Synthesis, Characterization, and Structure−Activity Relationships of Amidine-Substituted (Bis)benzylidene-Cycloketone Olefin Isomers as Potent and Selective Factor Xa Inhibitors[1,2]

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Factor Xa (FXa) is a trypsin-like serine protease that plays a key role in blood coagulation linking the intrinsic and extrinsic pathways to the final common pathway of the coagulation cascade. During our initial studies, we observed facile photochemical conversion of the known FXa/tPA inhibitor, BABCH [(*E*,*E*)-2,7-bis(4-amidinobenzylidene)cycloheptan-1-one, **1a**], to the corresponding (*Z*,*Z*) olefin isomer, **1c** (FXa K_i = 0.66 nM), which was over 25 000 times more potent than the corresponding (*E*,*E*) isomer (**1a**, FXa K_i = 17 000 nM). In order to determine the scope of this observation, we expanded on our initial investigation through the preparation of the olefin isomers in a homologous series of cycloalkanone rings, 4-substituted cyclohexanone analogues, and modified amidine derivatives. In most cases the order of potency of the olefin isomers was (*Z*,*Z*) > (*E*,*Z*) > (*E*,*E*) with the cycloheptanone analogue (**1c**) showing the most potent factor Xa inhibitory activity. In addition, we found that selectivity versus thrombin (FIIa) can be dramatically improved by the addition of a carboxylic acid group to the cycloalkanone ring as seen with **8c** (FXa K_i = 6.9 nM, FIIa K_i > 50 000 nM). Compounds with one or both of the amidine groups substituted with *N*-alkyl substituents or replaced with amide groups led to a significant loss of activity. In this report we have demonstrated the importance of the two amidine groups, the cycloheptanone ring, and the (*Z*,*Z*) olefin configuration for maximum inhibition of FXa within the BABCH template. The results from this study provided the foundation for the discovery of potent, selective, and orally active FXa inhibitors.

Introduction

Factor Xa (FXa), a trypsin-like serine protease, links the intrinsic and extrinsic pathways to the final common pathway in the blood coagulation cascade. The primary role of FXa is the proteolytic activation of prothrombin, after combining with factor Va and calcium on a phospholipid membrane to form the prothrombinase complex.[3] Thrombin then promotes blood clot formation by catalyzing the formation of polymerizable fibrin from fibrinogen in addition to activating platelets. Ex vivo data demonstrate that prothrombinase, not thrombin, is the major determinant of the procoagulant activity of human whole-blood clots.[4]

Progression of thrombosis plays an important role in the pathophysiology of unstable angina and myocardial infarction and may be a factor in the failure of coronary fibrinolysis. Progression of deep vein thrombosis can contribute to pulmonary embolism. Recent data indicate FXa may be more important than thrombin as a mediator of thrombus progression.[4,5] This hypothesis is consistent with studies showing persistent inhibition of procoagulant activity and thrombus progression by inhibitors of FXa.[6] These studies and others stimulated interest in novel approaches to nonpeptide-based inhibitors of human FXa as potential candidates for preclinical testing.[7]

The most clinically[8] advanced nonpeptide FXa inhibitor is (2*S*)-2-[4-[((3*S*)-1-acetimidoyl-3-pyrrolidinyl)oxo]-phenyl]-3-(7-amidino-2-naphthyl)propanoic acid hydrochloride pentahydrate, DX-9065a, which has a K_i for FXa in purified systems of 40 nM[9] and is efficacious in several species.[10] We recently reported the synthesis of *N*-[2-[5-[amino(imino)methyl]-2-hydroxyphenoxyl-3,5-diflu-oro-6-[3-(4,5-dihydro-1-methyl-1*H*-imidazol-2-yl)phenoxy]pyridin-4-yl]-*N*-methylglycine, ZK807834 (CI 1031), a novel inhibitor of FXa, which inhibits purified human FXa with a K_i of 0.11 nM.[7b] Preliminary studies of the selectivity, pharmacokinetics, oral availability, and efficacy have been reported.[11] In a direct comparison in rabbits, ZK-807834 was shown to be efficacious at a 25-fold lower dose than DX-9065a, consistent with the observed differences of the in vitro potencies.[12]

In our previous communication we reported that the known FXa/tPA inhibitor, BABCH [(*E*,*E*)-2,7-bis(4-amidinobenzylidene)cycloheptan-1-one, **1a**], could be photochemically converted to a mixture of the (*E*,*Z*) and (*Z*,*Z*) double bond isomers (Scheme 1). When these isomers were tested in the absence of light we found that the (*Z*,*Z*) isomer, **1c** (FXa K_i = 0.66 nM), was over 25 000 times more potent then the corresponding (*E*,*E*) isomer (**1a**, FXa K_i = 17 μM). The high potency and selectivity of **1c** was very encouraging, and the conformational rigidity of this system provided an excellent template leading to the design of a variety of heterocyclic-based FXa inhibitor templates.[13] In order to understand this large disparity between the FXa inhibitory activities of the geometrical isomers, we investigated other structurally related analogues. In this report we describe the synthesis, photoisomerization, and in vitro activities of a series homologous cycloalkanone rings,

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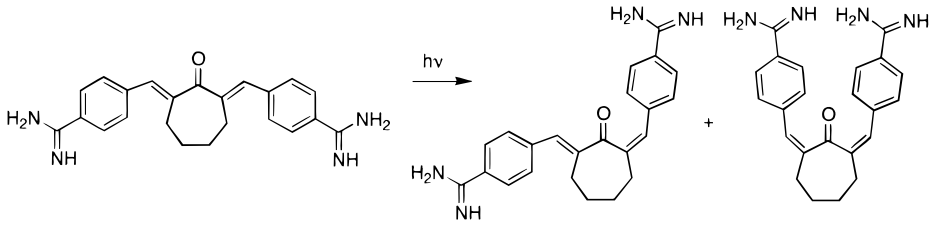
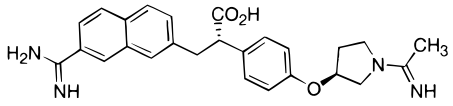
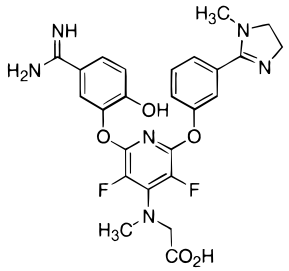
Scheme 1





DX-9065a
FXa K_i = 40 nM



ZK-807834
FXa K_i = 0.11 nM

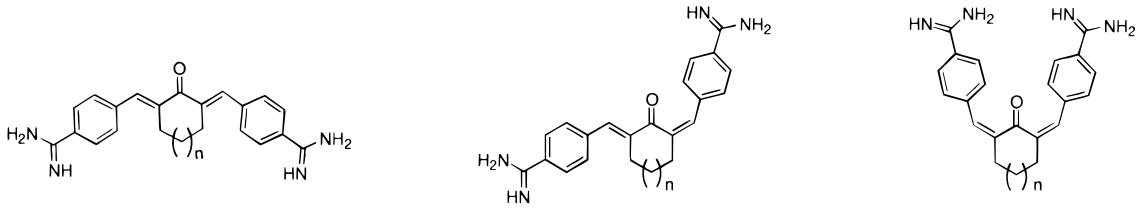
4-substituted cyclohexanone derivatives, and substituted amidine analogues (Tables 1−3).

Chemistry

The symmetrical (*E*,*E*)-bis-(amidinobenzylidene)cyclic ketone analogues in Table 1 (**1a**−**6a**) were prepared by acid-catalyzed condensation of 4-amidinobenzaldehyde (**7**) with the appropriate cycloalkanone (Scheme 2).[14] The poor solubility of the (*E*,*E*) isomers facilitated the purification of the lower ring analogues (**1a**−**3a**) which precipitated from the crude reaction mixture and were recrystallized to afford analytically pure samples as salts. The larger ring analogues (**4a**−**6a**) could not be effectively precipitated from a mixture that included the monobenzylidene and the corresponding (*E*,*Z*) isomer, and therefore they were purified by preparative reverse-phase HPLC. The ^{1}H NMR spectra are consistent with a symmetrical configuration, and the downfield chemical shift of the vinylic protons (δ = 7.08−7.69 ppm, Table 4) is consistent with the ^{1}H NMR spectra of other reported α,β-unsaturated cyclic ketones having (*E*,*E*) double bond configurations.[15] Unambiguous assignment of the (*E*,*E*) double bond geometry was confirmed by NOESY NMR studies of **1a**,[2] **3a**, and **4a**. In these studies, NOE cross peaks were observed between the cycloheptanone ring protons and the protons meta to the amidine substituent, with no observed NOE between the cycloheptanone ring protons and the vinylic protons.

Solutions of the (*E*,*E*) isomers (**1a**−**6a**) were irradiated with a 450 W high-pressure mercury lamp to produce an equilibrium mixture of the corresponding (*E*,*Z*) **1b**−**6b** and (*Z*,*Z*) isomers **1c**, **3c**−**6c** with a small amount of the starting (*E*,*E*) isomer. The isomerization could be effected on a preparative scale using either a 450 W high-pressure mercury lamp or a 150 W standard flood lamp. The reaction mixtures were irradiated until a constant product ratio was obtained as determined by HPLC, typically 2−4 h with the 450 W high-pressure mercury lamp. The product ratio could be determined by integration of the vinyl resonances in the ^{1}H NMR spectrum of the crude photolysis mixtures. For example, photolysis of **1a** afforded a product ratio of <5%, 55%, and 40% for **1a**, **1b**, and **1c**, respectively. The isomers were separated by preparative HPLC, and purified fractions were handled with care to exclude light and prevent reequilibration during isolation. Irradiation of **2a** failed to afford any of the corresponding (*Z*,*Z*) isomer,

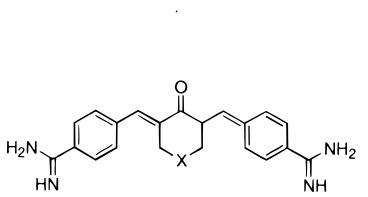
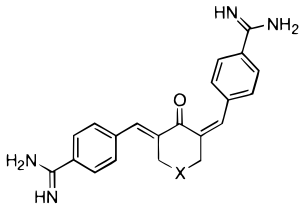
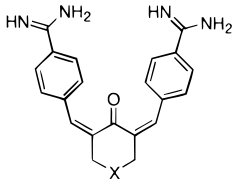
Table 1. In Vitro Inhibitory Activities of Bisarylamidine Cyclic Ketone Isomers Tested in the Absence of Light



		K_i (nM)[a]				K_i (nM)				K_i (nM)		
n	no.	FXa	FIIa	Trp	no.	FXa	FIIa	Trp	no.	FXa	FIIa	Trp
0	**2a**	4600	2200	590	**2b**	230	19200	730	**2c**	ND[b]	ND[b]	ND[b]
1	**3a**	5600	3000	170	**3b**	420	120	260	**3c**	18	810	440
2	**1a**	17000	6200	280	**1b**	200	130	29	**1c**	0.66	530	33
3	**4a**	1800	2100	310	**4b**	130	200	30	**4c**	4.6	300	100
4	**5a**	390	3700	1500	**5b**	320	350	340	**5c**	2.8	290	190
5	**6a**	960	730	3000	**6b**	510	150	2100	**6c**	110	740	2200

[a] K_i values for these competitive inhibitors are averaged from multiple determinations (n > 2), and the standard deviations are <30% of the mean. [b] Compound not available for assay.

