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Identification of Inhibitors of Drug-Resistant *Candida albicans* Strains from a Library of Bicyclic Peptidomimetic Compounds

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The screening of a library of small molecule peptidomimetics toward secreted aspartic proteinase-2 (SAP2) of *Candida albicans* allowed us to identify two compounds that showed in vitro inhibitory potency comparable to pepstatin A. In an experimental model of vaginal candidiasis, the two candidate compounds were as active as a therapeutic dose of fluconazole. Importantly, this activity was fully preserved when the challenger was a fluconazole-resistant strain of the fungus. Altogether, our data demonstrate SAP2 as a valid *C. albicans* target for the development of new drugs against this important human pathogen.

Introduction

Candida albicans is an opportunistic fungal pathogen that causes severe mucosal and systemic infections especially in immunocompromised subjects.[1] Although a certain number of antifungal agents are available, the need for new drugs against *C. albicans* is escalating because of both the widespread occurrence of mucosal infections caused by this fungus and the development of resistance against available drugs.[2] In fact, despite drug availability, *C. albicans* ranks as a highly frequent cause of morbidity, cost of hospitalization, and mortality.[3] Although the ability to cause disease is likely a complex process involving multiple interactions between *Candida* and the host, secretory aspartic proteinases (SAPs[a]) appear to be a major determinant in the virulence of this fungus, allowing it to adhere and invade host tissues.[4] Therefore, this family of enzymes offers a potential target for drug intervention in infections, mostly where current treatments fail owing to resistance to available drugs.[5] The recognized role of aspartic proteinase inhibitors for AIDS treatment and the homology between HIV and *Candida* proteinases[6] add further interest to this approach.

C. albicans expresses 10 distinct SAP genes (SAP1−10) in vitro and in vivo, but there is evidence that different SAP isoenzymes have different functions and can be differently expressed in different infections (e.g., mucosal versus systemic) or even at different stages of the same infection.[7] In particular, SAP2 is one of the most expressed enzymes implicated in host invasion. It has been recognized as a crucial virulence factor for vaginal infection,[8] and both reversible and irreversible inhibitors have been reported.[9]

With the aim of discovering new molecules active against drug-resistant *C. albicans*, we envisaged the possibility of targeting SAP2 with small-molecule peptidomimetic inhibitors. An efficient method for the generation of a library of peptidomimetics based on the 6,8-dioxa-3-azabicyclo[3.2.1]-octane scaffold was recently reported by our group. This method consists of a collection of amides obtained by the parallel combination of bicyclic methyl esters with various amines through a solvent-free synthetic reaction.[10] The availability of a wide variety of primary and secondary amines allowed us to synthesize a large and highly diverse small-molecule library based on this bicyclic scaffold. Dipeptide isosteres are particularly attractive because of the bicyclic acetal portion acting as a potential transition state analogue in the interaction with enzyme active sites.

Results and Discussion

For the first series of experiments, 17 pools, each consisting of 2−5 compounds, were selected to screen for in vitro inhibition of SAP2 activity. The pools were assembled in a way such that each pool contained similar scaffolds (Chart 1) decorated with different substituents at position 7 (Chart 2). The selected scaffolds differed either by the presence of the carbonyl group at position 2 or by hydrophobic substituents at the nitrogen atom and at positions 4 and 5.

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[a] Abbreviations: SAP, secreted aspartic proteinase; AIDS, acquired immune deficiency syndrome; HIV, human immunodeficiency virus; BTK, bicycles from L-tartaric acid and amino ketones; BTG, bicycles from L-tartaric acid and glycine; BtG, bicycles from D-tartaric acid and glycine; BTF, bicycles from L-tartaric acid and L-phenylalanine (the *O* in parentheses refers to the presence of a carbonyl group at the C-2 position of the bicyclic scaffold); OD, optical density; DMF, dimethylformamide; DCM, dichloromethane; BSA, bovine serum albumin; SD, standard deviation; YPD, yeast peptone dextrose; CFU, colony forming unit.

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Chart 1. Selected Pools of Compounds Containing Different Bicyclic Scaffolds

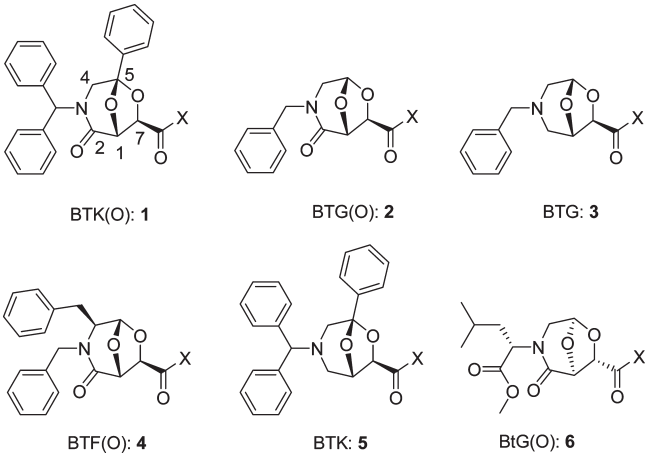
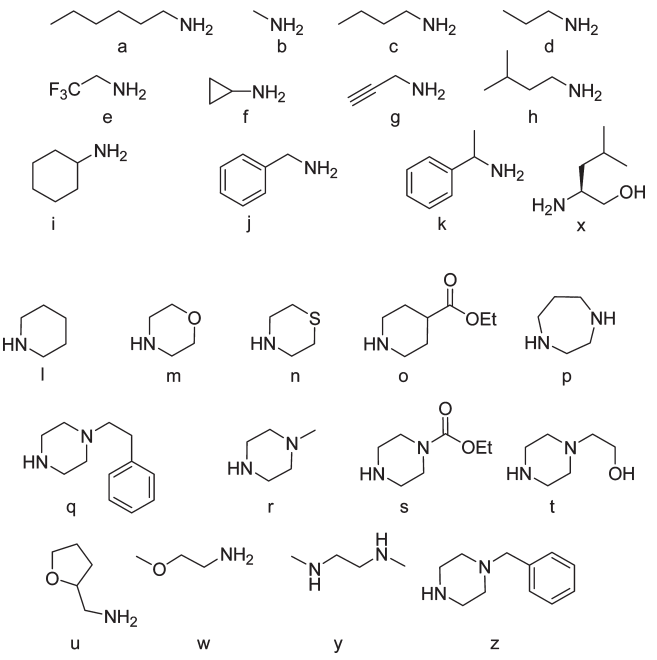


