Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 1829–1839

BIOORGANIC & MEDICINAL CHEMISTRY

Novel Morpholinone-Based D-Phe-Pro-Arg Mimics as Potential Thrombin Inhibitors: Design, Synthesis, and X-ray Crystal Structure of an Enzyme Inhibitor Complex

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Received 5 November 2001; accepted 7 January 2002

Abstract—A morpholinone structural motif derived from D(+)- and L(−)-malic acid has been used as a mimic of D-Phe-Pro in the thrombin inhibiting tripeptide D-Phe-Pro-Arg. In place of Arg the more rigid P1 truncated *p*-amidinobenzylamine (Pab) or 2-amino-5-aminomethyl-3-methyl-pyridine have been utilized. The synthetic strategy developed readily delivers these novel thrombin inhibitors used to probe the α-thrombin inhibitor binding site. The best candidate in this series of thrombin inhibitors exhibits an in vitro IC_{50} of 720 nM. The X-ray crystal structure of this candidate co-crystallized with α-thrombin is discussed.

Introduction

Undesired blood clotting is one of the major underlying events in a number of cardiovascular diseases, that is deep venous thrombosis, pulmonary embolism, unstable angina, restenosis following angioplasty, and arterial thrombosis.[1] Thrombin, a member of the trypsin family of serine proteases, plays a critical role in the blood coagulation cascade. The procoagulant properties of thrombin are exerted via the conversion of fibrinogen into a fibrin clot and from activation of zymogens upstream in the coagulation cascade.[2] Moreover, thrombin is the most potent stimulator of platelet aggregation known. Currently, intense efforts are under way to develop small molecule thrombin inhibitor drugs to exploit the potential of regulating hemostasis and thrombosis in disease.[3]

The classical motif of thrombin inhibitors is the D-Phe-Pro-Arg sequence[4] mimicking thrombin's natural substrate, fibrinogen. A number of drug candidates and clinical inhibitors like inogatran[5] (Fig. 2) have been developed based on this motif.[6]

It has recently been reported by Semple et al. on the design and synthesis of highly potent thrombin inhibitors[7] incorporating a novel 3-amino-1-carboxymethyl-2-piperidinone scaffold (**A**) extending from the proximal (P) to the distal (D) pocket of thrombin (Fig. 1).

The directional vectors from the sp^2 hybridized N1-atom and the sp^3-hybridized C3-atom of the piperidinone fit well into the P-D-pocket of thrombin, providing a β-sheet like hydrogen bond network with the backbone carbonyl of Ser214, as well as hydrogen bonds to Gly216. This binding is similar to that observed for thrombin inhibitors having a P2-proline

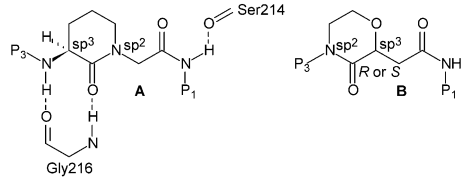

Figure 1. Comparison of the 3-amino-1-carboxymethyl-2-piperidinone scaffold **A** with the scaffolds (**B**) used in this report.

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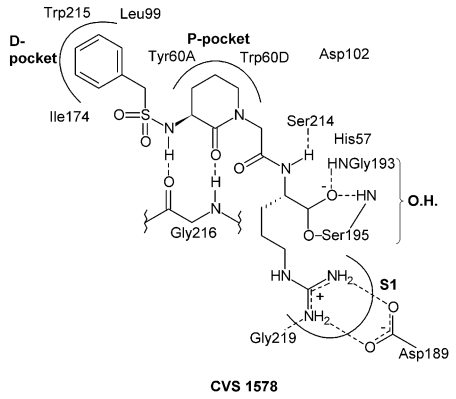
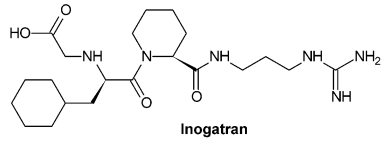




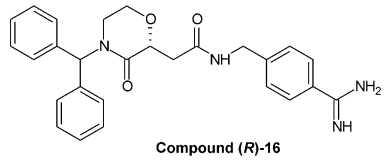


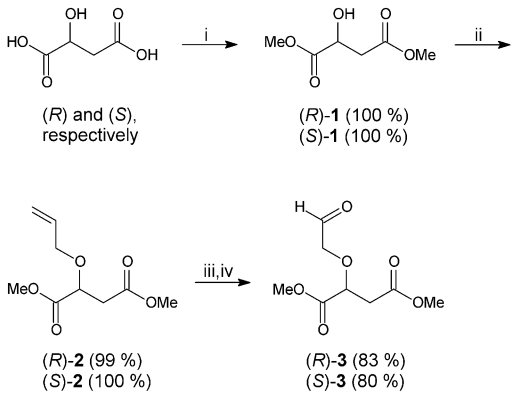
Figure 2. Structures of CVS 1578 ($IC_{50} = 6.2$ nM), inogatran ($IC_{50} = 22$ nM) and compound (*R*)-**16** ($IC_{50} = 720$ nM). The thrombin nomenclature and the important interactions between CVS 1578 and thrombin are briefly outlined. O.H. denotes oxyanion hole binding site.

residue such as PPACK.[8] It has been our aim to explore the thrombin binding pockets using novel and easily accessible templates and we reasoned that it may be of interest to investigate the effect of replacing the sp^2 N1-atom with a sp^3 C-atom and the sp^3 C3-atom with a sp^2 N-atom (Fig. 1). We were also intrigued to investigate the effect of excluding the hydrogen bond donating capability present between the 3-amino group in the 3-amino-1-carboxymethyl-2-piperidinone scaffold and the CO of Gly216. This led to the construction of the 2-(3-*oxo*-morpholin-2-yl)-acetic acid derivatives (**B**) as a D-Phe-Pro replacement (Fig. 1). Structural motifs based on **B** are readily available starting from commercially available (*R*)- or (*S*)-malic acid using the synthetic route developed.

In this report, we have focused on using the Arg mimic *p*-amidinobenzylamine in the P1 position and mainly lipophilic amines in the P3 position. Interestingly, the compounds in this series show promising affinity for thrombin. The best compound exhibits an in vitro thrombin activity (IC_{50}), of 720 nM.

Results and Discussion

Commercially available D(+)-malic acid (unnatural form) and L(−)-malic acid (natural form) were used as starting materials. D(+)-malic acid was treated with thionyl chloride in methanol to give the dimethyl ester (*R*)-**1**[9] in 100% yield. The hydroxyl group of (*R*)-**1** was



Scheme 1. Reagents: (i) $SOCl_2$, MeOH; (ii) allyl bromide, Ag_2O, toluene; (iii) OsO_4, *N*-methyl-morpholine-*N*-oxide monohydrate, THF/H_2O 3:1; (iv) $NaIO_4$, THF/H_2O 3:1.

alkylated with allyl bromide in toluene using silver(I) oxide[10] affording the alkene (*R*)-**2** in 99% yield.[11] (*R*)-**2** was treated with a catalytic amount of osmium tetroxide with *N*-methyl morpholine-*N*-oxide as reoxidant to give the corresponding diol, which, without further purification, was oxidatively cleaved with sodium periodate[12] providing the aldehyde (*R*)-**3** in 83% yield. The enantiomer (*S*)-**3** was synthesized according to the same method (Scheme 1). (*R*)-**3** and (*S*)-**3** were used as precursors for all the designed target compounds.

The aldehydes (*R*)-**3** and (*S*)-**3** were first reacted with benzylamine and phenethylamine in a reductive amination process using sodium borohydride and ground molecular sieves (3 Å).[13] (*R*)-**3** was also reacted with isobutylamine using the same procedure (Table 1). Not surprisingly, ring closure to the six-membered morpholinone derivatives (*R*)-**4**, (*S*)-**4**, (*R*)-**5**, (*S*)-**5**, and **6** occurred spontaneously during the reaction,[14] giving the desired products in yields ranging from 50 to 64% (Table 1).

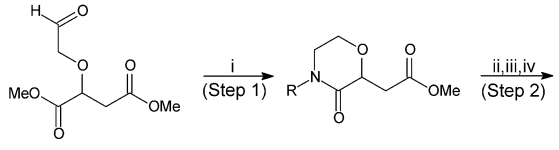
Hydrolysis of the monoesters with lithium hydroxide in water–dioxane afforded after work up the corresponding monoacids, which were coupled to 4-(benzyloxycarbonyl)-amidinobenzylamine [Pab(Z)][15] using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC), 1-hydroxybenzotriazole (HOBt), and triethylamine. The protecting group was cleaved off using catalytic hydrogenation, affording the benzamidines (*R*)-**7**, (*S*)-**7**, (*R*)-**8**, (*S*)-**8**, and **9** in yields ranging from 40 to 92% over the three steps (Table 1).

