, $J = 7.1$ Hz), 2.45 (s, 6H), 2.58 (t, 4H, $J = 7.1$ Hz), 2.72 (s, 4H), 3.22 (t, 4H, $J = 7.1$ Hz), 3.27 (s, 4H), 7.33 (d, 4H, $J = 7.9$ Hz), 7.71 (d, 4H, $J = 7.9$ Hz).

General Procedure B: Amalgam Deprotection. 8-(*p*-Toluenesulfonyl)-1,4-dithia-8,11-diazacyclotetradecane (5a). To a stirred solution of **4a** (3.3 g, 6.09 mmol) in a mixture of anhydrous THF (50 mL) and MeOH (50 mL) were added dibasic sodium phosphate (6.2 g, 43.67 mmol) and freshly prepared 3% sodium amalgam (50 g). The reaction mixture was heated to reflux for 44 h then allowed to cool to room temperature and the supernatant solution was decanted from the amalgam and evaporated to dryness. The residue was partitioned between $CHCl_3$ (100 mL) and brine (25 mL), the aqueous layer was separated and extracted with $CHCl_3$ (2 × 75 mL), and the combined organic extracts were dried ($MgSO_4$) and concentrated to give 1,4-dithia-8,11-diazacyclotetradecane (1.4 g, 98%) as a pale yellow oil: ^{1}H NMR ($CDCl_3$) δ 1.78 (m, 4H), 2.68−2.81 (m, 16H); FAB MS *m*/*z* 235 (M + H, 100). This was used without further purification.

The macrocycle from above (1.4 g, 5.98 mmol) was dissolved in anhydrous CH_2Cl_2 (50 mL) containing Et_3N (1.81 g, 17.95 mmol). To this solution was added a solution of *p*-toluenesulfonyl chloride (1.02 g, 5.58 mmol) in CH_2Cl_2 (50 mL) dropwise with stirring over 2 h at 0 °C. The reaction mixture was then allowed to warm to room temperature and stirred a further 2 h. Brine (20 mL) was added to the reaction mixture and the organic layer was separated, dried ($MgSO_4$), and evaporated to dryness to give the crude product as a white solid. Purification by column chromatography on silica gel (CH_2Cl_2/MeOH, 95:5) gave **5a** (1.5 g, 65%) as a white solid: ^{1}H NMR ($CDCl_3$) δ 1.75−1.84 (m, 2H), 1.91−2.04 (m, 2H), 2.43 (s, 3H), 2.62−2.73 (m, 4H), 2.75 (s, 4H), 2.78 (t, 2H, $J = 5.5$ Hz), 2.85 (t, 2H, $J = 5.5$ Hz), 3.12 (m, 4H), 7.31 (d, 2H, $J = 8.3$ Hz), 7.67 (d, 2H, $J = 8.3$ Hz); FAB MS *m*/*z* 389 (M + H, 100), 233 (16).

General Procedure C: Dimerization. 11,11′-[1,4-Phenylenebis(methylene)]bis[8-(*p*-toluenesulfonyl)-1,4-dithia-8,11-diazacyclotetradecane] (6a). To a stirred solution of **5a** (1.5 g, 3.86 mmol) in dry CH_3CN (75 mL) were added α,α′-dibromo-*p*-xylene (510 mg, 1.93 mmol) and potassium carbonate (1.61 g, 11.59 mmol) and the mixture was heated to reflux for 22 h. The reaction mixture was allowed to cool to room temperature then concentrated and the residue was partitioned between CH_2Cl_2 (100 mL) and H_2O (30 mL). The aqueous phase was separated and extracted with two further portions of CH_2Cl_2 (50 mL). The combined organic phases were dried ($MgSO_4$) and evaporated, and the residue was purified by column chromatography on silica gel (EtOAc/hexane, 1:1) to give **6a** (1.25 g, 74%) as a white solid: ^{1}H NMR ($CDCl_3$) δ 1.73 (m, 4H), 1.93 (m, 4H), 2.42 (s, 6H), 2.44−2.53 (m, 8H), 2.57 (t, 4H, $J = 7.3$ Hz), 2.69−2.76 (m, 12H), 3.13−3.27 (m, 8H), 3.56 (s, 4H), 7.23 (s, 4H), 7.27 (d, 4H, $J = 8.3$ Hz), 7.61 (d, 4H, $J = 8.3$ Hz); FAB MS *m*/*z* 879 (M + H, 100), 723 (70), 490 (25).

11,11′-[1,4-Phenylenebis(methylene)]bis[1,4-dithia-8,-11-diazacyclo tetradecane] Tetrahydrochloride (7a). Using general procedure B, deprotection of **6a** (450 mg, 0.51 mmol) with 1% sodium amalgam gave **7a** (free base) (262 mg, 90%) as a white solid: ^{1}H NMR ($CDCl_3$) δ 1.77 (m, 4H), 1.85 (m, 4H), 2.44 (t, 4H, $J = 5.7$ Hz), 2.51−2.61 (m, 12H), 2.66−2.81 (m, 16H), 3.52 (s, 4H), 7.22 (s, 4H).