Chart 2. Primary and Secondary Amines Used as the X Component



Compounds belonging to pools XVI and XVII (Table 1) were prepared using L-leucine methyl ester as the starting material to build the bicyclic scaffold (Scheme 1). Briefly, leucine methyl ester hydrochloride (**7**) was treated with bromoacetaldehyde dimethylacetal at 120 °C in DMF and in the presence of Et_3N and KI. Amine **8** was then converted into amide **9** through a coupling reaction with (2*S*,3*S*)-di-*O*-acetyltartaric anhydride, which was subsequently treated with thionyl chloride in MeOH to give the cyclic acetal **10**. Final trans-acetalization to give the bicyclic scaffold **11** was achieved by treating **10** in refluxing toluene for 30 min in the presence of H_2SO_4 over silica gel. Amides for pools XVI and XVII were synthesized by heating methyl ester **22** in the presence of neat amine at 60 °C for 18 h.

As reported in Table 1, pools I and II, differing by the substitution level at C-7, did not show any relevant inhibitory potency with respect to pepstatin A, indicating a detrimental effect of the phenyl ring at position 5 of the bicyclic scaffold. This detrimental effect was also noticed for pools XIV and XV. Compounds of pools III and IV showed moderate to low inhibitory potency, whereas pools V and VI, containing the same scaffold, showed good inhibitory activity. These results suggested that the above scaffold could be a good template for ligand discovery. They also indicated the C-7 substituents in pools V and VI as good variants for further analysis. The lack of efficacy of pools VII and VIII, in conjunction with the inactivity of pools XIV and XV, provided preliminary evidence that the carbonyl group at C-2 of the scaffold is crucial for SAP2 binding affinity. Pools IX and X−XIII contain the BTF(O) scaffold, which is derived from the combination of phenylalanine and tartaric acid derivatives. These pools showed interesting inhibitory potency, suggesting the compatibility of the phenyl ring at position 4 of the scaffold.

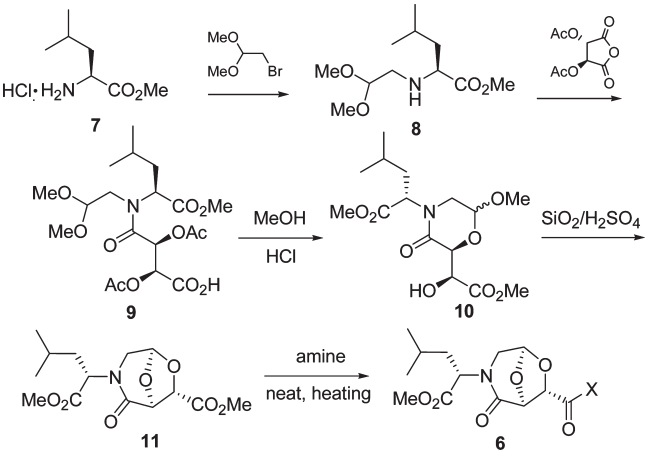
The variation of the substituent at the nitrogen atom was taken into account in generating the pools XVI and XVII with a Leu isostere at position 3 of the BTG(O) scaffold (Table 1). The fair inhibitory activity of these pools indicated the possibility of the modulation at the nitrogen atom of the bicyclic template with other nonpolar substituents. The results obtained with pools V and VI and with pools X and IX (which

Table 1. Selected Pools for the First Screening to SAP2

pool	compd	no. of elements	% inhibition[a]	normalized % inhibition[b]
I	**1a**, **1i**, **1k**	3	0	0
II	**1m**, **1s**, **1t**, **1o**	4	19	5
III	**2**-NH_2, **2b**, **2c**, **2j**, **2h**	5	18	4
IV	**2p**, **2s**	2	19	10
V	**2m**, **2n**, **2t**	3	92	31
VI	**2c**, **2a**, **2e**, **2k**	4	87	22
VII	**3d**, **3f**, **3g**, **3h**	4	13	3
VIII	**3l**, **3m**, **3n**, **3y**	4	6	2
IX	**4g**, **4c**, **4a**, **4e**, **4k**	5	42	8
X	**4m**, **4n**, **4t**	3	40	13
XI	**4d**, **4i**	2	73	36
XII	**4f**, **4h**, **4b**	3	87	29
XIII	**4p**, **4o**, **4s**, **4y**, **4x**	5	87	17
XIV	**5e**, **5d**, **5g**, **5c**	4	12	3
XV	**5l**, **5n**, **5o**, **5y**	4	23	6
XVI	**6m**, **6q**	2	80	40
XVII	**6r**, **6z**	2	57	28

[a] The inhibitory potency was determined using the ΔOD value, compared with the difference between ΔOD control proteinase − ΔOD (control proteinase + peptsatin A), which was taken as the 100% inhibition value. The concentration of each compound in the mixtures was 10 μM. [b] This value corresponds to the % inhibition normalized to the value of a single compound at 10 μM.

Scheme 1. Synthesis of Leucine-Derived Peptidomimetics of Pools XVI and XVII



contained, respectively, a different template but similar substituents) indicated that the scaffold BTG(O), together with the selected functional groups at position 7 of the molecule, is endowed with better structural requisites. Specifically, pool V (inhibition of 31%) showed better activity than pool X (inhibition of 13%), and pool VI showed higher potency (inhibition of 22%) as compared to pool IX (inhibition of 8%).

On the basis of initial evidence that a specific set of scaffolds may express a fair inhibitory activity, single compounds were screened to identify the best flanking units for scaffold decoration. This step also served to obtain more insights into the structure−activity relationship, hence facilitating the selection of the best hit compounds for in vivo experiments. The molecules possessing the *N*-benzyl-BTG(O) scaffold **2** were thus selected for the screening of single compounds and for assessing the preferred substituents at position 7 (Figure 1 and Table 2).