Table 2. In Vitro Inhibitory Activities of Bisarylamidine Cyclic Ketone Isomers Tested in the Absence of Light

		K_i (nM)[a]				K_i (nM)[a]				K_i (nM)[a]		
X	no.	FXa	FIIa	Trp	no.	FXa	FIIa	Trp	no.	FXa	FIIa	Trp
CH-CO_2H	**8a**	24400	21500	880	**8b**	ND[b]	ND[b]	ND[b]	**8c**	6.9	>50000	430
CH-CO_2Me	**9a**	>5000	4400	72	**9b**	>500	600	224	**9c**	4.8	1100	100
CH-Ph	**10a**	3300	630	56	**10b**	395	325	160	**10c**	5.2	1700	100
N-CO_2Et	**11a**	>5000	3300	125	**11b**	225	630	61	**11c**	2.5	380	20

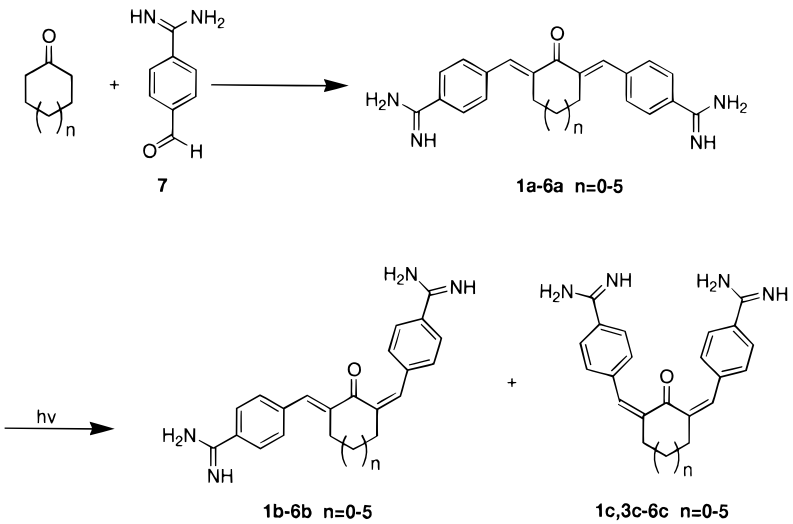
[a] K_i values for these competitive inhibitors are averaged from multiple determinations ($n \geq 2$), and the standard deviations are <30% of the mean. [b] Compound not available for assay.

Table 3. In Vitro Inhibitory Activities of Mono- and Bisarylamidine Cycloheptanone Analogues Tested in the Absence of Light

	(*E*,*E*) isomer K_i (nM)[a]				(*E*,*Z*) and (Z,E) isomers K_i (nM)				(*Z*,*Z*) isomer K_i (nM)		
no.	FXa	FIIa	Trp	no.	FXa	FIIa	Trp	no.	FXa	FIIa	Trp
13a	29000	12850	840	**13b**	500	360	155	**13c**	12	1600	30
14a	350	3700	500	**14b**	52	120	15	**14c**	18	1400	90
15a	>500	>500	>500	**15b**	1850	350	500	**15c**	50	110	690
17a	ND[b]	ND	ND	**17b**	>5000	NA[c]	NA	**17c**	>5000	2900	>5000
18a	>5000	>5000	>5000	**18b**	>5000	>5000	>5000	**18c**	1600	>5000	>5000
19a	1400	2100	410	**19b**	140	1000	200	**19c**	5.6	1400	44

[a] K_i values for these competitive inhibitors are averaged from multiple determinations ($n \geq 2$), and the standard deviations are <30% of the mean. [b] Compound not available for assay. [c] Compound not assayed on this protease.

Scheme 2



2c, which may be attributable to a competing dimerization reaction that has been reported for 2,5-difurfurylidenecyclopentanone.[15a]

The three isomeric adducts could be differentiated and unambiguously identified by their characteristic NMR spectra. The ^{1}H NMR spectra of the (*E*,*Z*) isomers (**1b**−**6b**) are significantly more complex, showing separate resonances for many of the hydrogen atoms, illustrating the unsymmetrical nature of these isomers. The ^{1}H NMR spectra of the (*Z*,*Z*) isomers (**1c**, **3c**−**6c**) contain the same elements of symmetry as the (*E*,*E*) isomers, but the upfield chemical shifts of the vinylic protons (δ = 6.82−6.96, Table 4) are consistent with related compounds containing the (*Z*,*Z*) double bond configuration.[15b] Studies with **1c**, **3c**, and **4c** showed NOE cross peaks between the vinylic and allylic protons on the cycloheptanone ring which unambiguously support the assignment of the (*Z*,*Z*) double bond configuration.

The 4-substituted cyclohexanone and piperidinone analogues in Table 2 were prepared in a similar fashion as outlined above. Condensation of the requisite cyclic ketones with 4-amidinobenzaldehyde afforded the appropriate (*E*,*E*) isomer. Subsequent photochemical isomerization, followed by preparative HPLC afforded the corresponding (*E*,*Z*) and (*Z*,*Z*) isomers. One exception was compound **8c** which could not be obtained pure after photoisomerization and chromatography. This compound was prepared by saponification of the purified ester analogue **9c**, followed by HPLC purification. Assignment of double bond geometry for this series was carried out by ^{1}H NMR and is consistent with the unsubstituted cycloalkanone series (Table 4).

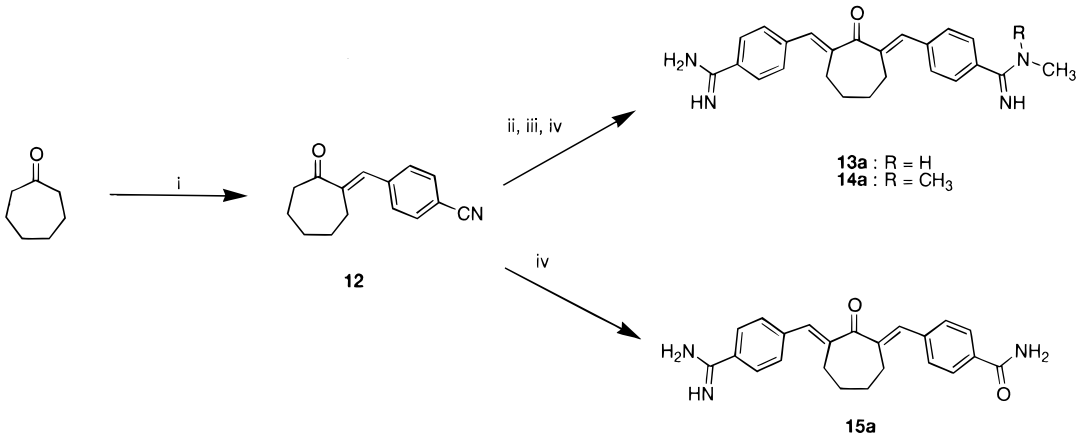
Table 4. Vinyl Proton ^{1}H NMR Chemical Shift Values of Bisarylamidine Cyclic Ketone Isomers

no.	δ^a	no.	δ^a	no.	δ^a
1a	7.35	**1b**	6.84, 7.63	**1c**	6.96
2a	7.56	**2b**	7.54, 7.7(m)[b]	**2c**	ND[c]
3a	7.69	**3b**	6.90, 7.42	**3c**	6.82
4a	7.08	**4b**	6.70, 7.65(m)[b]	**4c**	6.83
5a	7.10	**5b**	6.86, 7.65(m)[b]	**5c**	6.86
6a	7.20	**6b**	7.16, 7.65(m)[b]	**6c**	6.96
8a	7.85	**8b**	ND[c]	**8c**	6.85
9a	7.85	**9b**	6.95, 7.48	**9c**	6.85
10a	7.75	**l0b**	6.95, 7.45	**10c**	6.9
11a	7.75	**11b**	7.15, 7.55	**l1e**	7.13
13a	7.39	**13b**	6.82, 7.7(m)[b]	**13c**	6.98
14a	7.38	**14b**	6.8, 7.6(m)	**14c**	6.95, 7.00
15a	7.34	**15b**	6.74, 6.8, 7.6(m)[b]	**15c**	6.89, 6.96
17a	ND[c]	**17b**	6.73, 7.56	**17c**	6.84
18a	7.33	**18b**	6.78, 7.6	**18c**	6.93
19a	7.33	**19b**	6.8, 7.7(m)	**19c**	6.95

[a] Chemical shift of vinyl protons. [b] Chemical shift of vinyl proton could not be separated unambiguously from aromatic multiplet centered at listed resonance. [c] Compound not analyzed by ^{1}H NMR.