To a solution of the free base (112 mg, 0.196 mmol) in EtOH (8 mL) was passed HCl(g) resulting in the immediate formation of a white precipitate. The solution was concentrated to dryness and dried in vacuo to give **7a** hydrochloride (127 mg, 98%) as a white solid: ^{1}H NMR (D_2O) δ 1.94 (quintet, 8H, $J = 6.5$ Hz), 2.54 (t, 4H, $J = 6.5$ Hz), 2.61 (t, 4H, $J = 6.5$ Hz), 2.80 (s, 8H), 3.09−3.23 (m, 8H), 3.48 (s, 8H), 4.31 (s, 4H), 7.52 (s, 4H); ^{13}C NMR (D_2O) δ 23.00, 24.23, 27.79, 27.96, 31.11, 31.28, 39.58, 45.96, 46.56, 50.24, 58.78, 131.53, 132.29; FAB MS *m*/*z* 607 (MH + $H^{35}Cl$, 58), 571 (M + H, 72), 339 (75), 235 (86), 185 (100). Anal. ($C_{28}H_{50}N_4S_4$·4HCl) C, H, N, Cl.

11,11′-[1,4-Phenylenebis(methylene)]bis[1,4-dioxa-8,-11-diazacyclo tetradecane] Tetrahydrochloride Dihydrate (7b). Using general procedure B followed by formation of the hydrochloride salt (as described for **7a**), **6b** gave **7b** (96% overall yield) as a white solid: ^{1}H NMR (D_2O) δ 1.88 (m, 4H), 1.95 (m, 4H), 3.20 (t, 4H, $J = 5.3$ Hz), 3.26 (m, 4H), 3.47−3.66 (m, 24H), 4.40 (s, 4H), 7.57 (s, 4H); ^{13}C NMR (D_2O) δ 23.71, 24.72, 42.55, 49.58, 49.95, 52.76, 58.39, 69.08, 69.45, 70.11, 70.83, 131.15, 132.65; FAB MS *m*/*z* 543 (MH + $H^{35}Cl$, 66), 507 (M + H, 90), 307 (85), 203 (100). Anal. ($C_{28}H_{50}N_4O_4$·4HCl·2.5H_2O) C, H, N, Cl.

***N*-Diethoxyphosphoryl-3,3′-iminodipropionitrile (9).** To a solution of 3,3′-iminodipropionitrile (**8**) (2.0 g, 16 mmol) and triethylamine (2.7 mL) in dichloromethane (50 mL) was added dropwise with stirring, under argon, a solution of diethyl chlorophosphate (2.8 g, 16 mmol) in dichloromethane (20 mL) over approximately 30 min and the mixture was then allowed to stir overnight at room temperature. The mixture was washed with brine (50 mL), then dried (Na_2SO_4), and evaporated in vacuo giving **9** (2.7 g, 64%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 1.36 (t, 6H, $J = 7.2$ Hz), 2.66 (t, 4H, $J = 6.5$ Hz), 3.45 (m, 4H), 4.15 (m, 4H).

***N*-Diethoxyphosphoryl-3,3′-iminobis[propylamine] (10).** To a solution of **9** (1.0 g, 4 mmol) in methanol (50 mL, saturated with ammonia) was added Raney nickel (5.0 g, excess) and the mixture was hydrogenated at 45 Psi and room temperature for 48 h. The catalyst was filtered off and the solvent evaporated in vacuo to give **10** (0.95 g, 92%) as a colorless viscous oil: ^{1}H NMR ($CDCl_3$) δ 1.31 (t, 6H, $J = 7.2$ Hz), 1.65 (m, 4H), 2.75 (m, 4H), 3.05 (m, 4H), 4.15 (m, 4H).

***N*-Diethoxyphosphoryl-*N*′,*N*″-bis(*p*-toluenesulfonyl)-3,3′-iminobis[propylamine] (11).** To a solution of **10** (1.0 g, 4 mmol) and triethylamine (1.2 mL) in dichloromethane (50 mL) was added dropwise with stirring a solution of *p*-toluenesulfonyl chloride (1.6 g, 7 mmol, 2.2 equiv) in dichloromethane (25 mL) over approximately 15 min and the mixture was then allowed to stir at room temperature overnight. The mixture was washed with dilute hydrochloric acid (50 mL), saturated aqueous sodium bicarbonate (50 mL), and brine (50 mL), then dried (Na_2SO_4), and evaporated in vacuo to give a brown oil. The crude product was purified by column chromatography on silica gel (CH_2Cl_2:MeOH, 97:3) to give **11** (0.9 g, 43%) as a white solid: ^{1}H NMR ($CDCl_3$) δ 1.21 (t, 6H, $J = 7.2$ Hz), 1.65 (m, 4H), 2.42 (s, 6H), 2.91 (m, 8H), 3.85 (m, 4H), 7.27 (d, 4H, $J = 8.2$ Hz), 7.74 (d, 4H, $J = 8.2$ Hz).