All compounds showed moderate to good inhibitory potency, compared to pepstatin A. Among secondary amines, *N*-carboethoxypiperazine- and piperidine-derived compounds showed the highest inhibitory potency, as found for **2s** and **13**, respectively. Furthermore, the presence of hydroxylic groups around position 7 indicated a favorable effect, as shown by the remarkable inhibitory potency of **14** and **15**, containing the glycinol and leucinol moiety, respectively. The discrepant inhibitory activities of compounds **14**−**23**, which contain diverse amino alcohol derived substituents, suggested that the inhibitory potency could be affected by the hydrophobic content. The stereochemistry of the substituents did not influence the inhibitory potency, as suggested by the comparable inhibition values found for compounds **18**, **19** and **21**, **22**, corresponding to both enantiomers of proline and phenylalanine, respectively. More interestingly, N-benzylation of the leucinol derivative caused a significant loss of inhibition, as shown by the comparison of the activity of **23** (35%) with that of **15** (71%).

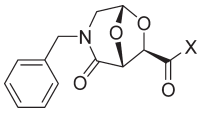
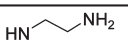
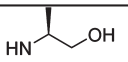
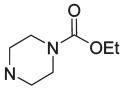
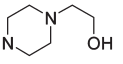
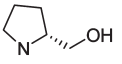
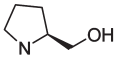
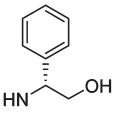
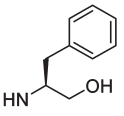
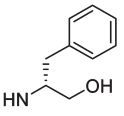
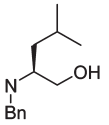
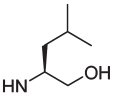


Figure 1. Selected scaffold for SAP2 inhibition.

The systematic evaluation of the library members for SAP2 inhibition resulted in the identification of compounds **2s**, **13**, **14**, and **15** as those possessing an inhibitory potency above 70% at 20 μM, thus being comparable to pepstatin A activity. The quantification of the inhibitory potency of two selected compounds indicated molecule **13** as the compound possessing slightly better binding capability than compound **15**, both expressing an IC_{50} value in the low micromolar range (Table 3).

Finally, the stereochemical scanning of all the stereoisomers of compounds **13** and **15** indicated a slight modulation of SAP2 inhibition by the stereochemical arrangement of the pharmacophoric elements, since moderate to good inhibition

Table 2. Array of Bn-BTG(O)-X Scaffolds toward SAP2

Compound	X	% Inhibition[a]	Compound	X	% Inhibition[a]
12		63	**16**		35
2s		74	**17**		23
2t		58	**18**		34
2p		63	**19**		48
2m		62	**20**		31
2n		58	**21**		30
13		74	**22**		29
14		71	**23**		35
15		71			

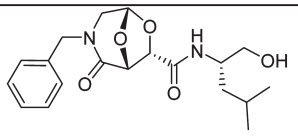
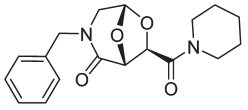
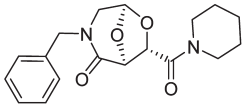
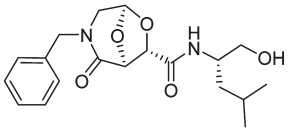
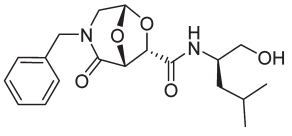
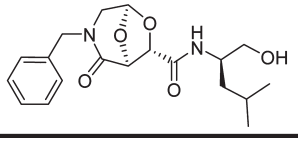
[a] The inhibitory potency was determined using the ΔOD value, compared with the difference between ΔOD control proteinase − ΔOD (control proteinase + peptsatin A), which was taken as the 100% inhibition value. The compounds were screened at 20 μM.

Table 3. Concentration-Dependent Inhibitory Potency of **13** and **15** toward SAP2

	% inhibition[a]	
concn, μM	**13**	**15**
20	80	74
10	67	61
2	50	6.9
0.2	19	
0.02	0	1.4

[a] The inhibitory potency was determined using the ΔOD value, compared with the difference between ΔOD control proteinase − ΔOD (control proteinase + peptsatin A), which was taken as the 100% inhibition value.

Table 4. Stereochemical Scanning of **13** and **15** toward SAP2[a]

Entry		% inhibition
15		71
13		74
24		50
25		33
26		29
27		60

[a] The inhibitory potency was determined using the ΔOD value, compared with the difference between ΔOD control proteinase − ΔOD (control proteinase + peptsatin A), which was taken as the 100% inhibition value. The compounds were screened at 20 μM.

was observed with all the compounds (Table 4). The two hit compounds **13** and **15** identified in the first run of screening were confirmed as the best stereoisomers in the hit identification for subsequent in vivo trials, as demonstrated by inhibition values of **24** compared to **13** and of **25**−**27** compared to **15**.

The optimization of enzymatic assays carried out on different pools of structurally analogous compounds allowed us to draw a preliminary structure−activity relationship of BTAa scaffolds, thus identifying some elements favoring or disfavoring SAP2 inhibitory activity. Specifically, it was found that the CO group at position 2 of the scaffold is crucial for binding activity, whereas position 7 can lead to a modulation of the inhibitory potency. The two enantiomeric BTG(O) and BtG(O) scaffolds did not determine dramatic differences in enzyme recognition, as demonstrated by the good inhibition expressed by pools XVI and XVII containing the enantiomeric BtG(O) scaffold. This suggests a "pseudosymmetric" interaction in the catalytic site of SAP2. BTF(O) scaffold showed some inhibitory potency, though highly influenced by the substituents at position 7 of the scaffold, thus indicating the possibility of placing a hydrophobic group at position 4-exo of the scaffold. On the contrary, the phenyl ring at the vicinal position 5 determined a loss of activity, suggesting a strict topological requirement for the aromatic group.

