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# Bivalent Inhibition of β-Tryptase: Distance Scan of Neighboring Subunits by Dibasic Inhibitors

Norbert Schaschke,[a,*] Andreas Dominik,[b] Gabriele Matschiner[c] and Christian P. Sommerhoff[c]

[a]*Max-Planck-Institut für Biochemie, D-82152 Martinsried, Germany*
[b]*Byk Gulden Lomberg Chemische Fabrik GmbH, D-78467 Konstanz, Germany*
[c]*Abteilung für Klinische Chemie und Klinische Biochemie, Klinikumsstandort Innenstadt der LMU München, D-80336 München, Germany*

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**Abstract**—Based on bifunctional diketopiperazines as templates and *m*-aminomethyl-phenylalanine as arginine mimetic, we have synthesized a new class of structurally related dibasic tryptase inhibitors with systematically increasing spacer length. These compounds were used to scan the distance between the active sites of two neighboring subunits of the β-tryptase tetramer. The $K_i$-values obtained are a function of the distance between the terminal amino groups and indicate optimal binding of inhibitors with 29–31 bonds between the amino groups. These experimental data are in full agreement with predictions derived from a novel modeling program that allows the docking of bivalent ligands. 

Human β-tryptase is a mast cell-specific serine protease that exhibits trypsin-like activity by hydrolyzing peptide bonds C-terminally of arginine and lysine residues.[1,2] A growing number of biological and immunological data suggests that tryptase plays a key role in the pathogenesis of diverse allergic and inflammatory disorders, most prominently asthma,[3,4] and thus is an interesting therapeutic target.[5] In particular, the enzyme acts as a (neuro)peptidase and is responsible for enhancing the contractility of the airway smooth muscle.[6] The X-ray structure of human β-tryptase[7,8] revealed that the enzyme consists of four quasi-identical subunits (A, B, C, and D) whose active sites are directed towards a central pore. Therefore, the four negatively charged S1 binding pockets are displayed in a defined spatial arrangement that should allow inhibition of the enzyme by dibasic ligands of appropriate length interacting simultaneously with two neighboring S1 subsites as depicted schematically in Figure 1. Compared to a monovalent binding, the advantage of such a bivalent interaction is the gain in affinity and selectivity achieved by exploiting the entropy effect.[9] For human β-tryptase, a variety of dibasic inhibitors has been described that take advantage of this particular feature of the enzyme.[10–15]

In this study, we have systematically investigated the acceptor properties of β-tryptase by performing a distance scan using a set of structurally related dibasic probing compounds. In addition, the ability of the compounds to bind in a mono- or bivalent fashion was analyzed using a novel modeling program that allows the docking of bivalent ligands.

Due to entropic reasons,[9] we have focused our scanning approach on the shortest distance between neighboring S1 pockets, that is those of subunits A and D (and their

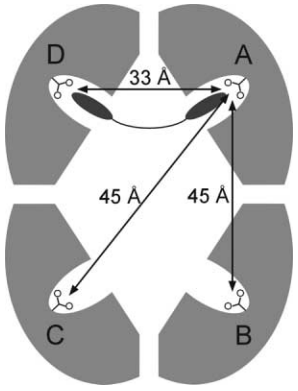


**Figure 1.** Schematic representation of the β-tryptase tetramer interacting with a bivalent inhibitor. The inter-S1 subsite distances are indicated and the side-chain carboxylate of Asp-189 at the bottom of each S1 pocket is shown.

*Corresponding author. Tel.: +49-89-8578-3910; fax: +49-89-8578-2847; e-mail: schaschk@biochem.mpg.de