8-Diethoxyphosphoryl-4,12-bis(*p*-toluenesulfonyl)-1-oxa-4,8,12-triazacyclotetradecane (13a). Using general procedure A, **11** (2.9 g, 5 mmol) and 2-bromoethyl ether (**12a**) (Aldrich; 1.16 g, 5 mmol) gave **13a** (1.1 g, 79%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 1.28 (t, 6H, $J = 7.0$ Hz), 1.95 (m, 4H), 2.42 (s, 6H), 3.15−3.25 (m, 12H), 3.65 (m, 4H), 3.95 (m, 4H), 7.35 (d, 4H, $J = 8.2$ Hz), 7.65 (d, 4H, $J = 8.2$ Hz).

General Procedure D: Diethoxyphosphoryl Deprotection. 4,12-Bis(*p*-toluenesulfonyl)-1-oxa-4,8,12-triazacyclotetradecane (14a). To a solution of **13a** (750 mg) in glacial acetic acid (4.0 mL) was added 30% HBr/acetic acid (Aldrich; 2.0 mL) and the reaction mixture stirred at room temperature for 2 h. Ether (100 mL) was added to precipitate a white solid which was allowed to settle to the bottom of the flask and the supernatant solution was decanted off. The solid was then washed by decantation with ether three times and the remaining traces of ether removed by evaporation under reduced pressure. The solid was partitioned between sodium hydroxide solution (10 mL, 10 N) and CH_2Cl_2 (150 mL) and the organic layer was separated, dried (Na_2SO_4), and evaporated in vacuo to give **14a** (410 mg, 69%) as a white solid: ^{1}H NMR ($CDCl_3$) δ 1.95 (m, 4H), 2.43 (s, 6H), 2.85 (t, 4H, $J = 6.5$ Hz), 3.28 (m, 8H), 3.65 (t, 4H, $J = 6.5$ Hz), 7.32 (d, 4H, $J = 8.2$ Hz), 7.67 (d, 4H, $J = 8.2$ Hz); FAB MS *m*/*z* 510 (M + H, 100), 329 (38), 307 (36).

8,8′-[1,4-Phenylenebis(methylene)]bis[4,12-bis(*p*-toluenesulfonyl)-1-oxa-4,8,12-triazacyclotetradecane] (15a). Using general procedure C, **14a** (403 mg, 0.79 mmol) and α,α′-dibromo-*p*-xylene (105 mg, 0.40 mmol) gave **15a** (393 mg, 89%) as a white solid: ^{1}H NMR ($CDCl_3$) δ 1.75 (m, 8H), 2.42 (s, 12H), 2.55 (m, 8H), 3.15−3.75 (m, 28H), 7.24 (s, 4H), 7.27 (d, 8H, *J*

= 8.2 Hz), 7.65 (d, 8H, *J* = 8.2 Hz); FAB MS *m/z* 1121 (M + H, 100), 965 (34), 611 (55), 354 (40).

General Procedure E: HBr/Acetic Acid Deprotection. 8,8′-[1,4-Phenylenebis(methylene)]bis[1-oxa-4,8,12-triazacyclotetradecane] Hexahydrobromide Tetrahydrate (16a). To a solution of **15a** (250 mg) in acetic acid (2.5 mL) was added hydrobromic acid (Aldrich; 48% aqueous, 1.5 mL) and the mixture was heated to reflux with stirring for 18 h. The mixture was allowed to cool and ether (50 mL) was added giving a white precipitate. The solid was allowed to settle to the bottom of the flask and the supernatant solution was decanted off. The solid was then washed by decantation with ether three times and the remaining traces of ether removed by evaporation under reduced pressure followed by drying in vacuo overnight gave **16a** as a white solid (221 mg, 62%): ^{1}H NMR (D_2O) δ 2.05 (m, 8H), 3.15−3.35 (m, 24H), 3.75 (m, 8H), 4.25 (s, 4H), 7.55 (s, 4H); FAB MS *m/z* 587 (M + H^{81}Br, 49), 585 (M + H^{79}Br, 49), 506 (M + H, 100), 307 (41). Anal. ($C_{28}H_{52}N_6O_2$·6HBr·H_2O·HOAc) C, H, N.

5,5′-[1,4-Phenylenebis(methylene)]bis[1,5,7-triazacyclotetradecane] Hexahydrobromide Pentahydrate (16b). ^{1}H NMR (D_2O) δ 1.60 (m, 8H), 2.81−3.22 (m, 16H), 4.19 (s, 4H), 4.30 (m, 8H), 7.31−7.65 (m, 12H); FAB MS *m/z* 583 (MH + H^{81}Br, 20), 581 (MH + H^{79}Br, 20), 501 (M + H, 36), 384 (20), 304 (58), 200 (66), 185 (10). Anal. ($C_{30}H_{56}N_6$·6HBr·5H_2O) C, H, N, Br.