In vivo experiments performed with the rat model of estrogen-dependent *C. albicans* vaginitis showed that the two hit compounds were the most effective inhibitors of the experimental infection. In particular, they caused a rapid decay of vaginal *C. albicans* burden. The kinetics of fungus clearance in rats intravaginally treated with compounds **13** and **15** was similar to the kinetics of the fungus clearance in rats treated with the comparator SAP2 inhibitor, pepstatin A. It was also similar to the kinetics of infection in rats treated with fluconazole (Table 5 and Figure 2). More importantly, the two compounds showed marked acceleration of fungus clearance in rats challenged with the fluconazole-resistant AIDS68 strain of *C. albicans*, similar to the activity shown against the fluconazole-susceptible strain of the fungus (Table 6 and Figure 3).

Conclusions

In conclusion, the screening of a library of bicyclic peptidomimetics for inhibition of SAP2 enzyme allowed us to select two hit compounds possessing in vitro activity comparable to pepstatin A, a prototypal inhibitor of aspartic proteinases. In a model of rat vaginal infection caused by fluconazole-susceptible or -resistant *C. albicans* strains, the two leads were highly effective, demonstrating the potential of the two peptidomimetics to cure both drug-susceptible and drug-resistant infection.

No precise insight into the mechanism of inhibition has been addressed here; therefore, it remains unknown whether our peptidomimetic inhibitors of SAP2 would express similar inhibitory activity against other members of SAP family.[13] However, some degree of inhibition of at least SAP1 and SAP3 activity is likely, given: (i) the high similarity of SAP2 to SAP1 and SAP3; (ii) the dominant role, as virulence factor, played by the latter two SAPs, together with SAP2, in our rat vaginits model.[13]

Overall, the results of this library screening allowed us to identify a new scaffold that is of interest in the research of novel aspartic proteinase inhibitors. The two peptidomimetics identified here as inhibitors of fluconazole-resistant *C. albicans* strains are being considered for subsequent trials in the drug development process.

Experimental Section

Chemistry. Pools of bicyclic compounds were prepared following the reported methods for the generation of BTK and BTAa scaffolds.[11] Libraries from libraries of peptidomimetics

Table 5. Acceleration of *Candida* SA40 Clearance in Rats Intravaginally Treated with **13** and **15** after Challenge (1, 24, 48 h)[a]

days	SA40 + **15**	SA40 + **13**	SA40 + pepstatin A	SA40	SA40 + fluconazole
0	>100	100 ± 0	>100	>100	100 ± 0
1	70.0 ± 1.3	61 ± 2.6	56.8 ± 2	>100	53.5 ± 4.4
2	57.6 ± 1.4	58 ± 1.8	51.0 ± 1.2	>100	49.7 ± 4.1
5	39.2 ± 3	35.8 ± 2.2	32.4 ± 2.5	80.0 ± 2.6	25.7 ± 1.7
7	30.6 ± 1.8	28.8 ± 1.2	28 ± 1.5	66.0 ± 2.1	11.7 ± 0.8
14	14.4 ± 1.6	14.0 ± 2.3	9.4 ± 1.4	26.2 ± 1.8	5.0 ± 2.4
21	8.0 ± 1.5	4.2 ± 2.1	5.0 ± 1.3	12.8 ± 1.2	1.25 ± 0.9
28	1.2 ± 0.7	2.2 ± 0.9	0	5.8 ± 1.6	0 ± 0

[a] All values are CFU/mL ×1000. SA40: untreated control. Starting day 1, all differences between **13**- and **15**-treated and untreated control are statistically significant ($P < 0.01$, Mann−Withney *U* test). No statistically significant difference is detected between pepstatin A- and **13**- or **15**-treated rats.

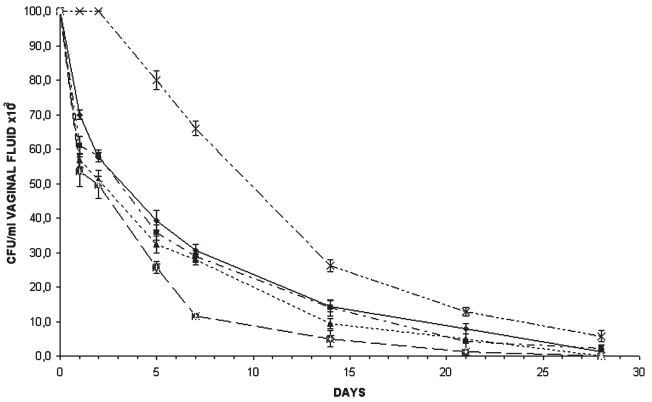


Figure 2. Vaginal infection with *C. albicans* SA40 in rats intravaginally treated with **13** and **15** after challenge (1, 24, 48 h): (◆) SA40 + **15**; (■) SA40 + **13**; (▲) SA40 + pepstatin A; (×) SA40; (∗) SA40 + fluconazole.

achieved by means of functionalization of the carboxylic group at position 7 have been reported.[10] Combustion analysis was used to determine purity. All tested compounds were >95% pure.

2-(2,2-Dimethoxyethylamino)-4(*S*)-methylpentanoic Acid Methyl Ester (8). A solution containing L-leucine methyl ester hydrochloride **7** (2.9 g, 16 mmol), bromoacetaldehyde dimethylacetal (1.9 mL, 2.7 g, 16 mmol), Et_3N (6.7 mL, 48 mmol), and a catalytic amount of KI in DMF (190 mL) was stirred at 120 °C for 3 days. The reaction mixture was concentrated under reduced pressure, diluted with water, and extracted with DCM. The organic layer was then washed with brine, dried over Na_2SO_4, and evaporated. The crude product was purified by column chromatography (silica gel, EtOAc/petroleum ether 1:1) to afford compound **8** as a yellow oil (1.2 g, 32% yield). $[\alpha]_D^{24}$ −3.32 (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 4.38 (t, J = 6 Hz, 1 H), 3.65 (s, 3 H), 3.30 (s, 3 H), 3.29 (s, 3 H), 3.24 (t, J = 6 Hz, 1 H), 2.68 (dd, J = 6 Hz, 1 H), 2.52 (dd, J = 6 Hz, 1 H), 1.71−1.55 (m, 2 H), 1.44−1.37 (m, 2 H), 0.86 (d, J = 4 Hz, 3 H), 0.83 (d, J = 4 Hz, 3 H). ^{13}C NMR ($CDCl_3$, 200 MHz): δ 175.9 (s), 103.6 (d), 59.9 (d), 54.0 (q), 53.1 (q), 51.7 (q), 49.3 (t), 42.8 (t), 25.0 (d), 22.8 (q), 22.5 (q). MS *m*/*z* 233 (0.5), 202 (7.2), 174 (33), 158 (14), 75 (100). IR ($CHCl_3$) 2915, 1729, 1130, 1065 cm^{-1}. Anal. Calcd for $C_{11}H_{23}NO_4$ (233.30): C, 56.63; H, 9.94; N, 6.00. Found: C, 57.09; H, 9.90; N, 6.14.