The aldehyde (*R*)-**3** was also allowed to react with more sterically hindered amines, that is cyclohexylamine, (dicyclohexylmethyl)-amine, (diphenylmethyl)-amine, and *tert*-butyloxycarbonyl protected (*S*)-phenylglycine, using reductive aminations as described above, but employing other reducing agents.[16] Treating (*R*)-**3** with cyclohexylamine, (dicyclohexylmethyl)-amine (synthesized according to the method described by Bowles et al.),[17] and (diphenylmethyl)-amine together with pyridine–borane complex and ground molecular sieves (3 Å)[18] afforded the diesters **10**, (*R*)-**11**, and (*R*)-**12** in yields ranging from 43 to 78% (Table 2). Compound

13 was synthesized in 82% yield using sodium triacetoxyborohydride as reducing agent[19] (Table 2). The compounds (*S*)-**11** and (*S*)-**12** were obtained from the aldehyde (*S*)-**3** according to the same procedure used for their respective enantiomers in 71 and 77% yield, respectively (Table 2). The absence of spontaneous ring closure can be attributed to steric hindrance.[20]

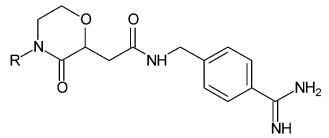
The diesters were hydrolyzed with lithium hydroxide in water-dioxane, followed by reacting the resulting diacids with *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluroniumhexafluorophosphate (HATU) and *N,N*-diisopropylethylamine (DIEA) for 1 h to accomplish the ring-closure. Pab(Z) was added and the mixtures were stirred overnight at room temperature. Finally, hydrogenation (deprotection) provided the desired benzamidine products **14**, (*R*)-**15**, (*S*)-**15**, (*R*)-**16**, (*S*)-**16**, and **17**[21] in 53–94% total yield from the corresponding diesters (Table 2). The benzamidine **18** was synthesized from benzyloxycarbonyl protected **17** by treatment with trifluoroacetic acid and triethylsilane in methylene chloride to remove the *tert*-butyl group in the phenylglycine part of the molecule, followed by hydrogenation to give **18** in 75% yield from Cbz-protected **17** (44% yield from **13** calculated over four steps) (Table 2).[22]

Table 1.



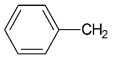
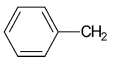
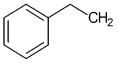
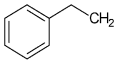
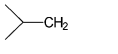
(*R*)-**3** and (*S*)-**3**, respectively

(*R*)-**4**, (*S*)-**4**, (*R*)-**5**, (*S*)-**5**, **6**



(*R*)-**7**, (*S*)-**7**, (*R*)-**8**, (*S*)-**8**, **9**

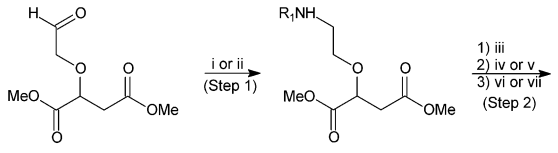
Reagents: (i) RNH_2, $NaBH_4$, mol sieves (3 Å), MeOH; (ii) LiOH, dioxane/H_2O; (iii) 4-(benzyloxycarbonyl)-amidinobenzylamine dihydrochloride, EDC, HOBt, Et_3N, DMF; (iv) H_2/Pd/C, EtOH

Prod	R	Yield (%), step 1	Yield (%), step 2
(*R*)-**7**		61[(*R*)-**4**]	81
(*S*)-**7**		64[(*S*)-**4**]	92
(*R*)-**8**		60[(*R*)-**5**]	83
(*S*)-**8**		50[(*S*)-**5**]	86
9[a]		51(**6**)	40

[a](*R*) configuration at the malic acid moiety of the compound.

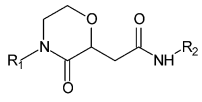
It has been shown that incorporation of aminopyridines in the P1 position may give rise to potent and orally available thrombin inhibitors.[23] To prepare an inhibitor having 1-(2-amino-3-methyl-pyridin-5-yl)-methylamine instead of *p*-amidinobenzylamine as P1 substituent the

Table 2.



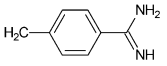
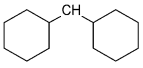
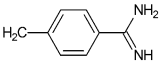
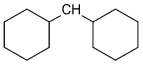
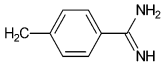
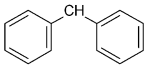
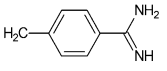
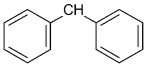
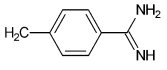
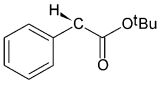
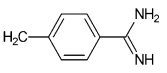
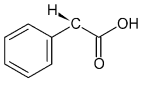
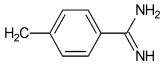
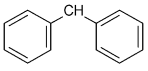
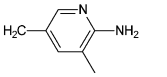
(*R*)-**3** and (*S*)-**3**, respectively

10, (*R*)-**11**, (*S*)-**11**, (*R*)-**12**, (*S*)-**12**, **13**



14, (*R*)-**15**, (*S*)-**15**, (*R*)-**16**, (*S*)-**16**, **17**, **18**, **19**

Reagents: (i) R_1NH_2, pyridine–borane complex, mol/sieves (3 Å), MeOH; (ii) R_1NH_2, $NaBH(OAc)_3$, mol/sieves (3 Å), THF; (iii) LiOH, dioxane/H_2O; (iv) 4-(benzyloxycarbonyl)-amidinobenzylamine dihydrochloride, HATU, DIEA, DMF; (v) 5-aminomethyl-3-methyl-2-(*N,N*-di-*tert*-butyloxycarbonylamino)-pyridine, HATU, DIEA, DMF; (vi) H_2/Pd/C, EtOH; (vii) CH_2Cl_2/TFA

Final prod	R_1	R_2	Yield (%), step 1	Yield (%), step 2
14[a]			58(**10**)	70
(*R*)-**15**			43[(*R*)-**11**]	94
(*S*)-**15**			71[(*S*)-**11**]	77
(*R*)-**16**			78[(*R*)-**12**]	53
(*S*)-**16**			77[(*S*)-**12**]	76
17[a,b]			82(**13**)	59
18[a,c]			82(**13**)	44
19[d]			78[(*R*)-**12**]	50

Unless otherwise noted, reagents (i) in step 1 and reagents (iii), (iv) and (vi) in step 2 were used.
[a](*R*) configuration at the malic acid moiety of the compound.
[b]Reagents (ii) used in step 1.
[c]Synthesized by treating Cbz protected **17** with a mixture of CH_2Cl_2, TFA and Et_3SiH, followed by the hydrogenation step (vi). The yield in step 2 was calculated from **13**.
[d]Reagents (v) and (vii) were used in step 2.

open diester (*R*)-**12** was hydrolyzed as described above, and the resulting diacid was treated with HATU and DIEA in DMF, followed by addition of the di-boc protected aminopyridine. The boc groups of the ring-closed product were removed by treatment with TFA in methylene chloride, providing the final product **19** in a total yield of 50% over three steps (Table 2). This gave us the opportunity to compare the biological activity of (*R*)-**16**, the most potent Pab-containing inhibitor in this series (Table 3), with the thrombin inhibiting properties of a compound (**19**) differing only in the P1 position.

Biological data

The thrombin inhibiting properties of the final products are summarized in Table 3.

Structure–activity relationship

As can be seen from examination of Table 3, only four compounds, that is **14**, (*R*)-**15**, (*R*)-**16** (Fig. 2), and **17**, have IC_{50} values ranging from 10 μM to slightly below 1 μM. To gain further information regarding the binding of these compounds in the thrombin active site compared to those reported by Krishnan et al.,[7c] compound (*R*)-**16** was cocrystallized with α-thrombin and subjected to X-ray analysis. Figure 2 shows the important interactions between the thrombin inhibitor CVS 1578 (IC_{50} = 6.2 nM) and the active site of thrombin.[7b] Clearly, a direct comparison of affinity with CVS 1578 may not seem relevant, since CVS 1578 through its aldehyde group is covalently bound to Ser195 and is able to form additional hydrogen bonds to the NH of Gly193 and to the CO of Gly216, as shown in Figure 2, whereas the present series of non-electrophilic inhibitors does not have these interactions. However, it has been shown that non-covalent inhibitors such as inogatran (IC_{50} = 22 nM)[5] (Fig. 2), can achieve high affinity to thrombin and interacts in a similar way forming an extensive hydrogen bond network with Ser214 and Gly216.