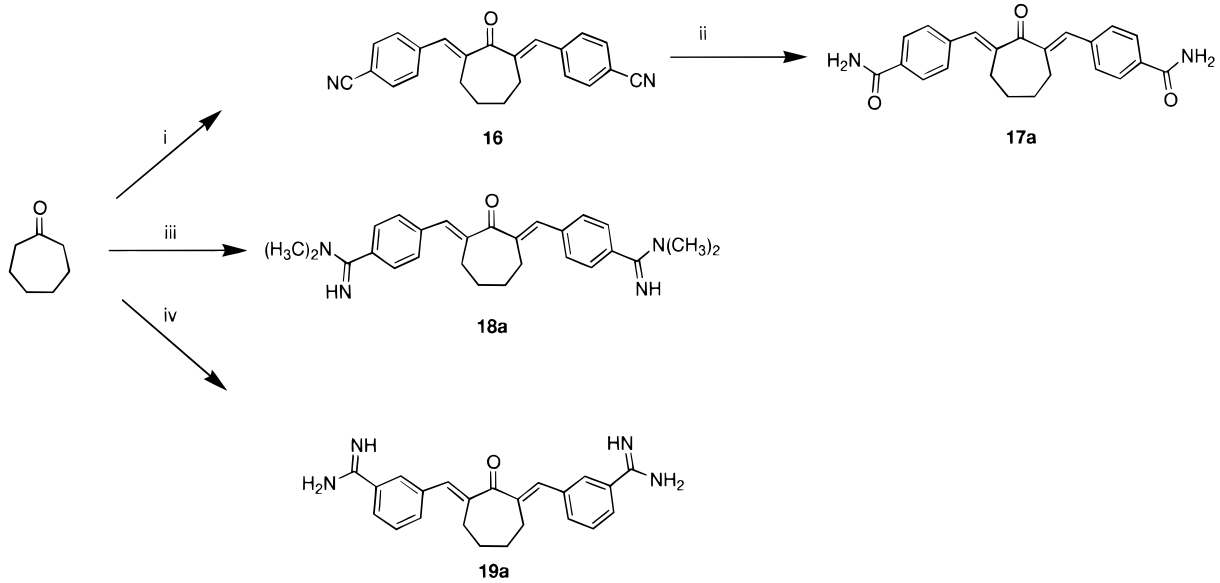
The symmetrical and unsymmetrical amide and alkylamidine (*E*,*E*) analogues were prepared as illustrated in Schemes 3 and 4. Condensation of cycloheptanone with a single equivalent of 4-cyanobenzaldehyde afforded (4-cyanobenzylidene)cycloheptanone (**12**). Conversion of nitrile to the substituted *N*-methyl and *N*,*N*-dimethylamidine via Pinner reaction was followed by condensation with 4-amidinobenzaldehyde to afford the corresponding (*E*,*E*) isomers **(13a** and **14a**, respectively). Condensation of **12** with **7** in the presence of phosphoric acid at 100 °C also effected conversion of the nitrile to afford the amide **15a**. Surprisingly, the major component isolated after condensation of cycloheptanone with an excess of 4-cyanobenzaldehyde in phosphoric acid at 100 °C was the bis-nitrile (**16**) and not the corresponding amide. The bis-amide (**17a**) was prepared by hydrolysis of **16** with sulfuric acid at 0−5 °C. The bis-dimethylamidine analogue **18a** was obtained by condensation of cycloheptanone with 4-dimethylamidinobenzaldehyde (**20**). In a similar manner, the bis-3-amidino analogue, **19a**, was prepared by condensation of cycloheptanone with 3-amidinobenzaldehyde.[16] Photolysis of the (*E*,*E*) isomers afforded an equilibrium mixture of isomers that were separated by preparative HPLC as described above. Due to the nonredundancy of the two aryl groups, photolysis of the unsymmetrical analogues (**13a**−**15a**)

Scheme 3[a]



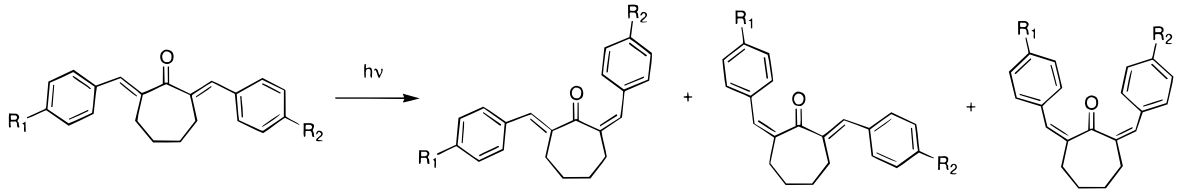
[a] Reagents: (i) 4-OHC-C_6H_4-CN, H_3PO_4; (ii) HCl, EtOH; (iii) MeNHR, EtOH, heat; (iv) H_3PO_4, **7**.

Scheme 4[a]



[a] Reagents: (i) 4-OHC-C_6H_4-CN, H_3PO_4; (ii) H_2SO_4; (iii) HCl, 4-OHC-C_6H_4-C(NH)NMe_2 (**20**); (iv) H_3PO_4, 3-OHC-C_6H_4-C(NH)NH_2.

Scheme 5



13a R_1 = C(NH)NH_2, R_2 = C(NH)$NHCH_3$
14a R_1 = C(NH)NH_2, R_2 = C(NH)$N(CH_3)_2$
15a R_1 = C(NH)NH_2, R_2 = $CONH_2$

13b R_1 = C(NH)NH_2, R_2 = C(NH)$NHCH_3$
14b R_1 = C(NH)NH_2, R_2 = C(NH)$N(CH_3)_2$
15b R_1 = C(NH)NH_2, R_2 = $CONH_2$

13c R_1 = C(NH)NH_2, R_2 = C(NH)$NHCH_3$
14c R_1 = C(NH)NH_2, R_2 = C(NH)$N(CH_3)_2$
15c R_1 = C(NH)NH_2, R_2 = $CONH_2$

produces two unique (*E*,*Z*) isomers in an approximate 1:1 ratio (Scheme 5). Due to the difficulties involved with the separation of these (*E*,*Z*) isomers, these analogues were tested as a mixture.

Results and Discussion

All of the compounds illustrated in Tables 1−3 were tested for their ability to inhibit human factor Xa in a purified enzyme system. In addition, all compounds were assayed against human thrombin and bovine trypsin. Assays were run in duplicate, and K_i values were determined as described previously.[17] All assays were run in the absence of light, and extra care was taken to minimize exposure of solutions to light before and during the bioassays to minimize isomerization of the inhibitors. The ability to avoid isomerization was verified by HPLC analysis of selected test compounds after completion of the assays.

The data in Table 1 illustrate that the order of potency in FXa for the double bond isomers of this series of cyclic ketone analogues is (*Z*,*Z*) > (*E*,*Z*) > (*E*,*E*). In addition, the FXa inhibitory activity of the (*Z*,*Z*) isomers varied significantly with the cycloalkanone ring size as seen by the 27-fold drop in activity for the cyclohexanone analogue (**3c**, FXa K_i = 18 nM) and the 170-fold lowered activity of the cyclodecanone analogue (**6c**, FXa K_i = 110 nM) when compared with **1c** (FXa K_i = 0.66 nM). The corresponding cyclooctanone and cyclononanone analogues (**4c** and **5c**, respectively) were within 7-fold of the activity of **1c**. A similar trend for potency of this series was seen with trypsin; however, the trypsin K_i values are 20−70 fold higher than the corresponding values for FXa. The measured K_i values were all within a factor of three for the (*Z*,*Z*) analogues against thrombin. These results suggest that the (*Z*,*Z*) inhibitors may adopt a similar binding mode in trypsin and FXa, but may bind in a different mode in thrombin.

The higher affinity of the (*Z*,*Z*) isomers for FXa as compared to trypsin suggests some favorable interactions in the FXa active site which are not conserved in the trypsin active site. Identification of the specific residues which are responsible for the higher potencies of the (*Z*,*Z*) compounds in FXa is complicated by a number of amino acid differences in the binding sites of these two proteases. The binding mode of **1c** in FXa has not been experimentally determined, and it is not known whether **1c** binds in the same mode in trypsin as in FXa. In our previous report, we proposed two possible binding modes to account for the potent FXa inhibitory activity of **1c**.[2] To gain further understanding of the binding mode for this series of (*Z*,*Z*) inhibitors, we attempted to resolve the crystal structure of **1c** in trypsin. Since photoisomerization may occur during crystal data collection, good resolution was needed to determine which isomer was bound in the active site of trypsin. Although both benzamidine rings were visible in the structure, occupying the S1 and S4 binding sites of the extended binding mode, the electron density for the central ring was absent in the solved structure resulting in an ambiguous determination of the double bond geometry of the inhibitor. Recently, the crystal structure of a commercial source of BABCH in trypsin was reported by another group at 1.7 Å resolution.[18] These investigators identified the trypsin-bound inhibitor as the (*E*,*Z*) isomer (**1b**) which binds in an extended mode in trypsin, consistent with their initial structural data.[18]

Table 5. In Vitro Inhibitory Activities of Bisarylamidine Cyclic Ketone Isomers Tested in the Absence of Light against tPA and uPA

	(*E*,*E*) isomer			(*E*,*Z*) isomer			(*Z*,*Z*) isomer	
no.	tPA[a]	uPA[b]	no.	tPA[a]	uPA[b]	no.	tPA[a]	uPA[b]
1a	39300	>30000	**1b**	720	10000	**1c**	16	20000
19a	66000	20000	**19b**	7300	15000	**19c**	61	>30000

[a] K_i values (nM) for these competitive inhibitors are averaged from multiple determinations ($n \geq 2$), and the standard deviations are <30% of the mean.[15] [b] IC_{50} values (nM).

The crystal structure of BABCH complexed with tissue-type plasminogen activator (tPA), another related serine protease, has been reported recently.[19] The observed binding mode in this structure is similar to that in the crystal structure of **1b** with trypsin, except for the geometry of the cycloheptanone ring. Although the bound inhibitor was originally thought to be the (*E*,*E*)-isomer (**1a**), the authors later proposed that it may in fact be **1c**, based on the (*Z*,*Z*) preference of FXa and its similarity to tPA.[18] These workers suggest that in tPA, Tyr 99 prevents the (*E*,*Z*)-isomer from binding well but allows the (*Z*,*Z*) isomer to bind. Tyr 99 is also found in FXa, but is Leu in trypsin and thrombin. The data in Table 5 show that tPA has a 45-fold preference for binding **1c** as compared to **1b**, and a 2500-fold preference for **1c** over **1a**. This is the same trend seen for FXa, although the magnitude of the (*Z*,*Z*) preference is lower for tPA than for FXa. Our data support the suggested similarity between FXa and tPA in their relative affinities for (*Z*,*Z*), (*E*,*Z*), and (*E*,*E*) isomers of BABCH, and the corresponding expectation that the isomer bound in the tPA crystal complex is **1c**.