(1*S*,5*R*,7*S*)-3-(1-Methoxycarbonyl-3(*S*)-methylbutyl)-2-oxo-6,8-dioxa-3-azabicyclo[3.2.1]octane-7-carboxylic Acid Methyl Ester (11). To a suspension of (2*S*,3*S*)-2,3-di-*O*-acetyltartaric anhydride (1 g, 4.7 mmol) in dry DCM (4.5 mL) was added, at 0 °C and under a nitrogen atmosphere, a solution of **8** (1 g, 4.7 mmol) in dry DCM (2.5 mL). The reaction mixture was stirred at room temperature overnight. After evaporation of the solvent, crude **9** was dissolved in MeOH (8 mL), and thionyl chloride (292 μL, 4 mmol) was added dropwise at 0 °C. The mixture was then allowed to reach 60 °C and stirred for 2 h. The solvent was removed, and the crude **10** was isolated as a yellow oil and used without further purification in the next step. A solution of **10** (1.63 g, 4.7 mmol) in toluene (8 mL) was quickly added to a refluxing suspension of SiO_2/H_2SO_4 (1 g), prepared as reported,[12] in toluene (12 mL). The mixture was allowed to react for 30 min, and then one-third of the solvent was distilled off. The hot reaction mixture was filtered through a pad of $NaHCO_3$ and after evaporation of the solvent, the crude product was purified by flash chromatography (silica gel, EtOAc/petroleum ether 1:2), affording **11** as a white solid (730 mg, 50% yield over three steps). $[\alpha]_D^{24}$ +22.0 (*c* 1.0, MeOH). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 5.88 (d, J = 2 Hz, 1 H), 5.09 (t, J = 8 Hz, 1 H), 4.87 (s, 1 H), 4.59 (s, 1 H), 3.72 (s, 3 H), 3.64 (s, 3 H), 3.50 (dd, J = 12.0, 2.0 Hz, 1 H), 3.11 (dd, J = 12.0, 2.0 Hz, 1 H), 1.67−1.60 (m, 2 H), 1.46−1.32 (m, 1 H), 0.88 (s, 3 H), 0.84 (s, 3 H). ^{13}C NMR ($CDCl_3$, 200 MHz): δ 170.8 (s), 168.7 (s), 165.6 (s), 100.0 (d), 77.8 (d), 77.3 (d), 52.8 (d), 52.4 (q), 52.3 (q), 48.1 (t), 36.6 (t), 24.7 (d), 23.3 (q), 21.3 (q). MS *m*/*z* 315 (11), 256 (100), 240 (4). Anal. Calcd for $C_{14}H_{21}NO_7$ (315.33): C, 53.33; H, 6.71; N, 4.44. Found: C, 53.09; H, 6.58; N, 4.59.

4′-Methyl-(2′*S*)-2′-[(1*R*,5*S*,7*S*)-7-(morpholine-4-carbonyl)-2-oxo-6,8-dioxa-3-azabicyclo[3.2.1]oct-3-yl]pentanoic Acid Methyl Ester (6m). Compound **11** (100 mg, 0.32 mmol) and morpholine (0.55 mL, 6.3 mmol) were stirred at 60 °C overnight. The reaction mixture was then diluted with methanol and eluted through Amberlyst 15. After solvent evaporation, the crude product was purified by column chromatography (silica gel, DCM/MeOH 20:1) giving pure **6m** (95 mg, 65% yield) as a yellow oil. $[\alpha]_D^{22}$ +29.0 (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 5.86 (d, J = 2 Hz, 1 H), 5.16 (s, 1 H), 5.06 (dd, J = 8 Hz, 1 H), 4.76 (s, 1 H), 3.70 (s, 3 H), 3.67−3.52 (m, 9 H), 3.15 (d, J = 12 Hz, 1 H), 1.75−1.67 (m, 2 H), 1.53−1.43 (m, 1 H), 0.94 (d, J = 6 Hz, 3 H), 0.92 (d, J = 6 Hz, 3 H). ^{13}C NMR ($CDCl_3$, 200 MHz): δ 170.8 (s), 99.8 (d), 84.6 (d), 78.0 (d), 66.8 (t), 66.6 (t), 52.8 (q), 52.5 (d), 48.6 (t), 46.0 (t), 42.7 (t), 36.8 (t), 24.8 (d), 23.3 (q), 21.6 (q). MS *m*/*z* 370 (14), 311 (60), 283 (19), 168 (100). IR ($CHCl_3$) 2932, 1735, 1668 cm^{-1}. Anal. Calcd for $C_{17}H_{26}N_2O_7$ (370.41): C, 55.13; H, 7.08; N, 7.56. Found: C, 55.17; H, 7.12; N, 7.52.