Table 3. Thrombin IC_{50} values (μM)

Compd	IC_{50}	Compd	IC_{50}	Compd	IC_{50}
(*R*)-**7**	>13	**14**	4.7	**17**	8.3
(*S*)-**7**	>13	(*R*)-**15**	1.1	**18**	>13
(*R*)-**8**	>13	(*S*)-**15**	>13	**19**	>13
(*S*)-**8**	>13	(*R*)-**16**	0.72		
9	>13	(*S*)-**16**	>13		

The overall alignment of (*R*)-**16** compares well with that of CVS 1578 (see the X-ray crystal structures in Figs 3 and 4). The amidine group of (*R*)-**16** forms as expected a strong salt bridge with Asp189 in the S1-pocket, very similar to that of the guanidine group of CVS 1578. The 3-*oxo*-morpholine ring of (*R*)-**16** nicely occupies the hydrophobic P-pocket (proximal pocket) of thrombin, which resembles the interaction of the piperidinone ring of CVS 1578 with the P-pocket, although (*R*)-**16** seems to move deeper into the P-pocket towards the S1′-site. Additionally, the NH of the P1–P2 amide group of (*R*)-**16** forms a week hydrogen bond (N–O distance 3.29 Å) with the carbonyl group of Ser214, while the corresponding distance of CVS 1578 is 3.01 Å. However, (*R*)-**16** lacks hydrogen bond contact between the 3-*oxo*-group and the NH of Gly216 (O–N distance 4.57 Å), which is a prominent feature of thrombin's interaction with CVS 1578 (O–N distance 3.35 Å). This is also a feature in most other non covalent bond inhibitors, including inogatran. In addition, the 3-amino group of the piperidinone ring of CVS 1578 forms a strong hydrogen bond to the C=O group of Gly216 (N–O distance 3.13 Å), a hydrogen bond donating capability absent in (*R*)-**16**. These observations, together with the absence of a covalent bond to Ser195, likely explain the lower affinity of our series of compounds. Furthermore, a comparison of the directional vector imposed by the sp^3-C2-atom of the 3-*oxo*-morpholine ring of (*R*)-**16**,

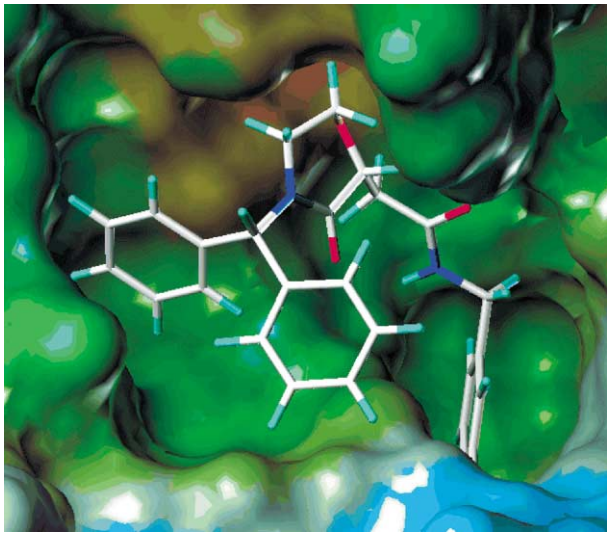


Figure 3. The Connolly surface map of the X-ray crystal structure of the α-thrombin–(*R*)-**16** complex at 2.0 Å resolution.

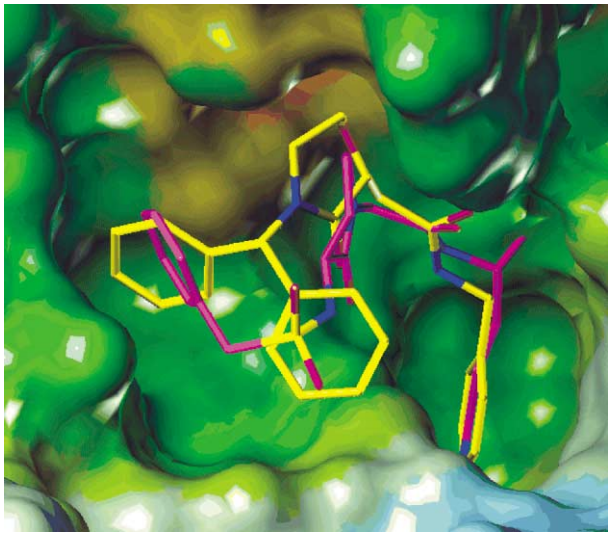


Figure 4. The superimposition of the α-thrombin–(*R*)-**16** (yellow) and α-thrombin–CVS 1578 (magenta) X-ray crystal structures.

resulting in the lack of the hydrogen bond to Gly216, and the vector imposed by the sp^2-N1-atom of the 2-piperidinone ring of CVS 1578 further contributes to the understanding of the modest potency of (*R*)-**16** (Figs 3 and 4).

One of the phenyl rings of the P3-diphenyl group of (*R*)-**16** occupies the D-pocket (distal pocket) and overlaps with the phenyl group of the benzyl-sulfonamide part of CVS 1578. However, the electron density of (*R*)-**16** in the X-ray structure of the thrombin–(*R*)-**16** complex is less well defined in this region, which indicates more than one possible conformation of the diphenyl group, where either of the phenyl groups points towards the hydrophobic surface of the D-pocket as schematically shown in Fig. 5. This is corroborated by the even less well-defined electron density of the phenyl group exposed to the surrounding water, as this phenyl group needs to occupy a distinctly separated position when the other phenyl group resides in the D-pocket.

Finally, the X-ray analysis of the thrombin-(*R*)-**16** complex reveals why (*R*)-**16** has higher affinity towards thrombin than (*S*)-**16** (an IC_{50} value of 0.72 μM vs >13 μM). (*S*)-**16** will form less favorable interactions with Trp60D, thereby either forcing Trp60D to move substantially, which has been observed by us when analyzing other thrombin inhibitors,[24] or inducing displacement of (*S*)-**16** relative to the P-pocket, which will penalize other favorable interactions with thrombin. Clearly, as indicated by the potency data, the preference for the 2-(*R*)-morpholine ring rather than the 2-(*S*)-configuration is valid for the whole series of compounds.

Independent of configuration, the compounds **7**–**9** all have IC_{50} values >13 μM, and are clearly less potent than (*R*)-**16**. Obviously, the preferred solution conformation of the P3-benzyl- (**7**), P3–2-phenylethyl- (**8**), and P3-isobutyl- (**9**) groups are not such that a favorable interaction with thrombin can be achieved, and that results in reduced potency compared to that of (*R*)-**16.** In contrast, the low energy conformation in solution of the diphenylmethyl group with respect to the C–N4 bond of the 3-*oxo*-morpholine ring is the one where the two phenyl groups bisect the morpholine ring with the methine hydrogen in the plane defined by the amide group (Fig. 5). This explanation is also valid for compounds **14**–**18**, which all have a di-substituted carbon attached to the N4-position of the 3-*oxo*-morpholine ring. The P3-groups in these compounds will all have a low energy conformation similar to the one of (*R*)-**16**, where the methine hydrogen will be in the plane of the lactam amide group and both carbons attached to the carbon of the *exo*-cyclic C–N4 bond bisect the morpholine ring. The dicyclohexylmethyl derivative (*R*)-**15** is almost as potent as (*R*)-**16**, which indicates that the cyclohexyl group fits as well into the D-pocket. We reasoned that an exchange of one of the phenyl groups of the diphenylmethyl group, that is the one that does not seem to contribute to the affinity, with the polar group 2-(*S*)-phenylacetic acid found in **18** might improve the potency by locating the phenyl group in the D-pocket and exposing the polar carboxyl group to the surrounding water. However, on the contrary, the affinity of **18** is decreased substantially (IC_{50} >13 μM), and the lipophilic tertiary butyl-ester **17**, although significantly less potent than (*R*)-**16** (IC_{50} = 8.3 μM vs IC_{50} = 720 nM), is more potent than **18**.

The reason for the positive effect of the additional phenyl group is at present unclear to us. It appears to be a general effect of increased hydrophobicity, which for reasons not clearly understood favors the enzyme–inhibitor interaction compared to that of the free inhibitor–water interactions.

Finally, compound **19** was prepared to see if a less basic P1-group in combination with the P2 morpholine template would prove useful. The 2-amino-5-aminomethyl-3-methyl-pyridine group has been incorporated in new classes of recently developed thrombin inhibitors.[23] However, this results in our case in a >10-fold decrease in activity of **19** compared to that of the corresponding *p*-amidinobenzylamine analogue (*R*)-**16**.

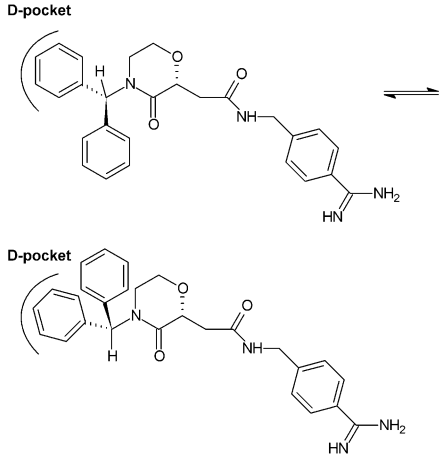


Figure 5. The different conformations of (*R*)-**16** in the active site of α-thrombin.