Table 5 shows that all of the geometrical isomers of **1** and **19** inhibit urokinase-type plasminogen activator (uPA) very weakly. In our earlier communication we proposed two potential binding modes for the BABCH olefin isomers in the active site of FXa and, based on

the 3D structure of uPA, neither of these binding modes would be favorable for these inhibitors, explaining the poor uPA inhibitory activity. Unlike the other serine proteases discussed previously, uPA has its S4 site effectively blocked by residues Leu 97B, Ala 98, and His 99,[20] so that an extended binding mode spanning the SI and S4 sites is not possible for uPA. In addition, the Glu 217 in FXa is an arginine residue in uPA, excluding the U-shaped binding mode.

The data in Table 1 clearly demonstrate that the (*E*,*E*) double bond isomers are significantly weaker inhibitors of factor Xa compared to the corresponding (*E*,*Z*) or (*Z*,*Z*) isomers. The difference is most pronounced in the five-, six-, and seven-membered rings (**1a**−**3a**) where the K_i values for these inhibitors are all within a factor of 40 of benzamidine (FXa K_i = 158 000 nM). Benzamidine has been shown to bind to serine protease inhibitors through a combination of a salt bridge interaction and hydrophobic interactions in the S1 pocket.[21] The high K_i values for **1a**−**3a** suggest that the interactions of these inhibitors with FXa are based primarily on one of the arylamidine substituents in the S1 pocket, with no other productive contacts of the inhibitor with the protease. This is consistent with the binding mode proposed in our recent communication,[2] with one of the arylamidine substituents bound in the S1 pocket, and the second arylamidine substituent being solvent exposed. The increased activity of the higher ring analogues, **4a**−**6a**, can be attributed to the increased flexibility of the cycloalkanone ring, allowing the inhibitors to potentially adopt conformation binding modes similar to the (*E*,*Z*) isomers. The lower ring analogues (**1a**−**4a**) are more potent inhibitors of trypsin when compared to FXa or thrombin, illustrating the difference between the binding pockets of these serine proteases. Interestingly, the selectivity difference between FXa and trypsin is reversed with the more flexible cyclononanone, and cyclodecanone analogues (**5a**, **6a**) demonstrate an approximate 4-fold increased inhibitory activity for FXa compared to trypsin.

The inhibitory activities for a series of 4-substituted cyclic analogues (**8**−**11**) are illustrated in Table 2. Consistent with the trend discussed above, the rank potency of FXa inhibitory activity for the geometrical isomers in this series is (*Z*,*Z*) > (*E*,*Z*) > (*E*,*E*). Each of the diverse substituents at the C-4 position of the (*Z*,*Z*) isomer, **8c**−**11c**, afforded a modest increase in the FXa inhibitory potency when compared with **3c**, suggesting that substitution at this position may favorably influence binding to FXa. In addition, the carboxylate analogue **8c** showed a dramatic increase in its thrombin selectivity (thrombin K_i > 50 000 nM). This selectivity toward thrombin appears to be based on a negative electrostatic interaction and not a steric interaction since neither the corresponding ester (**9c**) nor the benzyl or carbamate analogues (**10c** and **11c**, respectively) showed any significant difference in their thrombin activity.

Molecular dynamics simulations were performed to investigate the effects of a 4-carboxy substituent on (*Z*,*Z*) cyclohexanone binding to FXa and thrombin.[22] Complexes of FXa and thrombin with **3c** and its 4-carboxylate analogue **8c** were minimized and subjected to 500 ps of molecular dynamics to compare the effect of the carboxylate on inhibitor binding to these two proteases. The final structure of the FXa/**8c** complex is shown in Figure 1. In FXa, the binding modes of **3c** and **8c** were essentially the same, and they strongly resemble the extended binding mode previously found with MD simulations of **1c**.[2] Gln 192, which is Glu in thrombin, makes extensive hydrophobic contacts with **8c** and **3c** and forms a weak hydrogen bond with the carboxylate of **8c**. In contrast to the FXa results, the binding of **8c** in thrombin was significantly different from **3c**. During the simulation, the amidine−Asp 189 salt bridge in the S1 pocket began to break, indicating the instability of the bound structure. The inhibitor binding modes of **3c** and **8c** in FXa and **3c** in thrombin showed no such instability during the 500 ps simulations. Likewise, a 200 ps simulation of **10c**, which has a 4-phenyl substituent, in thrombin demonstrated no instability in its binding mode. These simulations support the data in Table 2 and suggest that the low affinity of **8c** for thrombin may be a result of an electrostatic repulsion between the carboxy group of the inhibitor and that of Glu 192, which is Gln in FXa and trypsin. The increased thrombin selectivity of Daiichi's compound, DX-9065a, was also significantly improved with the addition of a carboxylic acid group, and similar conclusions were reached from their modeling studies.[23] This observation provided us with insights that were advantageously used for the design of novel low molecular inhibitors with excellent thrombin selectivity.[7b]

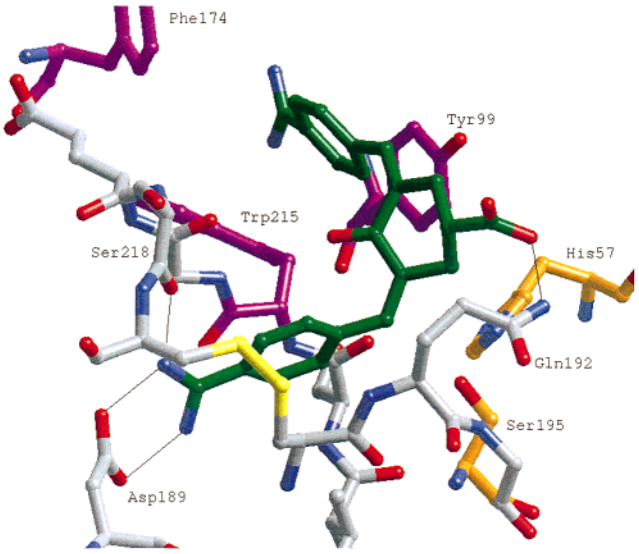


Figure 1. Model of binding mode of **8c** in FXa, from molecular dynamics simulation. The Cerius[2] program from MSI was used to create this figure.

The highly basic nature of the bisarylamidine inhibitors in Table 1 and 2 would present formidable obstacles toward the development of orally active agents. Replacement of one or both of the basic amidine substituents with a less polar substituent would increase the log *P* of the molecule and present a molecule that would have a better chance for absorption after oral administration. For example, the calculated logD (pH = 7.0) values for bisamidine **1c** and monoamidine **15c** are −2.93 and 0.45, respectively.[24] Therefore, we investigated a series of analogues where one or both of the amidine substituents are modified to better understand the structural requirements of the amidine substituents

with the hope that this data could be applied to other related amidine-based templates. The data from Table 3 again demonstrates the trend of potency for the different geometrical isomers ((*Z*,*Z*) > (*E*,*Z*) > (*E*,*E*)). In the (*Z*,*Z*) series, bis-replacement of the amidine substituent afforded inhibitors that were more than 3 orders of magnitude less active than **1c**, as seen with the bis-amide derivative **17c** (FXa K_i >5000 nM) and the bis-*N*,*N*-dimethylamidine analogue **18c** (FXa K_i = 1600 nM). This illustrates the importance of the salt bridge interaction of the amidine substituent in the S1 pocket for this series of inhibitors. Substitution of only one of the amidine groups with *N*-methyl or *N*,*N*-dimethyl (**13c** and **14c**, respectively), resulted in approximately 20-fold loss of activity against FXa when compared with **1c**, with minimal effect on the thrombin or trypsin inhibition. Further substitution of an amide group, **15c**, resulted in a 75-fold loss in activity against FXa and trypsin but showed a 10-fold increased potency toward thrombin.

Conclusions

SAR studies of these cycloalkanone series have shown that the in vitro FXa potency and selectivity are strongly dependent on the double bond geometry with the (*Z*,*Z*) isomers significantly more potent than the (*E*,*Z*) isomers. In general, the corresponding (*E*,*E*) isomers are weakly active inhibitors of FXa. Comparison of a series of (*Z*,*Z*) cycloalkanone analogues demonstrated that the FXa inhibitory activity varies significantly with the ring size with the cycloheptanone analogue (**1c**) as the most potent and selective compound from this series. Thrombin selectivity was achieved through addition of a carboxylate group to the 4-position of the cyclohexanone ring to afford compound **8c** (FXa K_i = 6.9 nM, thrombin K_i > 50 000 nM). In a series of (*Z*,*Z*)-cycloheptanone analogues, it was found that modification of both amidine substituents afforded inhibitors that were more than 3 orders of magnitude less active than **1c**, while modification of only one amidine substituent led to an 18−75-fold loss of potency. The (*Z*,*Z*) geometric isomers represent a new class of potent and selective inhibitors of FXa, and on the basis of good in vitro potency and selectivity, this conformationally rigid series has been used as a template for the design of a variety of chemically stable, potent, and selective inhibitors of FXa will be reported in future publications.

Experimental Section

General Methods. Proton magnetic resonance spectra (^{1}H NMR) were recorded in $CDCl_3$ or DMSO, as noted, on a Varian 300-MHz spectrometer (internal standard TMS, δ = 0). Elemental analyses were performed by Robertson Microlit Laboratories, Inc., Madison, NJ. HPLC analyses and preparative purifications were carried out using reverse-phase columns (Rainin, dynamax) using mixtures of acetonitrile and water, both of which contained 0.1% trifluoroacetic acid. Typically, the elution order for the three isomers was (*E*,*E*), (*E*,*Z*), and (*Z*,*Z*) isomer with the notable exception of **8a**, **8b**, and **8c** where the order is (*E*,*E*), (*Z*,*Z*), and (*E*,*Z*). Photochemical irradiation was carried out with a 150 W flood lamp or a medium-pressure, quartz, mercury vapor, 450 W lamp purchased from Ace Glass. Typically the irradiations were monitored by HPLC and continued until an equilibrium mixture of isomers was obtained.