(2*S*)-2-[(1*R*,5*S*,7*S*)-7-(4-Phenylethylpiperazine-1-carbonyl)-2-oxo-6,8-dioxa-3-azabicyclo[3.2.1]oct-3-yl]-4-methylpentanoic Acid Methyl Ester (6q). Compound **6q** was prepared as reported for **6m** starting from compound **11** (100 mg, 0.32 mmol) and 1-phenylethylpiperazine (1.2 mL, 6.3 mmol), giving the pure product (89 mg, 59% yield) as a yellow oil. $[\alpha]_D^{25}$ +21.3 (*c* 0.9, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.33−7.18 (m, 5 H), 5.88 (d, J = 2 Hz, 1 H), 5.17 (s, 1 H), 5.09 (dd, J = 8.0, 6.0 Hz, 1 H), 4.81 (s, 1 H), 3.72 (s, 3 H), 3.78−3.63 (m, 4 H), 3.57 (dd, J = 12.0, 2.0 Hz, 1 H), 3.18 (d, J = 12.0 Hz, 1 H), 2.88−2.80 (m, 2 H), 2.70−2.58 (m, 6 H), 1.78−1.70 (m, 2 H), 1.53−1.25 (m, 1 H), 0.98 (d, J = 6.0 Hz, 3 H), 0.94 (d, J = 6.0 Hz, 3 H). ^{13}C NMR ($CDCl_3$, 200 MHz): δ 170.6 (s), 166.5 (s), 164.8 (s), 138.5 (s), 128.4 (d), 128.3 (d), 126.1 (d), 99.5 (d), 77.7 (d), 76.9 (d), 59.5 (t), 52.6 (q), 52.4, 52.2 (t), 51.9 (d), 48.2, 44.3 (t), 41.3 (t), 36.5 (t), 32.4 (t), 24.4 (d), 22.8 (q), 21.2 (q). MS *m*/*z* 414 (1), 382 (95), 56(100). IR ($CHCl_3$) 2923, 1740, 1672 cm^{-1}. Anal. Calcd. for

Table 6. Acceleration of *Candida* AIDS68 Clearance in Rats Intravaginally Treated with **13** and **15** after Challenge (1, 24, 48 h)[a]

days	AIDS68 + **15**	AIDS68 + **13**	AIDS68 + pepstatin A	AIDS68 + fluconazole	AIDS68
0	> 100 ± 0	100 ± 0	> 100 ± 0	> 100 ± 0	> 100 ± 0
1	71.8 ± 1.3	61.6 ± 1.2	58.4 ± 1.0	100 ± 0	100 ± 0
2	62.6 ± 1.5	55.4 ± 1.7	52.0 ± 1.3	93.0 ± 4.3	100 ± 0
5	40.6 ± 1.4	26.8 ± 1.0	37.2 ± 1.6	61.0 ± 2.5	71.0 ± 1.6
7	23.2 ± 1.4	22.8 ± 2.2	30.0 ± 1.2	44.0 ± 2.9	50.0 ± 3.5
14	12.8 ± 1.2	11.2 ± 1.1	19.8 ± 0.8	18.7 ± 3.8	25.0 ± 1.6
21	3.4 ± 1.7	2.2 ± 0.9	3.8 ± 1.9	11.7 ± 0.7	10.7 ± 1.6
28	0 ± 0	0 ± 0	0 ± 0	0 ± 0	7.7 ± 3

[a] All values are CFU/mL ×1000. AIDS68: untreated control. Starting day 1, all differences between **13**- and **15**-treated and untreated control are statistically significant ($P < 0.01$, Mann−Withney U test). Also, starting from day 1 postchallenge, all values of **13**-, **15**-, and pepstatin A-treated rats denote statistically significant differences compared to same-day values of AIDS68-treated rats. No statistically significant difference is detected between pepstatin A- and **13**- or **15**-treated rats.

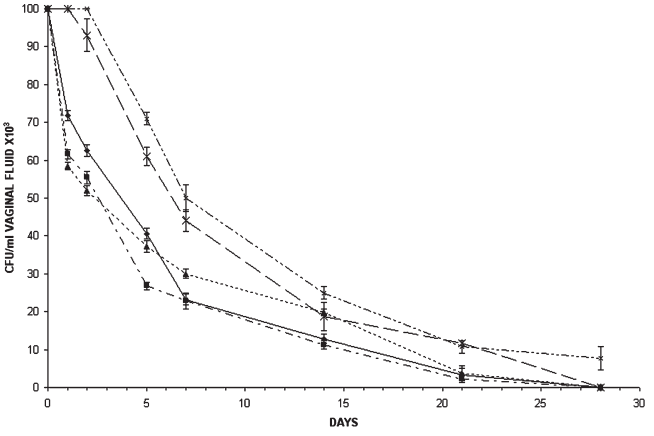


Figure 3. Vaginal infection with *C. albicans* AIDS68 in rats intravaginally treated with **13** and **15** after challenge (1, 24, 48 h): (◆) AIDS68 + **15**; (■) AIDS68 + **13**; (▲) AIDS68 + pepstatin A; (×) AIDS68; (∗) AIDS68 + fluconazole.

$C_{25}H_{35}N_3O_6$ (473.57): C, 63.41; H, 7.45; N, 8.87. Found: C, 63.28; H, 7.41; N, 8.79.

(2*S*)-2-[(1*R*,5*S*,7*S*)-7-(4-Methylpiperazine-1-carbonyl)-2-oxo-6,8-dioxa-3-azabicyclo[3.2.1]oct-3-yl]-4-methylpentanoic Acid Methyl Ester (6r). Compound **6r** was prepared as reported for **6m** starting from compound **11** (150 mg, 0.48 mmol) and 1-methylpiperazine (1.06 mL, 9.5 mmol) to give the pure product (128 mg, 72% yield) as a yellow oil. $[\alpha]_D^{25}$ +28.1 (*c* 0.9, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 5.85 (s, 1 H), 5.12 (s, 1 H), 5.05 (t, J = 8.0 Hz, 1 H), 4.77 (s, 1 H), 3.68 (s, 3 H), 3.62−3.51 (m, 5 H), 3.14 (d, J = 12.0 Hz, 1 H), 2.42−2.33 (m, 4 H), 2.72 (s, 3 H), 1.73−1.65 (m, 2 H), 1.49−1.42 (m, 1 H), 0.92 (d, J = 6.0 Hz, 3 H), 0.90 (d, J = 4.0 Hz, 3 H). ^{13}C NMR ($CDCl_3$, 200 MHz): δ 170.8 (s), 166.8 (s), 165.0 (s), 99.7 (d), 78.0 (d), 76.4 (d), 55.0, 54.6 (t), 52.8 (q), 52.5 (d), 48.6 (t), 46.1 (q), 45.4 (t), 42.3 (t), 36.9 (t), 24.8 (d), 23.3 (q), 21.6 (q). MS m/z 383 (23), 352 (2.4), 324 (9), 99 (55), 70 (100). IR ($CHCl_3$) 2866, 1738, 1670 cm^{-1}. Anal. Calcd. for $C_{18}H_{29}N_3O_6$ (383.44): C, 56.38; H, 7.62; N, 10.96. Found: C, 56.22; H, 7.58; N, 11.01.