Conclusion

Novel potential thrombin inhibitors based on malic acid have been prepared. These are easily accessible through the chemistry developed from commercially available starting materials. The best candidates of these 3-*oxo*-morpholine derivatives, having the morpholine ring in the P2 position, *p*-amidinobenzylamine in the P1 position, and lipophilic amines in the P3 position, were (*R*)-**16** with diphenylmethyl and (*R*)-**15** with dicyclohexylmethyl substituents in the P3 position (IC_{50} = 720 nM and 1.1 μM, respectively). To achieve higher potency within this series of compounds structural modifications leading to favorable hydrogen bonding interactions between the central 3-*oxo*-morpholine part of these molecules and Gly216 have to be investigated.

Experimental

Thrombin inhibition measurements

The thrombin inhibitor potency was measured with a chromogenic substrate method in a Plato 3300 robotic microplate processor (Rosys AG, CH-8634Hombrechtikon, Switzerland), using 96-well, half volume microtiter plates (Costar, Cambridge, MA, USA; Cat No 3690). Stock solutions of test substance in DMSO (72 μL), 10 mmol/L, were diluted serially 1:3 (24 + 48 μL) with DMSO to obtain 10 different concentrations, which were analyzed as samples in the assay, together with controls and blanks. The dilutions of each test substance were analyzed consecutively, row-wise on the microtiter plate, with wash-cycles between substances to avoid cross-contamination. 2 μL of test sample was diluted with 124 μL of assay buffer (0.05 mol/L Tris–HCl pH 7.4, ionic strength 0.15 adjusted with NaCl, BSA 1 g/L) and 12 μL of chromogenic substrate solution (S-2366, Cromogenix, Mölndal, Sweden), and finally 12 μL of α-thrombin solution (human α-thrombin, Sigma Chemical Co, St. Louis, MO, USA; Cat No T-6759) in buffer, was added, and the samples were mixed. The final assay concentrations were: Test substance 0.00068–13.3 μmol/L, S-2366 0.30 mmol/L, and α-thrombin 0.020 NIHU/mL. The linear absorbance increase during a 40 min incubation at 37 °C was used for calculation of percent inhibition for the test samples, as compared to blanks without inhibitor. The IC_{50} value, corresponding to the inhibitor concentration which caused 50% inhibition of the thrombin activity, was calculated from a log dose versus inhibition curve.

X-ray crystallography

Human α-thrombin was purchased from Enzyme Research Laboratories, Inc., South Bend, IN, USA, and hirugen from American Diagnostica, Inc., Greenwich, CT, USA. Hirugen–thrombin complex was prepared according to the method of Skrzypczak-Jankun et al.[25] The crystallization was done as described previously.[26] The X-ray diffraction data were collected on a MAR-II imaging plate system, MAR Research, Hamburg, Germany, using Cu K_α radiation from a rotating anode. The data was reduced and scaled using DENZO and SCALEPACK[27] programs. The hirugen–α-thrombin structure previously examined in our laboratory was used in the refinement of the (*R*)-**16**–α-thrombin complex structure. The refinement was performed using REFMAC (CCP4 package)[28] with subsequent runs of CNX.[29] Statistics for X-ray data collection and refinement are presented in Table 4.

General methods

NMR-spectra were recorded on a Bruker AF 250 instrument using $CDCl_3$, methanol-d_4, or D_2O with TMS as an internal standard. The NMR measurements of the benzamidine final products were performed on the free bases unless otherwise noted. Mass spectral data was obtained in positive ion mode using a double focusing Finnigan MAT900S equipped with electrospray interface. Resolution: 5000 (10% valley definition). Two PEG references were used, one on either side (on the mass scale) of the mass of the compounds that were analyzed. Spray voltage: 1.2 kV. The infusion rate was 1.2 μL/min. Temperature of the capillary heater: 230 °C. Optical rotations were measured in $CHCl_3$ or methanol solutions on a Perkin–Elmer 141 polarimeter. The optical rotations of the benzamidine final products were measured on their respective acetate salts. TLC was carried out on Merck precoated 60 F_{254} plates using UV light and charring with ethanol/sulfuric acid/acetic acid/*p*-anisaldehyde 90:3:1:2 for visualisation. Column chromatography was performed using silica gel 60 (0.040–0.063 mm, Merck). Organic phases were dried over anhydrous magnesium sulfate. Concentrations were performed under diminished pressure (1–2 kPa) at a bath temperature of 40°C. The benzamidine final products were sent as the acetate salts for elemental analysis, and sometimes some or all of the acetic acid was lost during drying/heating before the actual analysis. Some of the final compounds proved to be unsuitable for elemental analysis, perhaps because of degradation, and were instead analyzed by HRMS. The acetate salts of the final products were prepared by stirring in water/acetic acid 50:1 for 1 h, followed by freeze drying.

Table 4. Parameters and statistics for X-ray crystallography data collection and refinement

No of measurements	101,906
No of unique reflections	28,198
Data completeness (%)	95.9
R_{merge}[a]	0.062
No of atoms in refined model	2571
protein	2239
cofactor (hirugen)	90
inhibitor	34
solvent	208
Resolution range in refinement (Å)	15–1.86
r.m.s. deviation for bond length (Å)	0.005
angles (°)	1.37
R_{cryst}[b]	0.216
R_{free}	0.226

[a] $R_{merge} = S_h S_i(|I(h,i) - <I(h)>|)/S_h S_i I(h,i)$ where I(h,i) is the intensity value of the ith measurement of h, and $<I(h)>$ is the corresponding mean value of h for all i measurements of h.
[b] $R_{cryst} = S_{hkl}(|F_o - F_c|)/S_{hkl}|F_o|$. $|F_o|$ and $|F_c|$ are observed and calculated structure factor amplitudes, respectively.

General synthetic procedures

Procedure A. Reductive amination of aldehydes (typical procedure). To a solution of the aldehyde (175 mg, 0.85 mmol) in methanol (4 mL) were added ground molecular sieves (160 mg, 3 Å) and the amine (0.85 mmol). 15 min later, sodium borohydride (34 mg, 0.90 mmol) was added. The mixture was stirred at room temperature overnight, after which saturated aqueous ammonium chloride was added. The suspension was filtered through Celite and the filtrate was evaporated. The crude residue was purified by flash column chromatography.

Procedure B. As Procedure A, but with pyridine–borane complex as the reducing agent.

Procedure C. As Procedure A, but with sodium triacetoxyborohydride as the reducing agent, and with tetrahydrofuran as solvent.

Procedure D. Ester hydrolysis + peptide coupling + deprotection of benzyloxycarbonyl protecting group (typical procedure). The monoester (0.43 mmol) was dissolved in dioxane/water 1:1 (4 mL). Lithium hydroxide (1 M, 0.85 mL, 0.85 mmol) was added dropwise and the mixture was stirred at room temperature for 20 min. The solution was neutralized with 1 M hydrochloric acid and evaporated. The residue was suspended in dimethylformamide (5 mL) and 4-(benzyloxycarbonyl)amidinobenzylamine dihydrochloride [Pab(Z)·2HCl] (218 mg, 0.61 mmol), 1-hydroxybenzotriazole (HOBt) (86 mg, 0.64 mmol), and triethylamine (170 μL, 124 mg, 1.23 mmol) were added. The mixture was cooled to 0°C, followed by the addition of *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) (128 mg, 0.67 mmol). The reaction mixture was stirred at 0 °C for 1 h, and then at room temperature overnight. The dimethylformamide was evaporated and the crude product was purified through a short silica column. The appropriate fractions were collected and evaporated. The remainder was dissolved in ethanol (95%, 5 mL) and palladium on active carbon (10%, 40 mg) was added and the mixture was hydrogenated (atmospheric pressure) at room temperature for 1 h. The suspension was filtered and evaporated.

Procedure E. Ester dihydrolysis + ring closure + peptide coupling + deprotection of benzyloxycarbonyl protecting group (typical procedure). To a solution of the diester (0.14 mmol) in dioxane/water 1:1 (6 mL) was added lithium hydroxide (1 M, 0.56 mL, 0.56 mmol) dropwise. The mixture was stirred at room temperature for 40 min after which it was neutralized with 1 M hydrochloric acid and evaporated. The residue was suspended in dimethylformamide (6 mL) and the mixture was cooled to 0 °C in an ice bath. *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluroniumhexafluorophosphate (HATU) (117 mg, 0.31 mmol) and diisopropylethylamine (97 μL, 72 mg, 0.56 mmol) were added and the reaction mixture was stirred at 0 °C for 1 h. 4-(Benzyloxycarbonyl)amidinobenzylamine dihydrochloride [Pab(Z)·2HCl] (61 mg, 0.17 mmol) was added and the solution was stirred for an additional hour at 0°C and then at room temperature overnight. The dimethylformamide was evaporated and the residue was purified by flash column chromatography. The appropriate fractions were pooled and evaporated. The remainder was dissolved in ethanol (95%, 6 mL) and palladium on active carbon (10%, 20 mg) was added and the mixture was hydrogenated (atmospheric pressure) at room temperature for 1 h. The suspension was filtered and evaporated.