4-Chloro-2,6-bis(hydroxymethyl)pyridine (22a). To a stirred solution of dimethyl 4-chloropyridine-2,6-dicarboxylate (**21a**) (5 g, 21.83 mmol) in 200 mL of anhydrous EtOH was added sodium borohydride (3.31 g, 87.33 mmol) and the mixture gently refluxed under an argon atmosphere for 16 h. The solution was cooled to room temperature and concentrated to dryness. The residue was partitioned between ethyl acetate (50 mL) and H_2O (50 mL) and the aqueous layer was separated and extracted with ethyl acetate (×3). The combined organic extracts were dried ($MgSO_4$) and concentrated in vacuo to give **22a** (2.41 g, 64%) as a white solid: ^{1}H NMR (DMSO-d_6) δ 4.51 (d, 4H, *J* = 6.7 Hz), 5.53 (t, 2H, *J* = 6.7 Hz), 7.35 (s, 2H).

4-Chloro-2,6-bis(chloromethyl)pyridine (23a). To a stirred solution of **22a** (2.41 g, 13.93 mmol) and Et_3N (7.8 mL, 55.72 mmol) in CH_2Cl_2 (100 mL) and $CHCl_3$ (50 mL) under an argon atmosphere, cooled to 0 °C, was added methanesulfonyl chloride (3.2 mL, 41.79 mmol). The solution was stirred at 0 °C for 30 min, then allowed to warm to room temperature, and stirred for a further 36 h. The reaction mixture was quenched with water (50 mL) and the aqueous phase was separated and extracted with CH_2Cl_2. The combined organic extracts were dried ($MgSO_4$) and concentrated in vacuo to afford a red-orange oil. The product was purified by column chromatography on a short plug of silica gel (CH_2Cl_2) to give **23a** (1.9 g, 65%) as a pale yellow solid: ^{1}H NMR (DMSO-d_6) δ 4.77 (s, 4H), 7.69 (s, 2H).

15-Chloro-7-diethoxyphosphoryl-3,11-bis(*p*-toluenesulfonyl)-3,7,11,17-tetraazabicyclo[13.3.1]heptadeca-1(17),-13,15-triene (24d). Using general procedure A, **11** (1.8 g, 3.13 mmol) and **23a** (660 mg, 3.13 mmol) gave **24d** (640 mg, 29%) as a fluffy white solid: ^{1}H NMR ($CDCl_3$) δ 1.22 (t, 3H, *J* = 7.8 Hz), 1.25 (t, 3H, *J* = 7.8 Hz), 1.51 (quintet, 4H, *J* = 7.6 Hz), 2.43 (s, 6H), 2.70−2.83 (m, 4H), 3.11 (t, 4H, *J* = 7.6 Hz), 3.80−4.01 (m, 4H), 4.25 (4H), 7.33 (d, 4H, *J* = 9 Hz), 7.55 (s, 2H), 7.69 (d, 4H, *J* = 9 Hz); FAB MS *m/z* 713 (M + H, 100), 557 (55).

15-Chloro-3,11-bis(*p*-toluenesulfonyl)-3,7,11,17-tetraazabicyclo [13.3.1]heptadeca-1(17),13,15-triene (25d). Using general procedure D, **24d** (640 mg, 0.899 mmol) gave **25d** (440 mg, 85%) as a white solid: ^{1}H NMR ($CDCl_3$) δ 1.52 (quintet, 4H, *J* = 7.6 Hz), 2.31−2.37 (m, 4H), 2.44 (s, 6H), 3.17 (t, 4H, *J* = 7.6 Hz), 4.29 (s, 4H), 7.33 (d, 4H, *J* = 8.7 Hz), 7.43 (s, 2H), 7.72 (d, 4H); FAB MS *m/z* 577 (M + H, 100), 421 (16).

7,7′-[1,4-Phenylenebis(methylene)]bis[15-chloro-3,11-bis(*p*-toluenesulfonyl)-3,7,11,17-tetraazabicyclo[13.3.1]-heptadeca-1(17),13,15-triene] (26d). Using general procedure C, **25d** (430 mg, 0.746 mmol) and α,α′-dibromo-*p*-xylene (99 mg, 0.373 mmol) gave **26d** (280 mg, 60%) as a white solid: ^{1}H NMR ($CDCl_3$) δ 1.33−1.40 (m, 8H), 2.13 (br t, 8H, *J* = 6.8 Hz), 2.43 (s, 12H), 3.11 (br t, 8H, *J* = 7.5 Hz), 4.30 (s, 8H, 6.99 (s, 4H), 7.31 (d, 8H, *J* = 8.2 Hz), 7.49 (s, 4H), 7.69 (d, 8H, *J* = 8.2 Hz); FAB MS *m/z* 1255 (M + H, 100), 1099 (34), 678 (36), 575 (64).