(2*S*)-2-[(1*R*,5*S*,7*S*)-7-(4-Benzylpiperazine-1-carbonyl)-2-oxo-6,8-dioxa-3-azabicyclo[3.2.1]oct-3-yl]-4-methylpentanoic Acid Methyl Ester (6z). Compound **6z** was prepared as reported for **6m** starting from compound **11** (100 mg, 0.32 mmol) and 1-benzylpiperazine (1.1 mL, 6.3 mmol) to give the pure product (106 mg, 72% yield) as a yellow oil. $[\alpha]_D^{23}$ +20.1 (*c* 1.1, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.42−7.27 (m, 5 H), 5.88 (s, 1 H), 5.25−5.05 (m, 2 H), 4.79 (s, 1 H), 3.71 (s, 3 H), 3.63−3.53 (m, 7 H), 3.16 (d, J = 11.6 Hz, 1 H), 2.51−2.45 (m, 4 H), 1.76−1.68 (m, 2 H), 1.55−1.25 (m, 1 H), 0.96 (d, J = 5, 3 H), 0.93 (d, J = 6.2 Hz, 3 H). ^{13}C NMR ($CDCl_3$, 200 MHz): δ 170.8 (s), 166.8 (s), 165.0 (s), 129.1 (d), 128.3 (d), 127.3 (d), 99.7 (d), 78.0 (d), 76.4 (d), 62.9 (t), 52.9 (q), 52.7, 52.7 (t), 52.5 (d), 48.5 (t), 45.5, 42.4 (t), 36.8 (t), 24.8 (d), 23.3 (q), 21.6 (q). MS m/z 459 (10), 400 (1), 330 (1), 175 (19), 91 (100). IR ($CHCl_3$) 2940, 1740, 1672 cm^{-1}. Anal. Calcd for $C_{24}H_{33}N_3O_6$ (459.55): C, 62.73; H, 7.24; N, 9.14. Found: C, 62.80; H, 7.32; N, 9.50.

(1*S*,5*R*,7*R*)-3-Benzyl-2-oxo-6,8-dioxa-3-azabicyclo[3.2.1]octane-7-carboxylic Acid Benzyl-(1-hydroxymethyl-3-methylbutyl)amide (23). Amide **23** was prepared as reported,[10] starting from the corresponding (1*S*,5*R*,7*R*)-bicyclic ester (40 mg, 0.144 mmol) and *N*-benzyl-L-leucinol (300 mg, 1.44 mmol), giving the pure product after flash chromatography (EtOAc/petroleum ether 2:1). $[\alpha]_D^{24}$ +12.8 (*c* 1.2, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.30−7.11 (m, 10 H), 6.57 (d, J = 2.1 Hz, 1 H), 4.94 (s, 1 H), 4.64 (s, 1 H), 4.47 (s, 2 H), 3.97−3.25 (m, 8 H), 3.02 (d, J = 12.5 Hz, 1 H), 1.55 (m, 1 H), 1.30 (m, 2 H), 0.82−0.80 (m, 6 H). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 168.8 (s), 165.6 (s), 135.2 (s), 129.0, 128.7, 128.2, 127.8, 99.8 (d), 79.4 (d), 77.7 (d), 65.2 (t), 61.5 (t), 50.9 (t), 50.0 (d), 48.2 (t), 40.0 (t), 25.0 (d), 23.2 (q), 22.2 (q). MS m/z 452 (11), 219 (12), 105 (45), 91 (100). Anal. Calcd for $C_{26}H_{32}N_2O_5$ (452.54): C, 69.01; H, 7.13; N, 6.19. Found: C, 68.96; H, 7.08; N, 6.11.

(1*S*,5*S*,7*S*)-3-Benzyl-7-(piperidine-1-carbonyl)-6,8-dioxa-3-azabicyclo[3.2.1]octan-2-one (24). Amide **24** was prepared as reported for the enantiomer **13**, starting from the corresponding (1*R*,5*S*,7*S*)-bicyclic ester (40 mg, 0.144 mmol) and piperidine (143 μL, 1.44 mmol), giving the pure product after flash chromatography (EtOAc/petroleum ether 2:1). $[\alpha]_D^{24}$ −44.6 (*c* 1.3, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.28−7.14 (m, 5 H), 5.77 (d, J = 1.8 Hz, 1H), 5.07 (s, 1 H), 4.86 (s, 1 H), 4.60 (part A of AB system, J = 15.0 Hz, 1 H), 4.36 (part B of AB system, J = 15.0 Hz, 1 H), 3.43 (m, 3 H), 3.32 (dd, J = 12.1, 1.8 Hz, 1 H), 3.04 (d, J = 12.1 Hz, 1 H), 2.85 (d, J = 15.3 Hz, 1 H), 1.56 (m, 6 H). Anal. Calcd for $C_{18}H_{22}N_2O_4$ (330.38): C, 65,44; H, 6,71; N, 8,48. Found: C, 65.38; H, 6.65; N, 8.41.