Procedure F. Ester dihydrolysis + ring closure + peptide coupling + deprotection of Boc protecting groups. As Procedure E until the peptide coupling step, where 5-aminomethyl-3-methyl-2-(*N*,*N*-di-*tert*-butyloxycarbonylamino)-pyridine was used instead of Pab(Z)·2HCl. The removal of the two boc groups was achieved by stirring the coupled product in methylene chloride/trifluoroacetic acid 4:1 at room temperature for 3 h.

Synthetic experimentals

(*R*)-2-Hydroxy-succinic acid dimethyl ester [(*R*)-1]. Compound (*R*)-**1** was synthesized according to ref 9.

(*S*)-2-Hydroxy-succinic acid dimethyl ester [(*S*)-1]. Compound (*S*)-**1** was synthesized according to the method for the preparation of (*R*)-**1**.

(*R*)-2-Allyloxy-succinic acid dimethyl ester [(*R*)-2]. To a solution of (*R*)-**1** (0.938 g, 5.79 mmol) in toluene (10 mL) were added allyl bromide (6.25 g, 51.7 mmol) and silver(I) oxide (1.34 g, 5.78 mmol). After stirring for 16 h at room temperature the mixture was filtered through Celite and the solvent was evaporated to give the crude product (1.16 g, 99%) as a slightly yellow oil. No further purification was necessary. (*R*)-**2**: $[\alpha]_D^{22}$ +52.3 (*c* 1.8, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 250 MHz) δ 2.75 (dd, *J* = 7.3 Hz, *J* = 16.1 Hz, 1H), 2.83 (dd, *J* = 5.3 Hz, *J* = 16.1 Hz, 1H), 3.71 (s, 3H), 3.77 (s, 3H), 4.03 (dd, *J* = 6.0 Hz, *J* = 12.6 Hz, 1H), 4.23 (dd, *J* = 5.7 Hz, *J* = 12.6 Hz, 1H), 4.36 (dd, *J* = 5.3 Hz, *J* = 7.3 Hz, 1H), 5.21 (dd, *J* = 1.3 Hz, *J* = 10.2 Hz, 1H), 5.28 (dd, *J* = 1.3 Hz, *J* = 17.2 Hz, 1H), 5.82–5.99 (m, 1H); ^{13}C NMR ($CDCl_3$, 62.9 MHz) δ 37.8, 52.0, 52.2, 72.1, 74.2, 118.1, 133.8, 170.5, 171.9. Anal. calcd for $C_9H_{14}O_5$: C, 53.46; H, 6.98. Found: C, 53.37; H, 6.90.

(*S*)-2-Allyloxy-succinic acid dimethyl ester [(*S*)-2]. Compound (*S*)-**2** was prepared in 100% yield from (*S*)-**1** according to the method for the preparation of (*R*)-**2**. (*S*)-**2**: $[\alpha]_D^{22}$ −53.9 (*c* 1.6 $CHCl_3$). Anal. calcd for $C_9H_{14}O_5$: C, 53.46; H, 6.98. Found: C, 53.60; H, 7.30.

(*R*)-2-(2-*oxo*-Ethoxy)-succinic acid dimethyl ester [(*R*)-3]. To an ice-cold mixture of (*R*)-**2** (134 mg, 0.66 mmol) and *N*-methylmorpholine *N*-oxide monohydrate (175 mg, 1.29 mmol) in tetrahydrofuran/water 3:1 (5 mL) was added osmium tetroxide (0.02 M in *tert*-butanol, 0.70 mL, 0.014 mmol; the solution was stabilized with 1% *tert*-butylhydroperoxide). After 20 min the ice bath was removed and the mixture was stirred at room temperature overnight. Solid sodium hydrogen sulfite (165 mg) was added and the mixture was stirred for an additional 15 min. The mixture was filtered through silica and the solvents were evaporated providing the crude diol as a slightly green oil. The crude product was dissolved in tetrahydrofuran/water 3:1 (8 mL) and sodium periodate (282 mg, 1.32 mmol) was added. The mixture was stirred at room temperature for 30 min, after which the diol was completely cleaved. The mixture was filtered through silica and the solvents were evaporated. Flash column chromatography (ethyl acetate/toluene 2:1) provided the aldehyde (*R*)-**3** (112 mg, 83%) as a colorless oil. (*R*)-**3**: $[\alpha]_D^{22}$ +42.5 (*c* 1.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 250 MHz) δ 2.84 (dd, *J* = 7.3 Hz, *J* = 16.4 Hz, 1H), 2.93 (dd, *J* = 5.1 Hz, *J* = 16.4 Hz, 1H), 3.72 (s, 3H), 3.78 (s, 3H), 4.17 (d, *J* = 17.5 Hz, 1H), 4.33 (d, *J* = 17.5 Hz, 1H), 4.40 (dd, *J* = 5.1 Hz, *J* = 7.3 Hz, 1H), 9.71 (s, 1H); ^{13}C NMR ($CDCl_3$, 62.9 MHz)

δ 37.5, 52.1, 52.5, 76.3, 76.7, 170.4, 171.1, 200.0. Anal. calcd for $C_8H_{12}O_6$: C, 47.06; H, 5.92. Found: C, 46.88; H, 5.94.

(*S*)-2-(2-*oxo*-Ethoxy)-succinic acid dimethyl ester [(*S*)-3]. Compound (*S*)-**3** was prepared in 80% yield from (*S*)-**2** according to the method for the preparation of (*R*)-**3**. (*S*)-**3**: $[\alpha]_D^{22}$ −44.1 (*c* 0.5, $CHCl_3$). Anal. calcd for $C_8H_{12}O_6{\cdot}0.10CHCl_3$: C, 45.01; H, 5.64. Found: C, 44.84; H, 5.59.

((*R*)-4-Benzyl-3-*oxo*-morpholin-2-yl)-acetic acid methyl ester [(*R*)-4]. Compound (*R*)-**4** (a colorless syrup) was prepared in 61% yield from (*R*)-**3** and benzylamine according to Procedure A. Chromatography mobile phase: (Ethyl acetate/toluene 2:1). (*R*)-**4**: $[\alpha]_D^{22}$ +74.5 (*c* 0.9, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 250 MHz) δ 2.91 (dd, *J* = 6.6 Hz, *J* = 16.4 Hz, 1H), 3.02 (dd, *J* = 4.4 Hz, *J* = 16.4 Hz, 1H), 3.06 (ddd, *J* = 2.3 Hz, *J* = 2.9 Hz, *J* = 12.1 Hz, 1H), 3.50 (ddd, *J* = 4.4 Hz, *J* = 10.6 Hz, *J* = 12.1 Hz, 1H), 3.68 (s, 3H), 3.76 (ddd, *J* = 2.9 Hz, *J* = 10.6 Hz, *J* = 12.1 Hz, 1H), 3.95 (ddd, *J* = 2.3 Hz, *J* = 4.4 Hz, *J* = 12.1 Hz, 1H), 4.49 (d, *J* = 14.6 Hz, 1H), 4.53 (dd, overlapped, 1H), 4.73 (d, *J* = 14.6 Hz, 1H), 7.20–7.38 (m, 5H); ^{13}C NMR ($CDCl_3$, 62.9 MHz) δ 37.2, 45.9, 49.9, 51.9, 63.1, 74.4, 127.7, 128.2, 128.7, 136.2, 168.0, 171.1. Anal. calcd for $C_{14}H_{17}NO_4$: C, 63.87; H, 6.51; N, 5.32. Found: C, 63.68; H, 6.50; N, 5.32.

((*S*)-4-Benzyl-3-*oxo*-morpholin-2-yl)-acetic acid methyl ester [(*S*)-4]. Compound (*S*)-**4** was prepared in 64% yield from (*S*)-**3** and benzylamine according to Procedure A. (*S*)-**4**: $[\alpha]_D^{22}$ −75.0 (*c* 0.4 $CHCl_3$). Anal. calcd for $C_{14}H_{17}NO_4$: C, 63.87; H, 6.51; N, 5.32. Found: C, 63.50; H, 6.70; N, 5.20.

((*R*)-3-*oxo*-4-Phenethyl-morpholin-2-yl)-acetic acid methyl ester [(*R*)-5]. Compound (*R*)-**5** (a colorless syrup) was prepared in 60% yield from (*R*)-**3** and phenethylamine according to Procedure A. (*R*)-**5**: $[\alpha]_D^{22}$ +115.3 (*c* 1.6, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 250 MHz) δ 2.75–3.02 (m, 5H), 3.68 (ddd, *J* = 4.1 Hz, *J* = 10.6 Hz, *J* = 11.7 Hz, 1H), 3.60 (t, *J* = 7.5 Hz, 2H), 3.69 (s, 3H), 3.65–3.75 (m, overlapped, 1H), 3.88 (ddd, *J* = 1.9 Hz, *J* = 4.1 Hz, *J* = 12.0 Hz, 1H), 4.45 (dd, *J* = 4.0 Hz, *J* = 6.9 Hz, 1H), 7.12–7.34 (m, 5H); ^{13}C NMR ($CDCl_3$, 62.9 MHz) δ 33.4, 37.1, 47.5, 49.1, 51.8, 62.9, 74.3, 126.5, 128.6, 128.8, 138.8, 167.9, 171.1. Anal. calcd for $C_{15}H_{19}NO_4$: C, 64.97; H, 6.91; N, 5.05. Found: C, 64.75; H, 7.00; N, 5.05.