General Procedure F: Sulfuric Acid Deprotection. 7,7′-[1,4-Phenylenebis(methylene)]bis[15-chloro-3,7,11,-17-tetraazabicyclo [13.3.1]heptadeca-1(17),13,15-triene] Hexahydrobromide (27d). A solution of **26d** (270 mg, 0.215 mmol) in concentrated H_2SO_4 (3 mL) was stirred at 110 °C for 2 h. The dark brown solution was allowed to cool to room temperature and the pH adjusted to pH 14 with 10 N NaOH. The aqueous solution was extracted with $CHCl_3$ (3 × 20 mL) and the combined organic extracts were dried ($MgSO_4$) and concentrated in vacuo to give a pale yellow oil. This compound was converted to the hydrobromide salt using the following procedure:

To a stirred solution of the oil in anhydrous EtOH (5 mL) was passed HBr(g). The resulting tan solid was collected by filtration under argon and washed with acetic acid and then Et_2O. The solid was dissolved in H_2O (5 mL) and treated with charcoal (120 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 2 mL, after which glacial acetic acid was added resulting in the immediate formation of a white precipitate. The precipitate was collected by filtration, washed with Et_2O, and dried in vacuo giving **27d** (90 mg, 35%) as a white solid: ^{1}H NMR (D_2O) δ 2.10−2.24 (m, 8H), 3.00−3.12 (m, 8H), 3.12−3.24 (m, 8H), 4.21 (s, 4H), 4.40 (s, 8H), 7.39 (s, 4H), 7.53 (s, 4H); ^{13}C NMR (D_2O) δ 19.46, 43.22, 48.33, 48.75, 58.38, 125.09, 130.6, 132.1, 147.1, 151.6; FAB MS *m/z* 721 (MH + H^{81}Br, 51), 719 (MH + H^{79}Br, 38) 639 (M + H, 100), 372 (18). Anal. ($C_{34}H_{48}N_8Cl_2$·6HBr·HOAc) C, H, N, Hal.

7,7′-[1,4-Phenylenebis(methylene)]bis[3,7,11-triazabicyclo[13.3.1]heptadeca-1(17),13,15-triene] Hexahydrobromide Dihydrate (27a). Using general procedure E, **26a** (70 mg, 0.06 mmol) gave **27a** (63 mg, 90%) as a white solid: ^{1}H NMR (D_2O) δ 1.65−1.75 (m, 8H), 2.95−3.15 (m, 16H), 4.23 (s, 4H), 4.34 (s, 8H), 7.43 (s, 4H), 7.54−7.74 (m, 8H); FAB MS *m/z* 651 (MH + H^{81}Br, 18), 649 (MH + H^{79}Br, 18), 569 (M + H, 45), 535 (15), 370 (75), 338 (100). Anal. ($C_{36}H_{52}N_6$.6HBr·$2H_2O$) C, H, N, Br.

7,7′-[1,4-Phenylenebis(methylene)]bis[3,7,11,17-tetraazabicyclo[13.3.1]heptadeca-1(17),13,15-triene] Hexahydrobromide Hexahydrate (27b). Using general procedure E, **26b** (150 mg, 0.13 mmol) gave **27b** (60 mg, 39%) as a white solid: ^{1}H NMR (D_2O) δ 2.22 (m, 8H), 3.03 (m, 8H), 3.27 (m, 8H), 4.35 (s, 4H), 4.43 (s, 8H), 7.44 (d, 4H, *J* = 7.5 Hz), 7.49 (s, 4H), 7.85 (t, 2H, *J* = 7.5 Hz); FAB MS *m/z* 653 (MH + H^{81}-Br, 13), 651 (MH + H^{79}Br, 13), 571 (M + H, 48), 339 (58), 235 (100). Anal. ($C_{34}H_{50}N_8$·6HBr·$6H_2O$·0.5HOAc) C, H, N, Br.

7,7′-[1,4-Phenylenebis(methylene)]bis[3,7,11,15,17-pentaazabicyclo[13.3.1]heptadeca-1(17),13,15-triene] Heptahydrobromide (27c). Using general procedure F, **26c** (350 mg, 0.295 mmol) gave **27c** (220 mg, 62%) as a pale yellow solid: ^{1}H NMR (D_2O) δ 2.27 (m, 8H), 3.14 (m, 8H), 3.30 (m, 8H), 4.38 (s, 4H), 4.57 (s, 8H), 7.51 (s, 4H), 8.67 (s, 4H); ^{13}C NMR (D_2O) δ 19.45, 43.46, 46.92, 48.39, 58.78, 130.64, 132.52, 145.44, 146.54; FAB MS *m/z* 655 (MH + H^{81}Br, 12), 653 (MH + H^{79}-Br, 10), 573 (M + H, 20), 340 (13), 236 (56). Anal. ($C_{32}H_{48}N_{10}$·7HBr·HOAc) C, H, N, Br.