(1*R*,5*S*,7*S*)-3-Benzyl-2-oxo-6,8-dioxa-3-azabicyclo[3.2.1]octane-7-carboxylic Acid [1-Hydroxymethyl-3(*S*)-methylbutyl]amide (25). Compound **25** was prepared as reported for the diastereomer **15**, starting from the corresponding (1*R*,5*S*,7*S*)-bicyclic ester (40 mg, 0.144 mmol) by aminolysis with (*S*)-leucinol (190 μL, 1.44 mmol), giving the pure compound after flash chromatography (EtOAc/petroleum ether 2:1). $[\alpha]_D^{23}$ −19.9 (*c* 1.3, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.30−7.14 (m, 5 H), 6.52 (d, J = 7.7 Hz, 1 H), 5.80 (d, J = 2.2 Hz, 1 H), 4.99 (s, 1 H), 4.62 (s, 1 H), 4.50 (AB system, J = 15.1 Hz, 2 H), 3.97 (m, 1 H), 3.63 (dd, J = 11.0, 2.9 Hz, 1 H), 3.50 (dd, J = 11.0, 5.1 Hz, 1 H), 3.36 (d, J = 2.2 Hz, 1 H), 2.00 (br, 1 H), 1.56 (m, 1 H), 1.40 (m, 2 H), 0.89 (d, J = 2.2 Hz, 3 H), 0.86 (d, J = 1.8 Hz, 3 H). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 168.6 (s), 165.6 (s), 135.2 (s), 128.7 (d, 2 C), 127.8 (d, 2 C), 127.7 (d), 99.8 (d), 79.3 (d), 77.5 (d), 65.3 (t), 50.9 (t), 49.9 (d), 48.2 (t), 40.1 (t), 24.9 (d), 23.0 (q), 22.2 (q). MS m/z 362 (12), 332 (55), 219 (67), 92 (100). Anal. Calcd for $C_{19}H_{26}N_2O_5$ (362.42): C, 62.97; H, 7.23; N, 7.73. Found: C, 62.85; H, 7.28; N, 7.68.

(1*S*,5*R*,7*R*)-3-Benzyl-2-oxo-6,8-dioxa-3-azabicyclo[3.2.1]octane-7-carboxylic Acid [1-Hydroxymethyl-3(*R*)-methylbutyl]amide (26). Compound **26** was prepared as reported for **25** starting

from the corresponding (1*S*,5*R*,7*R*)-bicyclic ester (45 mg, 0.16 mmol) and (*R*)-leucinol (210 μL, 1.60 mmol), giving the pure compound after flash chromatography (EtOAc/petroleum ether 2:1) with the same spectroscopic data as for **27**. $[\alpha]_D^{24}$ +15.2 (*c* 1.2, $CHCl_3$). Anal. Calcd for $C_{19}H_{26}N_2O_5$ (362.42): C, 62.97; H, 7.23; N, 7.73. Found: C, 62.67; H, 7.18; N, 7.63.

(1*R*,5*S*,7*S*)-3-Benzyl-2-oxo-6,8-dioxa-3-azabicyclo[3.2.1]octane-7-carboxylic Acid [1-Hydroxymethyl-3(*R*)-methylbutyl]amide (27). Compound **27** was prepared as reported[10] for the enantiomer **15**, starting from the corresponding (1*R*,5*S*,7*S*)-bicyclic ester (36 mg, 0.13 mmol) by aminolysis with (*R*)-leucinol (168 μL, 1.30 mmol), giving the pure compound after flash chromatography (EtOAc/petroleum ether 2:1) with the same spectroscopic data as for **15**. $[\alpha]_D^{24}$ +16.7 (*c* 1.5, $CHCl_3$). Anal. Calcd for $C_{19}H_{26}N_2O_5$ (362.42): C, 62.97; H, 7.23; N, 7.73. Found: C, 63.01; H, 7.26; N, 7.64.

Biology. The enzymatic screening of peptidomimetic libraries was carried out by an established spectrophotometric method of measuring BSA hydrolysis by SAP2 and calculating the inhibition percentage with respect to the reference SAP2 inhibitor pepstatin A, at a fixed concentration, as detailed below.[13]

Proteinase Enzyme Assay. Spectrophotometric Method. The inhibition of proteinase activity by the various compounds of pool I was measured by a spectrophotometric assay in a comparison with pepstatin A activity, at the same concentration. Each assay contained 50 μL of sample in 0.4 mL of 1% (w/v) BSA in 50 mM sodium citrate, pH 3.2, and 50 μL of protease solution (1 μg/mL). After 30 min at 37 °C, 1 mL of 10% (w/v) trichloroacetic acid was added. The tubes were stored in ice for 30 min and then centrifuged (3000*g*) for 10 min. The absorbance of the supernatant was read at 280 nm. The control was 1% BSA in citrate buffer. One unit of the enzyme catalyzed a ΔA_{280} of 1 min^{-1}. With the pure proteinase the assay was proportional to enzyme concentration over the range ΔA_{280} = 0.1−0.4 and a limit detection of 1 μg.[13] The difference of optical density (ΔOD) of 1 mL of solution in 1 min was determined and referenced to the activity of peptstatin A in % units. For pools of compounds the relative inhibitory potency was normalized to the single compound. For all data corresponding to in vitro experiments the SD value was never above 5%. In order to select the best scaffold and the best substituents independently, each pool consisted of a few compounds having the same core scaffold and differing by the substituents.

In Vivo Assay. Experimental Vaginal Infection. A previously described rat vaginal model was adopted.[14] In brief, oophorectomized female Wistar rats (80−100 g; Charles River, Calco, Italy) were injected subcutaneously with 0.5 mg of estradiol benzoate (Estradiolo, Amsa Farmaceutici srl, Rome, Italy). Six days after the first estradiol the animals were inoculated intravaginally with $>10^7$ yeast cells in 0.1 mL of saline solution of each *C. albicans* strain tested. The inoculum was dispensed into the vaginal cavity through a syringe equipped with a multipurpose calibrated tip (Combitip; PBI, Milan, Italy). The yeast cells had been previously grown in YPD broth (yeast extract 1%, peptone 2%, dextrose 2%) at 28 °C on a gyrator shaker (200 rpm), harvested by centrifugation (1500 rpm), washed, counted in a hemocytometer, and suspended to the required number in saline solution. The number of cells in the vaginal fluid was counted by culturing 100 μL samples (using a calibrated plastic loop, Disponoic, PBI, Milan, Italy), taken from each animal, on Sabouraud agar containing chloramphenicol (50 μg/mL) as previously described. The rat was considered infected when at least 1 CFU was present in the vaginal lavage, i.e., a count of $>10^3$ CFU/mL. Compounds **13** and **15** were administered intravaginally at 10 μM at 1, 24, and 48 h after intravaginal *Candida albicans* challenge with two different strains, namely, SA40 and the pharmacoresistant AIDS68.[14] Positive (pepstatin A 10 μg, fluconazole 10 μg and negative (sterile saline solution) were similarly administered.

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