((*S*)-3-*oxo*-4-Phenethyl-morpholin-2-yl)-acetic acid methyl ester [(*S*)-5]. Compound (*S*)-**5** was prepared in 50% yield from (*S*)-**3** and phenethylamine according to Procedure A. (*S*)-**5**: $[\alpha]_D^{22}$ −117.9 (*c* 1.0, $CHCl_3$). Anal. calcd for $C_{15}H_{19}NO_4{\cdot}0.11$EtOAc: C, 64.61; H, 6.98 N, 4.88. Found: C, 64.50; H, 7.35; N, 4.80.

((*R*)-4-Isobutyl-3-*oxo*-morpholin-2-yl)-acetic acid methyl ester (6). Compound **6** (a colorless syrup) was prepared in 51% yield from (*R*)-**3** and isobutylamine according to Procedure A. **6**: $[\alpha]_D^{22}$ +62.6 (*c* 0.6, MeOH); ^{1}H NMR ($CDCl_3$, 250 MHz) δ 0.93 (d, *J* = 6.6 Hz, 6H), 1.99 (m, 1H), 2.83 (dd, *J* = 7.1 Hz, *J* = 16.4 Hz, 1H), 3.00 (dd, *J* = 4.1 Hz, *J* = 16.4 Hz, 1H), 3.10–3.34 (m, 3H), 3.56–3.75 (m, 1H), 3.71 (s, 3H), 3.75–3.89 (m, 1H), 4.20 (ddd, *J* = 2.1 Hz, *J* = 4.2 Hz, *J* = 12.0 Hz, 1H), 4.49 (dd, *J* = 4.1 Hz, *J* = 7.1 Hz, 1H); ^{13}C NMR ($CDCl_3$, 62.9 MHz) δ 19.9, 20.2, 26.3, 37.2, 47.2, 51.8, 54.3, 63.1, 74.4, 168.0, 171.1. Anal. calcd for $C_{11}H_{19}NO_4{\cdot}0.041CHCl_3$: C, 56.65; H, 8.20; N, 5.99. Found: C, 56.70; H, 8.35; N, 6.05.

***N*-{4-[Amino(imino)methyl]benzyl}-2-((*R*)-4-benzyl-3-*oxo*-morpholin-2-yl)-acetamide [(*R*)-7].** Compound (*R*)-**7** (a colorless solid) was prepared in 81% yield from (*R*)-**4** according to Procedure D. Chromatography mobile phase after the coupling step: Ethyl acetate/methanol 9:1. (*R*)-**7**: $[\alpha]_D^{22}$ +51.9 (*c* 0.8, MeOH); ^{1}H NMR (methanol-d_4, 250 MHz) δ 2.79 (dd, *J* = 7.7 Hz, *J* = 15.0 Hz, 1H), 2.92 (dd, *J* = 4.0 Hz, *J* = 15.0 Hz, 1H), 3.17 (ddd, *J* = 2.4 Hz, *J* = 3.0 Hz, *J* = 12.4 Hz, 1H), 3.48 (ddd, *J* = 4.4 Hz, *J* = 10.2 Hz, *J* = 12.4 Hz, 1H), 3.79 (ddd, *J* = 3.0 Hz, *J* = 10.2 Hz, *J* = 12.1 Hz, 1H), 3.98 (ddd, *J* = 2.4 Hz, *J* = 4.4 Hz, *J* = 12.1 Hz, 1H), 4.47 (s, 2H), 4.53 (d, *J* = 15.0 Hz, 1H), 4.57 (dd, overlapped, 1H), 4.68 (d, *J* = 15.0 Hz, 1H), 7.22–7.38 (m, 5H), 7.45 (d, *J* = 8.4 Hz, 2H), 7.72 (d, *J* = 8.4 Hz, 2H); ^{13}C NMR (methanol-d_4, 62.9 MHz) δ 39.6, 43.6, 47.3, 50.9, 63.8, 75.8, 128.5, 128.7, 128.8, 129.0, 129.8, 131.8, 137.6, 145.0, 167.8, 170.5, 172.6. Anal. calcd for $C_{21}H_{24}N_4O_3{\cdot}1.11$HOAc$\cdot0.43H_2O$: C, 61.31; H, 6.55; N, 12.32. Found: C, 61.35; H, 6.35; N, 12.40.

***N*-{4-[Amino(imino)methyl]benzyl}-2-((*S*)-4-benzyl-3-*oxo*-morpholin-2-yl)-acetamide [(*S*)-7].** Compound (*S*)-**7** was prepared in 92% yield from (*S*)-**4** according to Procedure D. (*S*)-**7**: $[\alpha]_D^{22}$ −53.3 (c 0.6, MeOH). Anal. calcd for $C_{21}H_{24}N_4O_3{\cdot}0.94$HOAc$\cdot1.43H_2O$: C, 59.39; H, 6.67; N, 12.11. Found: C, 59.40; H, 6.74; N, 12.08.

***N*-{4-[Amino(imino)methyl]benzyl}-2-((*R*)-3-*oxo*-4-phenethyl-morpholin-2-yl)-acetamide [(*R*)-8].** Compound (*R*)-**8** (a colorless solid) was prepared in 83% yield from (*R*)-**5** according to Procedure D. (*R*)-**8**: $[\alpha]_D^{22}$ +60.0 (*c* 0.2 MeOH); ^{1}H NMR (methanol-d_4, 250 MHz) δ 2.79 (dd, *J* = 7.7 Hz, *J* = 15.0 Hz, 1H), 2.83 (dd, *J* = 4.0 Hz, *J* = 15.0 Hz, 1H), 2.88 (t, *J* = 7.0 Hz, 2H), 3.09 (ddd, *J* = 2.4 Hz, *J* = 2.9 Hz, *J* = 12.1 Hz, 1H), 3.44 (ddd, *J* = 4.4 Hz, *J* = 10.1 Hz, 12.3 Hz, 1H), 3.60 (t, *J* = 7.0 Hz, 2H), 3.71 (ddd, *J* = 2.9 Hz, *J* = 10.1 Hz, *J* = 12.3 Hz, 1H), 3.91 (ddd, *J* = 2.4 Hz, *J* = 4.4 Hz, *J* = 12.1 Hz, 1H), 4.45 (s, 2H), 4.45 (dd, overlapped, 1H), 6.97–7.13 (m, 5H), 7.42 (d, *J* = 8.4 Hz, 2H), 7.72 (d, *J* = 8.4 Hz, 2H); ^{13}C NMR (methanol-d_4, 62.9 MHz) δ 34.1, 39.6, 43.6, 48.5, 50.1, 63.7, 75.7, 127.5, 128.4, 128.7, 129.0, 129.6, 129.9, 140.1, 144.7, 167.7, 170.4, 172.6. Anal. calcd for $C_{22}H_{26}N_4O_3{\cdot}0.95$HOAc$\cdot0.51H_2O$: C, 62.30; H, 6.74; N, 12.16. Found: C, 62.55; H, 6.60; N, 12.25.

***N*-{4-[Amino(imino)methyl]benzyl}-2-((*S*)-3-*oxo*-4-phenethyl-morpholin-2-yl)-acetamide [(*S*)-8].** Compound (*S*)-**8** was prepared in 86% yield from (*S*)-**5** according to Procedure D. (*S*)-**8**: $[\alpha]_D^{22}$ −62.1 (*c* 1.0 MeOH). Anal. calcd for $C_{22}H_{26}N_4O_3{\cdot}0.59$HOAc$\cdot1.78H_2O$: C, 60.26; H, 6.96; N, 12.13. Found: C, 60.31; H, 6.64; N, 12.31.

***N*-{4-[Amino(imino)methyl]benzyl}-2-((*R*)-4-isobutyl-3-*oxo*-morpholin-2-yl)-acetamide (9).** Compound **9** (a colorless solid) was prepared in 40% yield from **6** according to Procedure D. **9**: $[\alpha]_D^{22}$ +55.6 (*c* 0.8, MeOH); ^{1}H NMR (methanol-d_4, 250 MHz) δ 0.92 (d, *J* = 6.6 Hz, 6H), 2.02 (m, 1H), 2.71 (dd, *J* = 8.0 Hz, *J* = 15.0 Hz, 1H), 2.87 (dd, *J* = 4.0 Hz, *J* = 15.0 Hz, 1H), 3.19–3.31 (m, 3H), 3.52–3.67 (m, 1H), 3.75–3.90 (m, 1H), 4.03 (ddd, *J* = 2.6 Hz, *J* = 4.0 Hz, *J* = 12.1 Hz, 1H), 4.41–4.57 (m, 1H), 4.48 (s, 2H), 7.50 (d, *J* = 8.4 Hz, 2H), 7.75 (d, *J* = 8.4 Hz, 2H); ^{13}C NMR (methanol-d_4, 62.9 MHz) δ 20.2, 20.4, 27.4, 39.7, 43.6, 49.9, 55.3, 63.9, 75.7, 128.8, 129.0, 129.6, 146.3, 168.1, 170.6, 172.7. HRMS *m*/*z* calcd for $C_{18}H_{27}N_4O_3$ (MH^+): 347.2083. Found 347.2082.