7,7′-[1,4-Phenylenebis(methylene)]bis[15-phenyl-3,7,-11,17-tetraazabicyclo[13.3.1]heptadeca-1(17),13,15-triene] Octahydrobromide (27e). Using general procedure E, **26e** (420 mg, 0.314 mmol) gave **27e** (330 mg, 74%) as a white solid: ^{1}H NMR (D_2O) δ 2.24 (m, 8H), 3.10 (m, 8H), 3.28 (m, 8H), 4.33 (s, 4H), 4.48 (s, 8H), 7.36−7.45 (m, 6H), 7.48 (s, 4H), 7.60−7.66 (m, 4H), 7.71 (s, 4H); ^{13}C NMR (D_2O) δ 19.68, 43.40, 48.67, 49.55, 58.73, 122.76, 127.48, 129.69, 130.50, 130.56, 132.59, 136.32, 150.88, 151.83; FAB MS *m/z* 805 (MH + H^{81}Br, 5), 803 (MH + H^{79}Br, 4), 723 (M + H, 60), 415 (34), 311 (100). Anal. ($C_{46}H_{58}N_8$·8HBr·HOAc) C, H, N, Br.

7,7′-[1,4-Phenylenebis(methylene)]bis[15-methoxy-3,7,11,17-tetraazabicyclo[13.3.1]heptadeca-1(17),13,15-triene] Hexahydrobromide Dihydrate (27f). Using general procedure F, **26f** (160 mg, 0.128 mmol) gave **27f** (65 mg, 44%) as a white solid: IR (KBr) ν 1607 cm^{-1} (C═C−OMe); ^{1}H NMR (D_2O) δ 2.14−2.26 (m, 8H), 3.00−3.10 (m, 8H), 3.12−3.23 (m, 8H), 3.80 (s, 6H), 4.25 (s, 4H), 4.35 (s, 8H), 7.01 (s, 4H), 7.43 (s, 4H); ^{13}C NMR (D_2O) δ 19.25, 42.98, 48.21, 49.12, 55.99, 58.26, 110.80, 130.46, 132.16, 151.63, 167.99: FAB MS *m/z* 713 (MH + H^{81}Br, 41), 711 (MH + H^{79}Br, 40), 631 (M + H, 100), 617 (14), 416 (12), 368 (13). Anal. ($C_{36}H_{54}N_8$·6HBr·2.5H_2O) C, H, N, Br.

7,7′-[1,4-Phenylenebis(methylene)]bis[3,7,11,17-tetraazabicyclo[13.3.1]heptadeca-13,16-trien-15-one] Octahydrobromide (27g). Using general procedure E, **26f** (150 mg, 0.12 mmol) gave **27g** (95 mg, 64%) as a white solid: IR (KBr) ν 1629 cm^{-1} (C═O); ^{1}H NMR (D_2O) δ 2.16−2.29 (m, 8H), 3.06 (t, 8H, J = 7.6 Hz), 3.27 (t, 8H, J = 7.6 Hz), 4.31 (s, 8H), 4.34 (s, 4H), 6.85 (s, 4H), 7.48 (s, 4H); ^{13}C NMR (D_2O) δ 19.1, 42.94, 48.20, 49.03, 58.30, 112.19, 130.29, 132.24, 151.63, 165.71; FAB MS *m/z* 685 (MH + H^{81}Br, 5), 683 (MH + H^{79}Br, 5), 603 (M + H, 100), 460 (12), 329 (16). Anal. ($C_{34}H_{50}N_8O_2$·8HBr) C, H, N, Br.

2,6-Bis(*N*,*N*′-*p*-toluenesulfonyl-2-aminoethyl)pyridine (28). A stirred solution of 2,6-bis(bromomethyl)pyridine hydrobromide (**18**) (6.0 g, 17 mmol), NaCN (5.1 g, 104 mmol), and cetyltrimethylammonium bromide (633 mg, 1.7 mmol) in benzene (50 mL) and H_2O (25 mL) was heated to reflux for 4 h. Upon cooling, the aqueous layer was separated and extracted with benzene (50 mL) and dichloromethane (75 mL) and the combined organic phases were dried (Na_2SO_4) and evaporated in vacuo to give a brown solid. Filtration of a CH_2Cl_2 solution of the brown residue through a short bed of basic alumina gave the crude dinitrile (2.4 g, 86%) as a white solid: ^{1}H NMR ($CDCl_3$) δ 3.94 (s, 4H), 7.45 (d, 2H, J = 7.5 Hz), 7.85 (t, 1H, J = 7.5 Hz).