Double Bond Isomers of 2,7-Bis(4-amidinobenzylidene)-cycloheptan-1-one (1a, 1b, and 1c). A suspension of **7** (4.2g, 22.8 mmol) in 20 mL of 85% phosphoric acid was stirred as neat cycloheptanone (1.27g, 11.4 mmol) was added. The reaction was heated at 100 °C for 3 h, allowed to cool, and diluted with methanol (50 mL) and ether (500 mL). The resulting solid was isolated by decanting. Purification was accomplished by treating a methanolic solution with charcoal, filtering through Celite, and precipitating with ether to yield **1a** (0.6 g). An aqueous acetonitrile solution of **1a** (0.53 g, 1.2 mmol) was irradiated with the 450 W lamp for 2 h. Purification by preparative HPLC using a 20−30% gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) yielded two new compounds, **1b** (0.2 g) and **1c** (0.25 g), which were isolated as white solids after lyophilization. **1a:** NMR (DMSO, TMS) δ 1.92 (s, 4H), 2.67 (s, 4H), 7.35 (s, 2H), 7.72 (d, 4H), 7.92 (d, 4H), 9.50 (br s, 8H). Anal. ($C_{23}H_{24}N_4O\cdot 2HCl\cdot H_2O$) C, H, N. **1b:** NMR (DMSO, TMS) δ 1.90 (br s, 4H), 2.51 (s, 2H), 2.79 (br s, 2H), 6.84 (s, 1H), 7.35 (d, 2H) 7.63 (s, 1H), 7.72 (m, 4H), 7.91 (d, 2H), 9.09 (s, 2H), 9.19 (s, 2H), 9.29 (s, 2H), 9.40 (s, 2H). Anal. ($C_{23}H_{24}N_4O\cdot 2C_2HF_3O_2\cdot 0.5H_2O$) C, H, N, F. **1c:** NMR (DMSO, TMS) δ 1.90 (m, 4H), 2.58 (m, 4H), 6.96 (s, 2H), 7.44 (d, 4H), 7.71 (d, 4H), 9.17 (br m, 8H). Anal. ($C_{23}H_{24}N_4O\cdot 2C_2HF_3O_2$) C, H, N, F.

Double Bond Isomers of 2,7-Bis(4-amidinobenzylidene)-cyclopentan-1-one (2a, 2b). A suspension of **7** (4.2 g, 22.8 mmol) in 20 mL of 85% phosphoric acid was stirred as neat cyclopentanone (0.94, 11.1 mmol) was added. The reaction was heated at 100 °C for 3 h, allowed to cool, diluted with methanol, and cooled in an ice bath for 30 min. The resulting solid was isolated by filtration and dried to give **2a**. An aqueous acetonitrile solution of **2a** (0.1 g, 0.24 mmol) was irradiated with a 150 W lamp for 24 h. Purification by preparative HPLC using 17% acetonitrile (0.1% TFA) in water (0.1% TFA) gave 44 mg of a white solid, **2b**, after lyophilization. **2a:** NMR (DMSO, TMS) δ 2.52 (m, 2H), 3.24 (m, 2H), 7.56 (s, 2H), 8.0 (m, 8H), 9.50 (br s, 8H). Anal. ($C_{21}H_{20}N_4O\cdot 2HCl\cdot H_2O$) C, H, N, Cl. **2b:** NMR (DMSO, TMS) δ 1.38 (m, 1H), 2.30 (m, 1H), 2.45 (m, 1H), 2.88 (m, 1H), 4.69 (s, 1H), 7.54 (s, 1H), 7.7 (m, 9H), 9.3 (m, 8H). Anal. ($C_{21}H_{20}N_4O\cdot 1.9C_2HF_3O_2\cdot 2.5H_2O$) C, H, N, F.

Double Bond Isomers of 2,7-Bis(4-amidinobenzylidene)-cyclohexan-1-one (3a, 3b, 3c). A suspension of **7** (4.2 g, 22.8 mmol) in 20 mL of 85% phosphoric acid was stirred as cyclohexanone (1.11 g, 11.3 mmol) was added at once. The reaction was heated at 100 °C for 3 h. The reaction was allowed to cool, diluted with 50 mL of methanol, and stirred for 30 min. The resulting solid was isolated by filtration and titrated with methanol and ether to yield 0.54 g of **3a**.

A aqueous acetonitrile solution of **3a** was irradiated with a 450 W lamp for 3 h. Purification by preparative HPLC using a 17−27% gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) yielded 0.3 g of **3b** and 80 mg of **3c** as solids after lyophilization. **3a:** NMR (DMSO, TMS) δ 1.77 (m, 2H), 2.94 (s, 4H), 7.69 (s, 2H), 7.8 (m, 8H), 9.36 (s, 4H), 9.54 (s, 4H). Anal. ($C_{22}H_{22}N_4O\cdot 2HCl\cdot 0.25H_2O$) C, H, N, Cl. **3b:** NMR (DMSO, TMS) δ 1.04 (m, 2H), 2.80 (m, 2H), 2.92 (m, 2H), 6.90 (s, IH), 7.42 (s, 1H) 7.60 (d, 2H), 7.75 (m, 4H), 7.88 (d, 2H), 9.13 (d, 4H), 9.38 (d, 4H). Anal. ($C_{22}H_{22}N_4O\cdot 1.9C_2HF_3O_2\cdot H_2O$) C, H, N, F. **3c:** NMR (DMSO, TMS) δ 2.30 (m, 2H), 2.82 (m, 4H), 6.82 (s, 2H), 7.6 (m, 8H), 9.4 (m, 8H). Anal. ($C_{22}H_{22}N_4O\cdot 2C_2HF_3O_2\cdot 0.6H_2O$) C, H, N, F.

Double Bond Isomers of 2,7-Bis(4-amidinobenzylidene)-cyclooctan-1-one (4a, 4b, and 4c). A suspension of **7** (1.0 g, 5.4 mmol) in 2.4 N hydrochloric acid was stirred as neat cyclooctanone (0.34 g, 2.6 mmol) was added. The reaction was heated at 100 °C for 8 h and allowed to cool over 10 h. The slurry was concentrated, dissolved in methanol, treated with charcoal, and filtered through Celite. Purification by reverse-phase HPLC using 30% acetonitrile (0.1% TFA) in water (0.1% TFA) yielded three compounds, **4a**, **4b**, and the monoalkylation product. A solution of the (*E*,*Z*) isomer (**4b**, 0.15 g, 0.4 mmol) in 20 mL of a 1:1 mixture of acetonitrile and water was irradiated with a 450 W lamp for 8 h. Purification by preparative HPLC using 30% acetonitrile (0.1% TFA) in water (0.1% TFA) gave **4c**, 40 mg, which was isolated as a white solid

after lyophilization. **4a:** NMR (DMSO, TMS) δ 1.62 (m, 2H), 1.75 (m, 4H), 2.72 (m, 4H), 7.08 (s, 2H), 7.65 (d, 4H), 7.87 (d, 4H), 9.13 (s, 4H), 9.36 (s, 4H). Anal. ($C_{24}H_{26}N_4O \cdot 2.3C_2HF_3O_2 \cdot 0.9H_2O$) C, H, N, F. **4b:** NMR (DMSO, TMS) δ 1.57 (m, 2H), 1.70 m, 4H), 2.56 (m, 4H), 6.70 (s, 1H), 7.65 (m, 9H). Anal. ($C_{24}H_{26}N_4O \cdot 1.9C_2HF_3O_2 \cdot H_2O$) C, H, N, F. **4c:** NMR (DMSO, TMS) δ 1.60 (m, 2H), 1.71 (m, 4H), 2.53 (m, 4H), 6.83 (s, 2H), 7.65 (m, 8H). Anal. ($C_{24}H_{26}N_4O \cdot 2.5C_2HF_3O_2 \cdot 1H_2O$) C, H, N, F.

Double Bond Isomers of 2,7-Bis(4-amidinobenzylidene)-cyclononan-1-one (5a, 5b, and 5c). A suspension of **7** (2.0 g, 10.8 mmol) in 2.4 N hydrochloric acid was stirred as neat cyclononanone (0.6 mL, 5.4 mmol) was added. The reaction was heated at 100 °C for 6 d and allowed to cool over 10 h. The slurry was concentrated, dissolved in methanol, treated with charcoal, and filtered through Celite. Product was precipitated with the addition of 1 N hydrochloric acid and purified by reverse-phase HPLC using 30% acetonitrile (0.1% TFA) in water (0.1 % TFA) to give 0.42 g of **5a** as a white solid after lyophilization. A solution of **5a** (0.42g, 0.6 mmol) in 15 mL of a 1:3 mixture of acetonitrile and water was irradiated with a 450 W lamp for 24 h. Purification by preparative HPLC using 30% acetonitrile (0.1% TFA) in water (0.1% TFA) yielded 0.1 g of **5b** and 80 mg of **5c** as white solids after lyophilization. **5a:** NMR (DMSO, TMS) δ 1.38 (m, 4H), 1.63 (m, 4H), 2.78 (m, 4H), 7.10 (s, 2H), 7.70 (m, 4H), 7.8 (m, 4H), 9.05 (br s, 4H), 9.32 (m, 4H). Anal. ($C_{25}H_{28}N_4O \cdot 2.1C_2HF_3O_2 \cdot H_2O$) C, H, N, F. **5b:** NMR (DMSO, TMS) δ 1.8 (m, 8H), 2.45 (m, 2H), 2.63 (m, 2H), 6.86 (s, 1H), 7.65 (m, 9H). Anal. ($C_{25}H_{28}N_4O \cdot 2.3C_2HF_3O_2 \cdot 1.3H_2O$) C, H, N, F. **5c:** NMR (DMSO, TMS) δ 1.56 (m, 4H), 1.68 (m, 4H), 2.43 (m, 4H), 6.86 (s, 2H), 7.60 (m, 8H). Anal. ($C_{25}H_{28}N_4O \cdot 2.7C_2HF_3O_2 \cdot 2H_2O$) C, H, N, F.