(*R*)-2-(2-Cyclohexylamino-ethoxy)-succinic acid dimethyl ester (10). Compound **10** (a colorless glue) was prepared in 58% yield from (*R*)-**3** and cyclohexylamine according to Procedure B. **10**: $[\alpha]_D^{22}$ +41.3 (*c* 0.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 250 MHz) 0.90–1.30 (m, 4H), 1.50–1.95 (m, 6H), 2.28–2.48 (m, 1H), 2.61–2.81 (m, 4H), 3.48–3.59 (m, 1H), 3.62–3.70 (m, 1H), 3.65 (s, 3H), 3.71 (s, 3H), 3.67–3.80 (m, 1H), 4.28 (dd, *J* = 4.8 Hz, *J* = 8.0 Hz, 1H); ^{13}C NMR ($CDCl_3$, 62.9 MHz) δ 24.9, 26.0, 33.3, 37.6, 46.1, 51.8, 52.1, 56.4, 70.9, 75.2, 170.4, 171.7. Anal. calcd for $C_{14}H_{25}NO_5 \cdot 0.03CHCl_3$: C, 57.92; H, 8.67; N, 4.82. Found: C, 58.00; H, 8.32; N, 4.80.

(*R*)-2-{2-[(1,1-Dicyclohexyl-methyl)-amino]-ethoxy}-succinic acid dimethyl ester [(*R*)-11]. Compound (*R*)-**11** (a colorless glue) was prepared in 43% yield from (*R*)-**3** and (dicyclohexylmethyl)-amine[17] according to Procedure B. (*R*)-**11**: $[\alpha]_D^{22}$ +26.7 (*c* 0.3, MeOH); ^{1}H NMR ($CDCl_3$, 250 MHz) δ 0.90–1.33δ (m, 10H), 1.39–1.82 (m, 12H), 1.91–2.02 (m, 2H), 2.70–2.98 (m, 4H), 3.52–3.64 (m, 1H), 3.70 (s, 3H), 3.77 (s, 3H), 3.70–3.85 (m, 1H), 4.32 (dd, *J* = 5.1 Hz, *J* = 7.7 Hz, 1H); ^{13}C NMR ($CDCl_3$, 62.9 MHz) δ 26.5, 26.6, 26.8, 28.5, 30.9, 37.7, 40.2, 51.0, 52.0, 52.3, 68.6, 71.0, 75.4, 170.5, 172.0. Anal. calcd for $C_{21}H_{37}NO_5$: C, 65.77; H, 9.72; N, 3.65. Found: C, 65.61; H, 10.00; N, 3.65.

(*S*)-2-{2-[(1,1-Dicyclohexyl-methyl)-amino]-ethoxy}-succinic acid dimethyl ester [(*S*)-11]. Compound (*S*)-**11** was pepared in 71% yield from (*S*)-**3** and (dicyclohexylmethyl)-amine[17] according to Procedure B. (*S*)-**11**: $[\alpha]_D^{22}$ −26.2 (*c* 0.8, MeOH). Anal. calcd for $C_{21}H_{37}NO_5$: C, 65.77; H, 9.72; N, 3.65. Found: C, 65.59; H, 9.82; N, 3.63.

(*R*)-2-{2-[(1,1-Diphenyl-methyl)-amino]-ethoxy}-succinic acid dimethyl ester [(*R*)-12]. Compound (*R*)-**12** (a colorless solid) was prepared in 78% yield from (*R*)-**3** and α-diphenyl aminomethane according to Procedure B. (*R*)-**12**: $[\alpha]_D^{22}$ +21.9 (*c* 1.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 250 MHz) δ 2.20 (s, 1H), 2.67–2.87 (m, 4H), 3.58 (s, 3H), 3.55–3.68 (m, 1H), 3.72 (s, 3H), 3.81–3.91 (m, 1H), 4.37 (dd, *J* = 4.0 Hz, *J* = 8.2 Hz, 1H), 4.85 (s, 1H), 7.14–7.49 (m, 10H); ^{13}C NMR ($CDCl_3$, 62.9 MHz) δ 37.7, 47.4, 51.9, 52.2, 67.1, 70.8, 75.4, 126.9, 127.2, 128.4, 144.1, 170.6, 171.9. Anal. calcd for $C_{21}H_{25}NO_5$: C, 67.91; H, 6.78; N, 3.77. Found: C, 67.76; H, 6.79; N, 3.91.

(*S*)-2-{2-[(1,1-Diphenyl-methyl)-amino]-ethoxy}-succinic acid dimethyl ester [(*S*)-12]. Compound (*S*)-**12** was prepared in 77% yield from (*S*)-**3** and α-diphenyl aminomethane according to Procedure B. (*S*)-**12**: $[\alpha]_D^{22}$ −20.2 (*c* 1.5, $CHCl_3$). Anal. calcd for $C_{21}H_{25}NO_5$: C, 67.91; H, 6.78; N, 3.77. Found: C, 67.69; H, 6.86; N, 3.74.

(*R*)-2-{2-[((*S*)-1-*tert*-Butoxycarbonyl-1-phenyl-methyl)-amino]-ethoxy}-succinic acid dimethyl ester (13). Compound **13** (a colorless oil) was prepared in 82% yield from (*R*)-**3** and (*S*)-2-amino-2-phenyl-acetic acid *tert*-butyl ester (the *tert*-butyl ester of (*S*)-phenylglycine) according to Procedure C. **13**: $[\alpha]_D^{22}$ +73.5 (*c* 0.8, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.38 (s, 9H), 2.45 (bs, 1H), 2.60–2.71 (m, 1H), 2.73–2.88 (m, 3H), 3.53–3.63 (m, 1H), 3.69 (s, 3H), 3.74 (s, 3H), 3.78–3.89 (m, 1H), 4.28 (s, 1H), 4.33 (dd, *J* = 5.1 Hz, *J* = 8.0 Hz, 1H), 7.25–7.42 (m, 5H); ^{13}C NMR ($CDCl_3$, 62.9 MHz) δ 27.9, 37.7, 46.9, 52.0, 52.2, 66.0, 71.0, 75.5, 81.3, 127.4, 127.7, 128.5, 138.8, 170.5, 171.9, 171.9. Anal. calcd for $C_{20}H_{29}NO_7$: C, 60.74; H, 7.39; N, 3.54. Found: C, 60.72; H, 7.38; N, 3.62.

***N*-{4-[Amino(imino)methyl]benzyl}-2-((*R*)-4-cyclohexyl-3-*oxo*-morpholin-2-yl)-acetamide (14).** Compound **14** (a colorless solid) was prepared in 70% yield from **10** according to Procedure E. **14**: $[\alpha]_D^{22}$ +40.0 (*c* 0.2, MeOH); ^{1}H NMR (D_2O, 250 MHz) δ 0.95–1.80 (m, 10H), 2.82 (dd, *J* = 4.4 Hz, *J* = 15.0 Hz, 1H), 2.90 (dd, *J* = 5.9 Hz, *J* = 15.0 Hz, 1H), 3.24–3.44 (m, 2H), 3.72–3.86 (m, 1H), 3.98–4.18 (m, 2H), 4.43 (s, 2H), 4.45 (dd, overlapped, 1H), 7.45 (d, *J* = 8.4 Hz, 2H), 7.72 (d, *J* = 8.4 Hz, 2H); ^{13}C NMR (D_2O, 62.9 MHz) δ 27.5, 27.7, 27.8, 31.1, 31.3, 40.7, 43.4, 45.3, 56.3, 65.4, 76.4, 129.5, 130.3, 130.5, 146.9, 169.3, 171.6, 175.0. Anal. calcd for $C_{20}H_{28}N_4O_3 \cdot 1.17HOAc \cdot 1.50H_2O$: C, 57.12; H, 7.66; N, 11.93. Found: C, 57.15; H, 7.35; N, 12.10.