To a solution of the dinitrile from above (4.3 g, 27 mmol) in MeOH (75 mL, saturated with ammonia) was added Raney nickel (10.0 g, excess) and the mixture was hydrogenated at 45 psi and room temperature for 48 h. The catalyst was removed by filtration through Celite and the filtrate was evaporated in vacuo to give the pyridyldiamine as a brown viscous oil (3.7 g, 83%): ^{1}H NMR ($CDCl_3$) δ 2.91 (m, 4H), 3.01 (m, 4H), 7.01 (d, 2H, J = 7.5 Hz), 7.55 (t, 1H, J = 7.5 Hz). This was used without further purification.

To a stirred solution of the brown oil and Et_3N (7.0 mL) in CH_2Cl_2 (75 mL) was added dropwise a solution of *p*-toluenesulfonyl chloride (8.7 g, 49 mmol) in CH_2Cl_2 (25 mL) over approximately 15 min and the reaction mixture was then allowed to stir at room temperature overnight. The solution was washed with saturated aqueous sodium bicarbonate (50 mL) and brine (50 mL), then dried (Na_2SO_4), and evaporated in vacuo to give a pale yellow viscous oil. The product was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 98:2) to give **28** (8.0 g, 75%) as a white solid: ^{1}H NMR ($CDCl_3$) δ 2.41 (s, 6H), 2.89 (t, 4H, J = 6.5 Hz), 3.25 (m, 4H), 5.65 (t, 2H), 6.95 (d, 2H, J = 7.5 Hz), 7.25 (d, 4H, J = 8.2 Hz), 7.45 (t, 1H, J = 7.5 Hz), 7.71 (d, 4H, J = 8.2 Hz).

3,9-Bis(*p*-toluenesulfonyl)-3,6,9,15-tetraazabicyclo[11.3.1]pentadeca-1(15),11,13-triene (32). Macrocyclization of **18** and **31** using general procedure A (68% yield) followed by deprotection of the diethoxyphosphoryl group using general procedure D (80% yield) gave **32** as a white solid: ^{1}H NMR ($CDCl_3$) δ 2.44 (s, 6H), 2.65 (m, 4H), 3.35 (m, 4H), 4.31 (s, 4H), 7.15 (d, 2H, J = 7.5 Hz), 7.35 (d, 4H, J = 8.2 Hz), 7.65 (d, 4H, J = 8.2 Hz), 7.85 (t, 1H, J = 8.2 Hz); FAB MS *m/z* 537 (M + Na, 26), 515 (M + H, 100), 359 (9).

4,10-Bis(*p*-toluenesulfonyl)-4,7,10,17-tetraazabicyclo[13.3.1]heptadeca-1(17),13,15-triene (33). Macrocyclization of **28** and **30** using general procedure A (23% yield) followed by deprotection of the diethoxyphosphoryl group using general procedure D (92% yield) gave **33** as a white solid: ^{1}H NMR ($CDCl_3$) δ 2.42 (s, 6H), 2.55 (m, 4H), 2.99 (m, 4H), 3.15 (m, 4H), 3.65 (m, 4H), 7.05 (d, 2H, J = 7.5 Hz), 7.31 (d, 4H, J = 8.2 Hz), 7.55 (t, 1H, J = 7.5 Hz), 7.67 (d, 4H, J = 8.2 Hz).

4,12-Bis(*p*-toluenesulfonyl)-4,8,12,19-tetraazabicyclo[15.3.1]nonadeca-1(19),15,17-triene (34). Macrocyclization of **28** and **29** using general procedure A (48% yield) followed by deprotection of the diethoxyphosphoryl group using general procedure D (97% yield) gave **34** as a white solid: ^{1}H NMR ($CDCl_3$) δ 1.55 (m, 4H), 2.42 (s, 6H), 2.35 (t, 4H, J = 6.2 Hz), 3.05 (t, 4H, J = 6.2 Hz), 3.15 (t, 4H, J = 6.2 Hz), 3.60 (t, 4H, J = 6.2 Hz), 7.05 (d, 2H, J = 7.5 Hz), 7.35 (d, 4H, J = 8.2 Hz), 7.55 (t, 1H, J = 7.5 Hz), 7.75 (d, 4H, J = 8.2 Hz); FAB MS *m/z* 571 (M + H, 100), 415 (20).

6,6′-[1,4-Phenylenebis(methylene)]bis[3,6,9,15-tetraazabicyclo[11.3.1]pentadeca-1(15),11,13-triene]Hexahydrobromide Trihydrate (35a). General procedure F gave **35a** (33% yield) as a white solid: ^{1}H NMR (D_2O) δ 2.65 (m, 8H), 3.05 (m, 8H), 3.75 (s, 4H), 4.45 (s, 8H), 7.20 (d, 4H, J = 7.5 Hz), 7.26 (s, 4H), 7.85 (t, 2H, J = 7.5 Hz); FAB MS *m/z* 597 (MH + H^{81}Br, 9), 595 (MH + H^{79}Br, 9), 515 (M + H, 57), 440 (48), 223 (100). Anal. ($C_{30}H_{42}N_8$·6HBr·3H_2O·0.75HOAc) C, H, N.