Double Bond Isomers of 2,7-Bis(4-amidinobenzylidene)-cyclodecan-1-one (6a, 6b, and 6c). A suspension of **7** (2.0 g, 10.8 mmol) in 2.4 N hydrochloric acid was stirred as neat cyclodecanone (0.8 mL, 5.4 mmol) was added. The reaction was heated at 100 °C for 3 d and allowed to cool over 10 h. The slurry was concentrated, dissolved in methanol, treated with charcoal, filtered through Celite, and precipitated with ether. Purification by reverse-phase HPLC using a gradient of 20−75% acetonitrile (0.1% TFA) in water (0.1% TFA) gave the (*E*,*E*) isomer (**6a**), 0.5 g, after concentration and lyophilization. A solution of **6a** (0.5 g, 0.7 mmol) in 15 mL of a 2:1 mixture of acetonitrile and water was irradiated with a 450 W lamp for 10 h. Purification by preparative HPLC using 30% acetonitrile (0.1% TFA) in water (0.1% TFA) gave 0.36 g of **6b** and 20 mg of **6c** as a white solid after lyophilization. **6a:** NMR (DMSO, TMS) δ 1.36 (m, 6H), 1.69 (m, 4H), 2.82 (m, 4H), 7.20 (s, 2H), 7.7 (m, 4H), 7.9 (m, 4H), 9.30 (s, 4H), 9.39 (s, 4H). Anal. ($C_{26}H_{30}N_4O \cdot 2.1C_2HF_3O_2 \cdot H_2O$) C, H, N, F. **6b:** NMR (DMSO, TMS) δ 1.5 (m, 10H), 2.82 (m, 2H), 2.63 (m, 2H), 7.16 (s, 1H), 7.65 (m, 9H), 9.5 (m, 8H). Anal. ($C_{26}H_{30}N_4O \cdot 2.1C_2HF_3O_2 \cdot 0.5H_2O$) C, H, N, F. **6c:** NMR (DMSO, TMS) δ 1.36 (m, 4H), 1.55 (m, 6H), 2.35 (m, 4H), 6.96 (s, 2H), 7.65 (m, 8H)), 9.5 (m, 8H). Anal. ($C_{26}H_{30}N_4O \cdot 3.2C_2HF_3O_2 \cdot H_2O$) C, H, N, F.

4-Amidinobenzaldehyde (7). A solution of 4-cyanobenzaldehyde (50 g, 0.37 mol) and methanol (44 mL, 1.09 mol) in 200 mL of a 1:3 mixture of ether and *p*-dioxane was cooled in an ice bath as hydrogen chloride gas was bubbled into the solution. The reaction was allowed to warm to ambient temperature over 16 h before solvent was removed by concentration. The residue was dissolved in 350 mL of methanol, saturated with ammonia gas, and heated at reflux for a total of 1.5 h with additional ammonia added in portions. The reaction was cooled to ambient temperature, the volume was reduced, and diluted with 1 L of acetone. The solid ammonium chloride was removed by filtration, and solvent was removed under reduced pressure. The residue was dissolved in 0.2 N aqueous hydrochloric acid solution, extracted with ethyl acetate (4×), concentrated, and lyopholyzed to yield 25 g of a white solid. **7:** NMR (DMSO, TMS) δ 8.1 (m, 4H), 9.5 (s, 2H), 9.70 (s, 2H), 10.13 (s, 1H).

Double Bond Isomers of 2,7-Bis(4-amidinobenzylidene)-4-carboxycyclohexan-1-one (8a and 8c). A slurry of ethyl 4-oxocyclohexancarboxylate (0.34 g, 2 mmol) and **7** (0.74 g, 4 mmol) in 5 mL of 4 N hydrochloric acid was heated at reflux for 50 min. Desired product precipitated upon cooling to yield **8a**, 0.4g, as a yellow solid. **8a:** NMR (DMSO, TMS) δ 2.85 (m, 1H), 3.1 (m, 4H), 7.7 (s, 2H), 7.85 (m, 8H), 9.18 (s, 4H), 9.32 (s, 4H). Anal. ($C_{23}H_{22}N_4O_3 \cdot 2HCl \cdot 1.65H_2O$) C, H, N, Cl.

A solution of **9c** (40 mg, 0.056 mmol) in 1 mL of aqueous THF was stirred at 0 °C as 0.8 mL of an aqueous lithium hydroxide solution (12.8 mg, 0.3 mmol) was added. After 30 min at 0 °C and 30 min at ambient temperature, the reaction was acidified with trifluoroacetic acid. Purification by preparative HPLC using a 5−35% gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) gave 0.03 g of the corresponding acid. **8c:** NMR (DMSO, TMS) δ 3.0 (m, 5H), 6.85 (s, 2H), 7.75 (m, 8H), 9.2 (m, 8H). Anal. ($C_{23}H_{22}N_4O_3 \cdot 2.35C_2HF_3O_2 \cdot 1H_2O$) C, H, N, F.

Double Bond Isomers of 2,7-Bis(4-amidinobenzylidene)-4-methoxycarboxy-cyclohexan-1-one (9a, 9b, and 9c). A solution of **8a** (0.52g, 1 mmol) in 50 mL of methanol was stirred at ambient temperature for 3 d. Solvent was removed, and residue was recrystallized from ethyl acetate to give 0.47 g of **9a** as a white solid. A solution of **9a** (0.52g, 1 mmol) in 300 mL of 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid was stirred and irradiated with a 450 W lamp for 4 h. Purification by preparative HPLC using a 5−35% gradient of acetonitrile (0.1% TFA) in water (0.1 % TFA) gave 0.12 g of **9b** and 0.1 g of **9c** after lyophilization. **9a:** NMR (DMSO, TMS) δ 2.95 (m, 1H), 3.1 (m, 4H), 3.58 (s, 3H), 7.7 (s, 2H), 7.85 (m, 8H), 9.3 (s, 4H), 9.56 (s, 4H). Anal. ($C_{24}H_{24}N_4O_3 \cdot 2HCl \cdot 2.4H_2O$) C, H, N, Cl. **9b:** NMR (DMSO, TMS) δ 2.95 (m, 1H), 3.1 (m, 4H), 3.62 (s, 3H), 6.95 (s, 1H), 7.48 (s, 1H), 7.6 (d, 2H), 7.72 (m, 4H), 7.88 (d, 2H), 9.1 (d, 4H), 9.36 (d, 4H). Anal. ($C_{24}H_{24}N_4O_3 \cdot 2C_2HF_3O_2 \cdot 0.8H_2O$) C, H, N, F. **9c:** NMR (DMSO, TMS) δ 3.0 (m, 4H), 3.2 (m, 1H), 3.58 (s, 3H), 6.85 (s, 2H), 7.75 (m, 8H), 9.3 (m, 8H). Anal. ($C_{24}H_{24}N_4O_3 \cdot 2.4C_2HF_3O_2 \cdot 1.5H_2O$) C, H, N, F.

Double Bond Isomers of 2,7-Bis(4-amidinobenzylidene)-4-phenyl-cyclohexan-1-one (10a, 10b, and 10c). A suspension of 7 (4.2 g, 22.8 mmol) and 4-phenylcyclohexanone (2 g, 11.5 mmol) in 45 mL of 2 N hydrochloric acid was stirred and heated at 100 °C for 2 h. The reaction was allowed to cool, diluted with 50 mL of methanol, and concentrated. The resulting solid was recrystallized from ethanol to give **10a**. A mixture of **10a** (0.5 g, 0.8 mmol) and 222 mL of a 50:50:1 mixture of acetonitrile, water, and trifluoroacetic acid was irradiated with 450 W lamp for a total of 5 h. Purification by preparative HPLC using a 20−45−95% gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) gave 40 mg of **10b** and 150 mg of **10c** as solids after lyophilization. **10a:** NMR (DMSO, TMS) δ 3.05 (m, 3H), 3.24 (m, 2H), 7.25 (m, 5H), 7.75 (m, 6H), 7.95 (m, 4H), 9.36 (s, 4H), 9.54 (s, 4H). Anal. ($C_{28}H_{26}N_4O$-EtOAc$\cdot$2.05HCl$\cdot$1.5H_2O) C, H, N, Cl. **10b:** NMR (DMSO, TMS) δ 3.1 (m, 5H), 6.95 (s, 1H), 7.22 (m, 1H) 7.35 (m, 4H), 7.45 (s, 1H), 7.6 (d, 2H), 7.7 (d, 2H), 7.84 (m, 4H), 9.18 (d, 4H), 9.38 (d, 4H). Anal. ($C_{28}H_{26}N_4O \cdot 2C_2HF_3O_2 \cdot 1.5H_2O$) C, H, N, F. **10c**: NMR (DMSO, TMS) δ 3.1 (m, 5H), 6.9 (s, 2H), 7.22 (m, 1H) 7.4 (m, 8H), 7.7 (m, 4H), 9.24 (d, 4H), 9.42 (d, 4H). Anal. ($C_{28}H_{26}N_4O \cdot 2C_2HF_3O_2 \cdot 0.3H_2O$) C, H, N, F.

Double Bond Isomers of 3,5-Bis(4-amidinobenzylidene)-*N*-ethoxycarbony-piperid-4-one (1a, 11b, and 11c). A suspension of **7** (3.26 g, 17.7 mmol) and *N*-ethoxycarbonylpiperidone (1 g, 5.8 mmol) in 45 mL of 2 N hydrochloric acid was stirred and heated at reflux for 4 h. The reaction was allowed to cool and was concentrated. Purification by precipitation with ethyl acetate from a methanolic solution gave 2.1 g of **11a**. A mixture of **11a** (0.5 g, 0.9 mmol) in 402 mL of a 100:100:1 mixture of acetonitrile, water, and trifluoroacetic acid was irradiated with a 450 W lamp for a total of 4 h. Purification by preparative HPLC using a 20−45−95% gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) gave 64 mg of **11b** lyophilization and 50 mg of **11c** as white solids after lyophilization. **11a:** NMR (DMSO, TMS) δ 0.9 (t, 3H), 3.84 (q, 2H), 4.75 (m, 4H), 7.65 (d, 4H), 7.75 (s, 2H), 7.9 (s, 4H), 9.06 (s, 4H), 9.39 (s, 4H). Anal. ($C_{24}H_{25}N_5O_3 \cdot 0.2$EtOAc$\cdot$2HCl$\cdot$

$0.5H_2O$) C, H, N, Cl. **11b:** NMR (DMSO, TMS) δ 1.1 (m, 3H), 4.08 (m, 2H), 4.56 (s, 2H), 4.74 (s, 2H), 7.15 (s, 1H), 7.55 (s, 1H) 7.75 (m, 6H), 7.95 (d, 2H), 9.38 (m, 8H). Anal. ($C_{24}H_{25}N_5O_3 \cdot 1.97C_2HF_3O_2 \cdot 1.8H_2O$) C, H, N, F. **11c:** NMR (DMSO, TMS) δ 1.22 (t, 3H), 4.12 (q, 2H), 4.52 (m, 4H), 7.13 (s, 2H), 7.62 (d, 4H), 7.75 (d, 4H), 9.2 (s, 4H), 9.35 (s, 4H). Anal. ($C_{24}H_{25}N_5O_3 \cdot 2C_2HF_3O_2 \cdot 1.2H_2O$) C, H, N, F.