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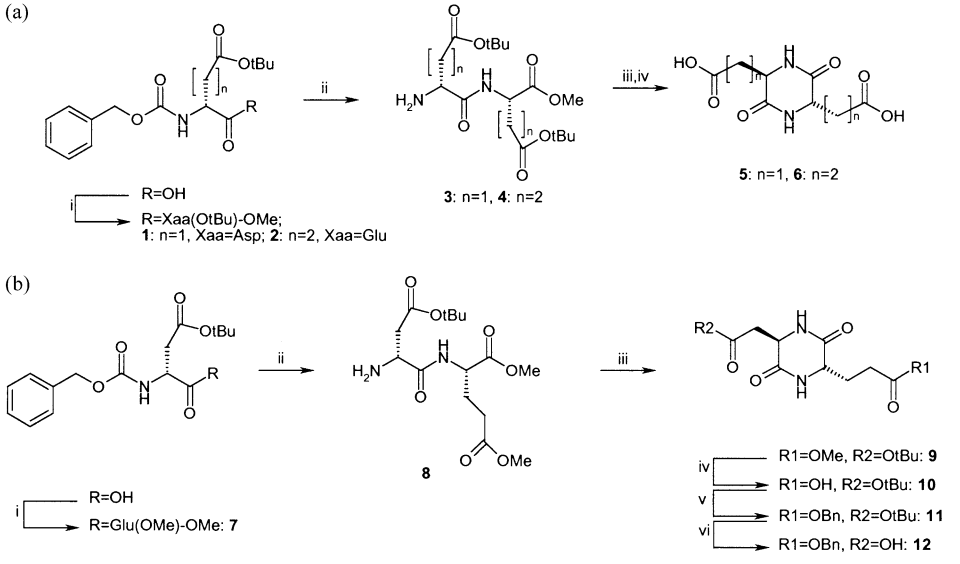
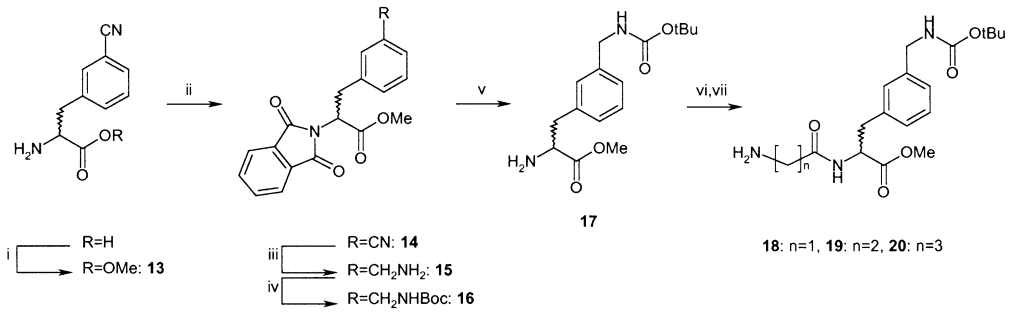
equivalents B and C), comprising 33 Å according to the crystal structure (Fig. 1).[7,8] As central scaffold for the display of two binding heads via spacers of increasing length diketopiperazines were chosen. In particular, those derived from aspartic and glutamic acid as well as mixed ones were utilized to minimize the scaffold's contribution to the distance between the binding heads. Diketopiperazines derived from amino acids can be divided stereochemically into those composed exclusively of L- or D-amino acids with *cis*-geometry in respect to the plane of the ring and mixed ones with *trans*-geometry. By inspecting the X-ray structure of the β-tryptase tetramer, diketopiperazines with *trans*-geometry were expected to allow a better positioning of the binding heads into the S1 subsites of neighboring subunits. Among the arginine mimetics, *m*- and *p*-aminomethyl-phenylalanine were investigated as potential binding heads. Based on the ~15-times higher inhibitory potency of Ac-DL-Phe(3-$H_2N$-$CH_2$)-OMe ($K_i = 13.7$ μM) compared to Ac-DL-Phe(4-$H_2N$-$CH_2$)-OMe ($K_i = 213$ μM) for the β-tryptase tetramer, the more potent *meta*-derivative was used in the present study, and to adjust the optimal spacer length, ω-amino acids were utilized.

To realize the distance scan, a building block strategy was applied. The symmetric scaffolds c[D-Asp-L-Asp-] (**5**) and c[D-Glu-L-Glu-] (**6**) were synthesized as outlined in Scheme 1a. In addition, the asymmetric scaffold c[D-Asp-L-Glu-] with orthogonal side-chain protection was obtained from Z-D-Asp(OtBu)-OH and H-L-Glu(OMe)-OMe following essentially the procedure used for the symmetric piperazine-2,5-diones (Scheme 1b). Subsequently, the side-chain protection pattern of **9** was transformed in three steps to yield **12** as building block suitable for the final inhibitor assembly. The binding head was obtained starting from H-DL-Phe(3-CN)-OH in five steps as outlined in Scheme 2. To vary systematically the distance between the headgroups, two types of dibasic inhibitors were synthesized: Symmetric ones were obtained as summarized in Scheme 3a, and an asymmetric inhibitor was synthesized utilizing the orthogonally protected scaffold **12** to complete the distance scan (Scheme 3b).