***N*-{4-[Amino(imino)methyl]benzyl}-2-[(*R*)-4-(1,1-dicyclohexyl-methyl)-3-*oxo*-morpholin-2-yl]-acetamide [(*R*)-15].** Compound (*R*)-**15** (a colorless solid) was prepared in 94% yield from (*R*)-**11** according to Procedure E. (*R*)-**15**: $[\alpha]_D^{22}$ +30.0 (*c* 0.3, MeOH); ^{1}H NMR (on the acetate salt) (methanol-d_4, 250 MHz) δ 0.90–1.41 (m, 10H), 1.60–1.90 (m, 13H), 1.93 (s, 3H), 2.68 (dd, *J* = 8.6 Hz, *J* = 14.8 Hz, 1H), 2.88 (dd, *J* = 3.9 Hz, *J* = 14.8 Hz, 1H), 3.41–3.56 (m, 1H), 3.69–3.86 (m, 1H), 3.95–4.07 (m, 1H), 4.25–4.36 (m, 1H), 4.50 (d, *J* = 4.4 Hz, 2H), 4.57 (dd, *J* = 3.9 Hz, *J* = 8.6 Hz, 1H), 7.54 (d, *J* = 8.4 Hz, 2H), 7.77 (d, *J* = 8.4 Hz, 2H); ^{13}C NMR (on the acetate salt) (methanol-d_4, 62.9 MHz) δ 23.5, 27.7, 27.9, 28.0, 28.1, 30.6, 30.8, 31.9, 32.1, 39.0, 39.2, 40.7, 43.9, 45.4, 50.6, 62.2, 64.4, 76.0, 128.5, 129.4, 129.5, 147.5, 168.6, 171.4, 173.1, 180.0. HRMS *m*/*z* calcd for $C_{27}H_{41}N_4O_3$ (MH^+): 469.3181. Found 469.3197.

***N*-{4-[Amino(imino)methyl]benzyl}-2-[(*S*)-4-(1,1-dicyclohexyl-methyl)-3-*oxo*-morpholin-2-yl]-acetamide [(*S*)-15].** Compound (*S*)-**15** was prepared in 77% yield from (*S*)-**11** according to Procedure E. (*S*)-**15**: $[\alpha]_D^{22}$ −30.3 (*c* 0.6,

MeOH); HRMS *m/z* calcd for $C_{27}H_{41}N_4O_3$ (MH^+): 469.3179. Found: 469.3186.

***N*-{4-[Amino(imino)methyl]benzyl}-2-[(*R*)-4-(1,1-diphenyl-methyl)-3-*oxo*-morpholin-2-yl]-acetamide [(*R*)-16].** Compound (*R*)-**16** (a colorless solid) was prepared in 53% yield from (*R*)-**12** according to Procedure E. (*R*)-**16**: $[\alpha]_D^{22}$ +10.0 (*c* 0.1, MeOH); ^{1}H NMR (D_2O, 250 MHz) δ 2.85 (dd, *J* = 4.4 Hz, *J* = 15.4 Hz, 1H), 2.97 (dd, *J* = 5.8 Hz, *J* = 15.4 Hz, 1H), 3.02–3.14 (m, 2H), 3.85–4.04 (m, 2H), 4.42 (s, 2H), 4.59–4.66 (m, 1H), 6.77 (s, 1H), 7.10–7.24 (m, 4H), 7.28–7.46 (m, 8H), 7.64 (d, *J* = 8.4 Hz, 2H); ^{13}C NMR (D_2O, 62.9 MHz) δ 38.8, 43.3, 44.5, 62.1, 63.4, 74.9, 128.2, 128.5, 128.7, 129.3, 130.0, 138.1, 138.2, 145.2, 169.0, 170.8, 172.8. Anal. calcd for $C_{27}H_{28}N_4O_3 \cdot 2.27HOAc \cdot 0.30EtOH$: C, 63.63; H, 6.46; N, 9.24. Found: C, 63.99; H, 6.27; N, 8.89.

***N*-{4-[Amino(imino)methyl]benzyl}-2-[(*S*)-4-(1,1-diphenyl-methyl)-3-*oxo*-morpholin-2-yl]-acetamide [(*S*)-16].** Compound (*S*)-**16** was prepared in 76% yield from (*S*)-**12** according to Procedure E. (*S*)-**16**: $[\alpha]_D^{22}$ −10.9 (c 0.6, MeOH); HRMS *m/z* calcd for $C_{27}H_{29}N_4O_3$ (MH^+): 457.2240. Found: 457.2243.

***N*-{4-[Amino(imino)methyl]benzyl}-2-[(*R*)-4-((*S*)-1-*tert*-butoxycarbonyl-1-phenyl-methyl)-3-*oxo*-morpholin-2-yl]-acetamide (17).** Compound **17** (a colorless solid, partially epimerized in the Phe-Gly part of the molecule with a ratio of approximately 2:1 in favor of the desired product) was prepared in 59% yield from **13** according to Procedure E. **17**: $[\alpha]_D^{22}$ +26.4 (*c* 0.7, MeOH); ^{1}H NMR (methanol-d_4, 250 MHz) δ 1.48 (s, 7H), 1.50 (s, 2H), 2.69–2.86 (m, 2H), 2.88–2.99 (m, 1H), 3.52–3.73 (m, 2H), 3.83–4.00 (m, 1H), 4.41–4.53 (m, 2H), 4.54–4.65 (m, 1H), 6.09 (s, 1H), 7.25–7.34 (m, 2H), 7.35–7.50 (m, 3H), 7.53 (d, *J* = 8.4 Hz, 2H), 7.72 (d, *J* = 8.4 Hz, 2H); ^{13}C NMR (methanol-d_4, 62.9 MHz) δ 28.2, 39.6, 39.8, 43.6, 43.7, 45.3, 45.5, 62.6, 63.9, 64.2, 75.8, 76.1, 83.8, 128.6, 128.8, 129.8, 130.0, 130.6, 130.7, 134.5, 134.6, 134.7, 145.6, 167.9, 170.3, 170.4, 171.0, 171.2, 172.4. HRMS *m/z* calcd for $C_{26}H_{33}N_4O_5$ (MH^+): 481.2451. Found 481.2452.

***N*-{4-[Amino(imino)methyl]benzyl}-2-[(*R*)-4-((*S*)-1-carboxy-1-phenyl-methyl)-3-*oxo*-morpholin-2-yl]-acetamide (18).** Compound **18** (a colorless solid) was synthesized from Cbz-protected **17** in 75% yield by first removing the *tert*-butyl group by stirring in a solution of dichloromethane/trifluoroacetic acid 2:1 in the presence of 3 molar equivalents of triethylsilane for 3 h, followed by evaporation and purification by flash column chromatography (ethyl acetate/methanol 4:1 with 1% acetic acid), followed by the hydrogenation step described in Procedure E. (The total yield of **18** from **13** was 44%.) **18**: $[\alpha]_d^{22}$ +66.7 (*c* 0.3, MeOH); ^{1}H NMR (methanol-d_4, 250 MHz) δ 2.76–3.00 (m, 3H), 3.56–3.81 (m, 2H), 3.85–4.01 (m, 1H), 4.53 (s, 2H), 4.59 (dd, *J* = 4.6 Hz, *J* = 8.0 Hz, 1H), 6.25 (s, 1H), 7.28–7.39 (m, 5H), 7.54 (d, *J* = 8.4 Hz, 2H), 7.75 (d, *J* = 8.4 Hz, 2H); ^{13}C NMR (methanol-d_4, 62.9 MHz) δ 39.8, 43.7, 44.5, 63.7, 64.0, 75.6, 128.1, 129.0, 129.2, 129.5, 129.9, 131.1, 137.6, 147.0, 168.2, 170.4, 173.1, 176.2; HRMS *m/z* calcd for $C_{22}H_{25}N_4O_5$ (MH^+): 425.1825. Found 425.1813.

***N*-(6-Amino-5-methyl-pyridin-3-ylmethyl)-2-[(*R*)-4-(1,1-diphenyl-methyl)-3-*oxo*-morpholin-2-yl]-acetamide (19).** Compound **19** (a colorless solid) was prepared in 50% yield from (*R*)-**12** according to Procedure F. **19**: $[\alpha]_D^{22}$ +3.1 (*c* 0.3, MeOH); ^{1}H NMR (on the acetate salt) (methanol-d_4, 250 MHz) δ 2.00 (s, 3H), 2.18 (s, 3H), 2.70–2.90 (m, 2H), 3.01–3.30 (m, 2H), 3.81–4.07 (m, 2H), 4.25 (s, 2H), 4.61 (dd, *J* = 4.8 Hz, *J* = 6.6 Hz, 1H), 7.00 (s, 1H), 7.18–7.45 (m, 10H), 7.60 (s, 1H), 7.67 (s, 1H); ^{13}C NMR (methanol-d_4, 62.9 MHz) δ 16.8, 39.8, 40.6, 44.8, 61.8, 64.1, 76.0, 122.1, 125.5, 128.9, 129.6, 130.4, 136.4, 139.3, 142.6, 155.7, 170.8, 172.6. HRMS *m/z* calcd for $C_{26}H_{29}N_4O_3$ (MH^+): 445.2240. Found 445.2233.

Acknowledgements

We would like to thank AstraZeneca R&D Mölndal for financial support. We would also like to thank Erika Gyzander and the Biochemistry Group, AstraZeneca R&D Mölndal, for providing the biological testings, and Karl-Erik Karlsson, AstraZeneca R&D Mölndal, for performing the HRMS measurements.

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