6,6′-[1,3-Phenylenebis(methylene)]bis[3,6,9,15-tetraazabicyclo[11.3.1]pentadeca-1(15),11,13-triene]Hexahydrobromide Trihydrate (35b). General procedure F gave **35b** (45% yield) as a white solid: ^{1}H NMR (D_2O) δ 2.66 (m, 8H), 3.11 (m, 8H), 3.76 (s, 4H), 4.45 (s, 8H), 7.26−7.29 (m, 8H), 7.78 (t, 2H, J = 7.5 Hz); FAB MS *m/z* 597 (MH + H^{81}Br, 9), 595 (MH + H^{79}Br, 9), 515 (M + H, 100). Anal. ($C_{30}H_{42}N_8$·6HBr·3H_2O·HOAc) C, H, N, Br.

7,7′-[1,4-Phenylenebis(methylene)]bis[4,7,10,17-tetraazabicyclo[13.3.1]heptadeca-1(17),13,15-triene] Octahydrobromide Tetrahydrate (36). General procedure F gave **36** (72% yield) as a white solid: ^{1}H NMR (D_2O) δ 2.75 (m, 8H), 3.05−3.65 (m, 28H), 6.88 (s, 4H), 7.15 (d, 4H, J = 7.5 Hz), 7.65 (t, 2H, J = 7.5 Hz); FAB MS *m/z* 653 (MH + H^{81}Br, 22), 651 (MH + H^{79}Br, 22), 571 (M+H, 31), 339 (21), 235 (100). Anal. ($C_{34}H_{50}N_8$·8HBr·4H_2O·0.5HOAc) C, H, N, Br.

8,8′-[1,4-Phenylenebis(methylene)]bis[4,8,12,19-tetraazabicyclo[15.3.1]nonadeca-1(19),15,17-triene] Nonahydrobromide Trihydrate (37). General procedure E gave **37** (65% yield) as a white solid: ^{1}H NMR (D_2O) δ 2.13 (m, 8H), 3.06−3.39 (m, 32H), 4.41 (s, 4H), 7.13 (d, 4H, J = 7.5 Hz), 7.51 (s, 4H), 7.65 (t, 2H, J = 7.5 Hz); FAB MS *m/z* 709 (MH + H^{81}Br, 33), 707 (MH + H^{79}Br, 33), 627 (M + H, 83), 367 (100). Anal. ($C_{38}H_{58}N_8$·8HBr·3.75H_2O) C, H, N.

Anti-HIV Activity Assays. Inhibition of HIV-1 (III_B) and HIV-2 (ROD) replication assays was performed as previously described.[2−5] 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-diphenyltetrazolium 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 noncytotoxic. 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.

Assay for Inhibition of Syncytium Formation.[3,20] Persistently HIV-1 (III_B)-infected HUT-78 cells (washed twice to remove free virus) were cocultured in 96-well microtrays in the presence of various concentrations of the test compounds with uninfected MOLT-4 cells, both cell types at a ratio of 5 × 10^5 cells each in a total volume of 200 μL. After incubation for

20−24 h at 37 °C in a CO_2-controlled atmosphere, syncytia were evaluated microscopically. The 50% inhibitory concentration was defined as the compound concentration required to inhibit syncytium formation by 50%.

Analysis of Inhibition of CXCR4-Specific mAb 12G5 Binding to CXCR4 by Bis-azamacrocycles. The specific effects on CXCR4 were determined as described previously.[9] Briefly, the lymphocytic SUP-T1 cells were incubated with compounds or PBS for 15 min at room temperature. The cells were washed with PBS and the 12G5 mAb (R&D Systems) was added for 30 min at room temperature. The cells were washed twice, then incubated with FITC-conjugated goat anti-mouse Ab (Caltag Labs) for 30 min at room temperature, and then washed twice with PBS. The percentage of positive cells and mean fluorescence intensity (NFI) values are indicated in each histogram and were analyzed by a FACScan flow cytometer (Becton Dickinson).

Measurement of Intracellular Calcium Concentrations. The determinations of intracellular calcium concentrations $[Ca^{2+}]_i$ were carried out as previously described.[9,21] SUP-T1 cells were loaded on a Fura-2 (Molecular Probes). Fura-2 fluorescence was measured in a luminescence spectrophotometer fitted with a water-thermostatable, stirred four-position cuvette holder (Perkin-Elmer). Cells were first stimulated with dilution buffer (control) or test compounds [AMD3100 or AMD3329 (compound **36**)] at different concentrations. As a second stimulus, SDF-1α (R&D Systems) was used at an optimal concentration to induce a maximal $[Ca^{2+}]_i$ increase. The second stimulus was added 100 s after the first stimulus.

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