2-(4-Cyanobenzylidene)cycloheptanone (12). A mixture of 4-cyanobenzaldehyde (10 g, 76 mmol) and cycloheptanone (29 mL, 76 mmol) in 100 mL of 85% phosphoric acid is heated for 1.5 h at 100 °C. The mixture is cooled to room temperature and filtered. The filtrate is poured into water (800 mL), and the resulting solid is isolated by filtration, washed with water, and dried. Purification by flash chromatography using a mixture of ether:hexane (1:2) as the eluant afforded 4.8 g of **12**. **12:** NMR ($CDCl_3$, TMS) δ 1.84 (m, 6H), 2.62 (m, 2H), 2.73 (m, 2H), 7.43 (m, 3H), 7.71 (d, 2H).

Double Bond Isomers of 2-(4-Amidinobenzylidene)-7-(4-(*N*-methylamidinobenzylidene)-cycloheptan-1-one (13a, 13b, and 13c). A solution of **12** (2.00 g, 9 mmol) in a mixture of anhydrous ethanol (17 mL) and anhydrous dioxane (34 mL) was cooled to 0 °C and saturated with anhydrous hydrogen chloride gas. The reaction mixture is allowed to stand for 60 h at 0−5 °C and then slowly added to 800 mL of a 3:1 mixture of ether:hexane. The resulting solid is isolated by filtration and dried in vacuo to afford the ethyl imidate as a solid. An ether solution of ethyl imidate (1.5 g) was washed with 10% aqueous sodium bicarbonate, dried, and concentrated. The residue is added to a solution of methylamine hydrochloride (0.42 g, 6.2 mmol) in 40 mL of methanol and heated for 3 h at 95 °C in a sealed vessel. The reaction mixture is cooled and the solvent removed. Purification of the residue by flash chromatography eluting with a 14:2:1:1 mixture of ethyl acetate:methanol:water:aqueous ammonium hydroxide afforded 2-(*N*-methylamidinobenzylidene)-cycloheptan-1-one as a solid.

A suspension of **7** in 15 mL of 85% phosphoric acid is stirred as 2-(*N*-methylamidinobenzylidene)-cycloheptan-1-one (1.24 g, 4.3 mmol) is added. The mixture is heated for 3.5 h at 100 °C and then cooled to room temperature. The reaction mixture is then diluted with 20 mL of methanol and ether. The resulting solid was isolated by decanting the liquid and purified by ether precipitation from a methanolic hydrochloric acid solution to afford **13a**. A solution of **13a** (0.27 g, 0.6 mmol) in methanol (55 mL) was irradiated with a 450 W lamp for 3 h. The solution is concentrated and purified by preparative reverse-phase HPLC, eluting with a 1:4 mixture of acetonitrile (0.1% TFA) in water (0.1% TFA) to give 0.1 g of **13b** and 97 mg of **13c** after lyophilization. **13a:** NMR (DMSO, TMS) δ 1.96 (m, 4H), 2.67 (m, 4H), 3.02 (m, 3H) 7.39 (s, 2H), 7.8 (m, 8H), 9.02 (s, 1H), 9.27 (s, 2H), 9.39 (s, 2H), 9.55 (s, 1H), 9.85 (m, 1H). Anal. ($C_{24}H_{26}N_4O \cdot 2C_2HF_3O_2 \cdot 1.25H_2O$) C, H, N, F. **13b:** NMR (DMSO, TMS) δ 1.92 (m, 4H), 2.55 (m, 2H), 2.71 (m, 2H), 3.02 (m, 3H), 6.82 (s, 1H), 7.6 (m, 9H), 9.45 (m, 7H). Anal. ($C_{24}H_{26}N_4O \cdot 2C_2HF_3O_2 \cdot 2H_2O$) C, H, N. **13c:** NMR (DMSO, TMS) δ 1.95 (m, 4H), 2.58 (m, 4H), 3.02 (m, 3H), 6.98 (s, 2H), 7.45 (m, 4H), 7.65 (m, 2H), 7.73 (m, 2H), (8.92 s, 1H), 9.15 (s, 2H), 9.34 (s, 2H), 9.42 (s, 1H), 9.78 (m, 1H). Anal. ($C_{24}H_{26}N_4O \cdot 2.2C_2HF_3O_2 \cdot 1.5H_2O$) C, H, N, F.

Double Bond Isomers of 2-(4-Amidinobenzylidene)-7-(4-(*N*,*N*-Dimethylamidinobenzylidene)-cycloheptan-1-one (14a, 14b, and 14c). A solution of **12** (1.2 g, 5.3 mmol) in 100 mL of anhydrous ethanol was cooled to 0 °C and saturated with anhydrous hydrogen chloride gas. The reaction mixture is allowed to warm to room temperature over 16 h and concentrated under reduced pressure. The residue was dissolved in 150 mL of absolute ethanol, treated with dimethylamine, and heated at reflux for 1 h. The reaction mixture is cooled and concentrated. The residue was dissolved in water, acidified with 1 N HCl, washed with ether, and neutralized with 1 N NaOH. Ether extraction gave 1.1 g of 2-(*N*,*N*-dimethylamidinobenzylidene)-cycloheptan-1-one. NMR (DMSO, TMS) δ 1.74 (m, 6H), 2.69 (m, 2H), 3.3 (m, 2H), 7.3 (s, 1H), 7.4 (m, 4H).

A mixture of **7** (1.0 g, 5.4 mmol) in 15 mL of 85% phosphoric acid was stirred as 2-(*N*,*N*-dimethylamidinobenzylidene)-cycloheptan-1-one (1.1 g, 5.4 mmol) was added. The mixture is heated for 3.5 h at 100 °C, then cooled to room temperature. The reaction mixture is then diluted with 20 mL of methanol and slowly added to a stirring ether solution. After 16 h, the solvent is decanted. Purification by preparative reverse-phase HPLC, eluting with a gradient of 15−40% of acetonitrile (0.1% TFA) in water (0.1% TFA), gave 160 mg of the (*E*,*E*) isomer (**14a**) and 60 mg of **14b** as solids. A solution of **14b** (0.2 g, 0.6 mmol) in methanol (20 mL) was irradiated with a 450 W lamp for 3 h. The solution is concentrated, and the residue purified by preparative reverse-phase HPLC, eluting with 20% of acetonitrile (0.1% TFA) in water (0.1% TFA). The (*Z*,*Z*) isomer (**14c**), 20 mg, was isolated as an amber solid after lyophilization. **14a:** NMR (DMSO, TMS) δ 1.90 (m, 4H), 2.5 (m, 2H), 2.65 (m, 2H), 3.02 (s, 3H), 3.25 (s, 3H) 7.38 (s, 2H), 7.75 (m, 6H), 7.95 (d, 2H), 9.02 (s, 1H), 9.3 (m, 5H). Anal. ($C_{25}H_{28}N_4O \cdot 3C_2HF_3O_2 \cdot 0.3H_2O$) C, H, N, F. **14b:** NMR (DMSO, TMS) δ 1.90 (m, 4H), 2.5 (m, 2H), 2.65 (m, 2H), 2.95 (s, 1.5H), 3.02 (s, 1.5H), 3.22 (s, 1.5H), 3.27 (s, 1.5H), 6.8 (s, 1H), 7.6 (m, 11H), 8.95 (m, 1H), 9.56 (m, 5H). Anal. ($C_{25}H_{28}N_4O \cdot 2.2C_2HF_3O_2 \cdot 1.0H_2O$) C, H, N, F. **14c:** NMR (DMSO, TMS) δ 1.97 (m, 4H), 2.61 (m, 4H), 2.98 (s, 3H), 3.22 (s, 3H), 6.95 (s, 1H) 7.00 (s, 1H), 7.65 (m, 6H), 7.76 (d, 2H), 8.93 (s, 1H), 9.3 (m, 5H). Anal. ($C_{25}H_{28}N_4O \cdot 2.5C_2HF_3O_2 \cdot 2.5H_2O$) C, H, N.

Double Bond Isomers of 2-(4-Amidinobenzylidene)-7-(4-carboxamidebenzylidene)-cycloheptan-1-one (15a, 15b, and 15c). A suspension of **7** (2.18 g, 11.8 mmol) in 16 mL of 85% phosphoric acid is stirred as **12** (2.66 g, 12 mmol) is added. The mixture is heated for 4 h at 100 °C and then cooled to 50 °C. The reaction mixture is slowly poured into water (75 mL). The resulting solid is isolated by filtration, washed sequentially with methanol and ether, and dried in vacuo to afford 1.9 g of **15a** as the phosphoric acid salt. The hydrochloride salt is obtained by treating a methanolic suspension (30 mL) with anhydrous hydrogen chloride gas and precipitating the salt with ether (300 mL). The resulting precipitate is isolated by filtration and dried in vacuo to afford **15a**. A solution of **15a** (0.50 g, 1.2 mmol) in methanol (5 mL) was irradiated with a 450 W lamp for 3 h. The solution is concentrated and purified by preparative reverse-phase HPLC, eluting with 23% of acetonitrile (0.1% TFA) in water (0.1% TFA), to give 97 mg of **15b** and 44 mg of **15c** after lyophilization. **15a:** NMR (DMSO, TMS) δ 1.94 (m, 4H), 2.69 (m, 4H), 7.34 (s, 2H), 7.46 (s, 1H), 7.60 (d, 2H), 7.74 (d, 2H), 7.95 (m, 4H), 8.10 (s, 1H), 9.28 (s, 2H), 9.49 (s, 2H). Anal. ($C_{23}H_{23}N_3O_2 \cdot HCl \cdot 0.5H_2O$) C, H, N, Cl. **15b:** NMR (DMSO, TMS) δ 1.95 (m, 4H), 2.42 (m, 2H), 2.70 (m, 2H), 6.74 (s, 0.5H), 6.80 (s, 0.5H), 7.67 (m, 11 H), 9.42 (m, 4H). Anal. ($C_{23}H_{23}N_3O_2 \cdot 1.2C_2HF_3O_2 \cdot H_2O$) C, H, N, F. **15c:** NMR (DMSO, TMS) δ 1.94 (m, 4H), 2.60 (m, 4H), 6.89 (s, 1H), 6.96 (s, 1H), 7.38 (d, 2H), 7.40 (s, 1H), 7.45 (d, 2H), 7.76 (m, 4H), 7.97 (s, 1H), 9.03 (s, 2H), 10.29 (s, 2H). Anal. ($C_{23}H_{23}N_3O_2 \cdot 1.2C_2HF_3O_2 \cdot H_2O$) C, H, N.

Double Bond Isomers of 2,7-Bis(4-aminocarboxybenzylidene)cycloheptan-1-one (17b and 17c). A mixture of 4-cyanobenzaldehyde (20.0 g, 153 mmol) and cycloheptanone (29 mL, 77 mmol) in 200 mL of 85% phosphoric acid is heated for 1.5 h at 100 °C. The mixture is cooled to room temperature. The resulting solid was isolated by filtration and washed with water and methanol to afford 2,7-bis-(4-cyanobenzylidene)-cycloheptanone (**16**). A solution of **16** (2.00 g, 6 mmol) in 15 mL of concentrated sulfuric acid is stirred for 3 h at room temperature. The reaction mixture is poured into 200 mL of water. The resulting solid was isolated by filtration, washed sequentially with water and methanol, and dried to afford the bis-benzamide, **17a**, as a solid.

A suspension of **17a** (0.60 g, 1.6 mmol) in methanol (40 mL) was irradiated with a 450 W lamp for 3 h. Purification by preparative reverse-phase HPLC, eluting with a gradient of 20−45% of acetonitrile (0.1% TFA) in water (0.1% TFA), yielded 61 mg of **17b** and 59 mg of **17c** after drying. **17b:** NMR (DMSO, TMS) δ 1.82 (m, 4H), 2.42 (m, 2H), 2.70 (m, 2H), 6.73 (s, 1H), 7.26 (d, 2H), 7.37 (br s, 1H), 7.48 (br s, 1H) 7.56 (s,

1H), 7.61 (d, 2H), 7.80 (d, 2H), 7.96 (m, 3H), 8.08 (br s, 1H). Anal. ($C_{23}H_{22}N_2O_3$) C, H, N. **17c:** NMR (DMSO, TMS) δ 1.82 (m, 4H), 2.60 (m, 4H), 6.84 (s, 2H), 7.38 (m, 6H), 7.78 (d, 4H), 7.97 (br s, 2H). Anal. ($C_{23}H_{22}N_2O_2 \cdot 0.3H_2O$) C, H, N.

Double Bond Isomers of 2,7-Bis[4-(*N*,*N*-dimethyl)-amidinobenzylidenelcycloheptan-1-one (18a, 18b, and 18c). A slurry of cycloheptanone (0.56 mL, 4.8 mmol) and **20** (2.04g, 9.6 mmol) in 15 mL of 3 N hydrochloric acid was heated at 100 °C for 3 h. The mixture was concentrated and purified by preparative reverse-phase HPLC to yield 190 mg of the (*E*,*E*) isomer, **18a**, and 30 mg of the (*E*,*Z*) isomer, **18b.** A solution of **18b** (50 mg, 0.1 mmol) in methanol (15 mL) was irradiated with a 450 W lamp for 3 h. Purification by preparative reverse-phase HPLC, eluting with a gradient of 20−45% of acetonitrile (0.1% TFA) in water (0.1% TFA), yielded 10 mg of **18c** after drying. **18a:** NMR (DMSO, TMS) δ 1.92 (m, 4H), 2.7 (m, 4H), 3.0 (s, 6H), 3.2 (s, 6H), 7.33 (s, 2H), 7.68 (m, 8H), 9.1 (s, 2H), 9.4 (s, 2H). Anal. ($C_{27}H_{32}N_4O \cdot 3.2C_2HF_3O_2 \cdot H_2O$) C, H, N, F. **18b:** NMR (DMSO, TMS) δ 1.92 (s, 4H), 2.5 (s, 2H), 2.76 (s, 2H), 2.98 (s, 3H), 3.2 (m, 3H), 3.22 (s, 3H), 3.26 (s, 3H), 6.78 (s, 1H), 7.46 (m, 4H), 7.6 (s, 1H), 7.68 (m, 4H), 8.92 (s, 2H), 9.3 (s, 2H). Anal. ($C_{27}H_{32}N_4O \cdot 3C_2HF_3O_2$) C, H, N. **18c:** NMR (DMSO, TMS) δ 1.82 (m, 4H), 2.56 (m, 4H), 3.0 (s, 6H), 3.2 (s, 6H), 6.93 (s, 2H), 7.54 (m, 8H), 8.92 (s, 2H), 9.3 (s, 2H). Anal. ($C_{27}H_{32}N_4O \cdot 2.75C_2HF_3O_2$) C, H, N, F.

Double Bond Isomers of 2,7-Bis(3-amidinobenzylidene)-cyclonoheptan-1-one (19a, 19b, and 19c). A suspension of 3-amidinobenzaldehyde (2.32 g, 12.5 mmol) in 12 mL of 85% phosphoric acid is stirred as cycloheptanone (0.70 g, 6.2 mmol) was added. The mixture is heated for 3.5 h at 100 °C, then cooled to room temperature. The dark red reaction mixture is diluted with methanol (20 mL) and ether (75 mL). The resulting solid was isolated by filtration, washed with methanol/ethyl acetate (1:1), and dried in vacuo to afford 1.2 g of **19a**. The hydrochloride salt was obtained by treatment of a methanolic solution with anhydrous hydrogen chloride gas and isolated by filtration. A solution of **19a** (0.4 g, 0.9 mmol) in 500 mL of methanol was irradiated with a 150 W lamp for 14 d. Purification by preparative HPLC using a 15−22% gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) gave 27 mg of **19b** and 57 mg of **19c** after lyophilization. **19a:** NMR (DMSO, TMS) δ 1.95 (m, 4H), 2.71 (m, 4H), 7.33 (s, 2H), 7.8 (m, 6H), 7.93 (s, 2H), 9.38 (s, 4H), 9.53 (s, 4H). Anal. ($C_{23}H_{24}N_4O \cdot 2HCl$) C, H, N, Cl. **19b:** NMR (DMSO, TMS) δ 1.92 (m, 4H), 2.50 (m, 2H), 2.76 (m, 2H) 6.80 (s, 1H), 7.70 (m, 9H), 9.19 (s, 4H), 9.38 (d, 4H). Anal. ($C_{23}H_{24}N_4O \cdot 2C_2HF_3O_2 \cdot 1.4H_2O$) C, H, N. **19c:** NMR (DMSO, TMS) δ 1.92 (m, 4H), 2.62 (m, 4H), 6.95 (s, 2H), 7.66 (m, 8H), 9.23 (s, 4H), 9.38 (s, 4H). Anal. ($C_{23}H_{24}N_4O \cdot 2C_2HF_3O_2 \cdot H_2O$) C, H, N.

4-(*N*,*N*-Dimethylamidino)benzaldehyde (20). A solution of 4-(methoxyimidate)benzaldehyde (10 g, 50 mmol) in 18 mL of trimethylorthoformate and 100 mL of methanol was stirred for 20 h. The reaction was filtered into ether (500 mL). The resulting precipitate was isolated by filtration to give 5.6 g of the corresponding acetal hydrochloride salt after drying. An ethanolic solution of the acetal hydrochloride salt (5 g) was treated with dimethylamine and heated at 90 °C for 2.5 h. Solvent was removed under reduced pressure. The residue was dissolved in 100 mL of 1 N hydrochloric acid and was heated at 60 °C for 3 h. The reaction was concentrated and lyophilyzed to yield 2.8 g of **20**. **20:** NMR (DMSO, TMS) δ 2.95 (s, 3H), 3.25 (s, 3H), 7.8 (d, 2H), 8.10 (d, 2H), 9.4 (s, lH), 9.58 (s, 1H), 10.12 (s, 1H).

Biological Measurements. Serine Protease Inhibition Assays. Care was taken during the assays to minimize exposure of cyclic ketone analogue solutions to light. Human factor Xa and human thrombin were from Enzyme Research Laboratories, Inc. (South Bend, IN), bovine trypsin was from Roche Molecular Biochemicals (Indianapolis, IN), and tissue plasminogen activator (tPA) and urokinase (uPA) were both from Sigma (St. Louis, MO). All five serine proteases were assayed in 150 mM NaCl, 2.5 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.5, and 0.1% PEG 6000, in a final assay volume of 200 μL at room temperature (~23 °C) in a THERMOmax 96-well microplate reader (Molecular Devices, Sunnyvale, CA). All substrates were Chromogenix substrates from Kabi Pharmacia Hepar, Inc. (Franklin, OH). The final concentration of enzyme and substrate used are as follows: factor Xa (1 nM) and S-2222 (164 μM), thrombin (16 nM) and S-2302 (300 μM), trypsin (16 nM) and S-2266 (127 μM), tPA (10 nM) and S-2288 (371 μM), and uPA (2 nM) and S-2444 (32 μM). These bisamidine-type inhibitors were found to have competitive mechanism against the arginine containing substrates used (data not shown); therefore, the K_m for each substrate was determined in these assay conditions, and the substrate concentration equal to K_m was used to assay varying concentrations of the inhibitors to determine the K_i. In the case of the very potent compound **1c**, the K_i for factor Xa was determined by both decreasing the enzyme concentration to 0.1 nM and by fitting data obtained at 0.3 and 1 nM enzyme to a modification of the Morrison equation.[17]

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