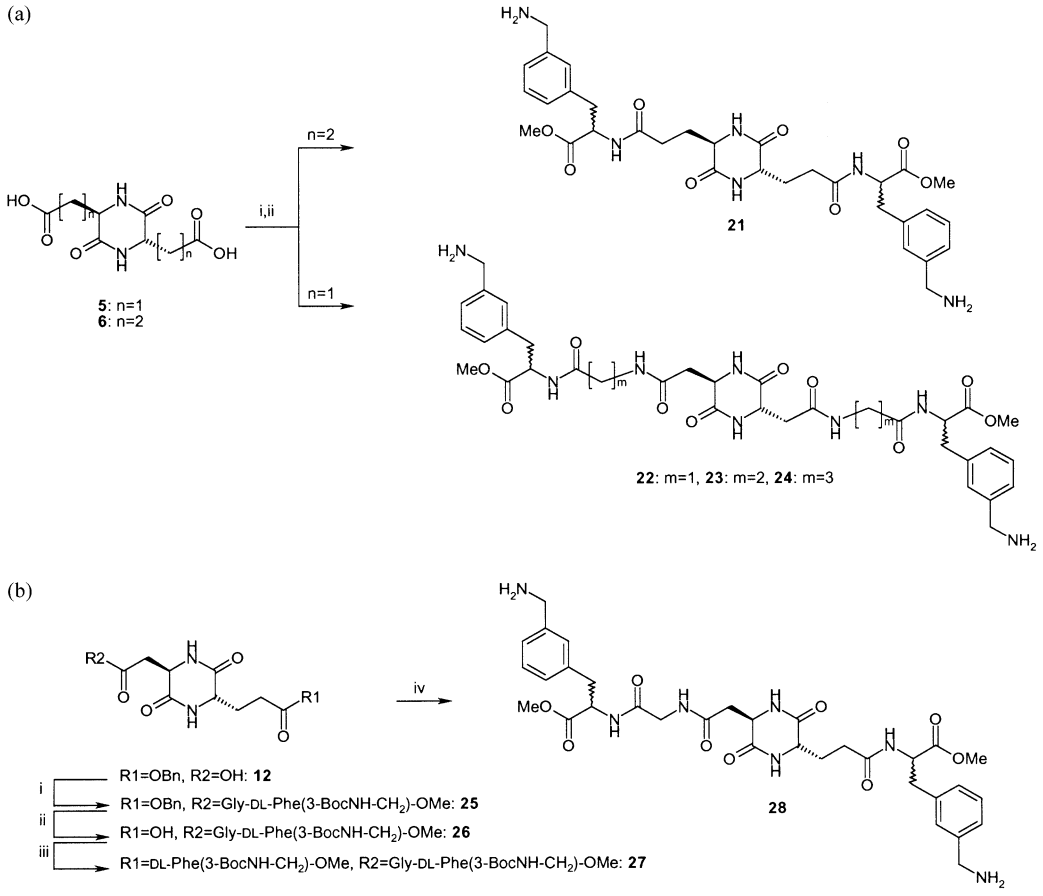
The inhibition kinetic data[16] for the interaction of the dibasic distance probes with the β-tryptase tetramer are summarized in Table 1. The results clearly show a relation between the distance of the binding heads and the




**Scheme 1.** (a) Synthesis of the symmetric scaffolds. Reaction conditions: (i) H-L-Xaa(OtBu)-OMe/DIEA/EDC/HOBt, $CHCl_3$; (ii) 10% Pd–C/$H_2$, MeOH; (iii) T, MeOH; (iv) 95% aq TFA, 0 °C→rt. (b) Synthesis of the asymmetric scaffold. Reaction conditions: (i) H-L-Glu(OMe)-OMe/DIEA/EDC/HOBt, $CHCl_3$; (ii) 10% Pd–C/$H_2$, MeOH; (iii) T, MeOH; (iv) NaOH, THF/$H_2O$ (2:1); (v) (a) $Cs_2CO_3$, MeOH; (b) $C_6H_5$–$CH_2$–Br, DMF; (vi) 95% aq TFA, 0 °C→rt.




**Scheme 2.** Synthesis of the binding head and its spacer functionalization. Reaction conditions: (i) MeOH/$SOCl_2$, −5 °C; (ii) *N*-ethoxycarbonylphthalimide/$Na_2CO_3$, dioxane/$H_2O$ (1:1); (iii) 10% Pd-C/$H_2$, AcOH; (iv) $(Boc)_2O$/$NaHCO_3$, dioxane/$H_2O$ (1:1); (v) $H_2N$–$NH_2$×AcOH, MeOH, 50 °C; (vi) *Z*-NH–$(CH_2)_n$–COOH/DIEA/EDC/HOBt, $CHCl_3$; (vii) 10% Pd–C/$H_2$, MeOH.

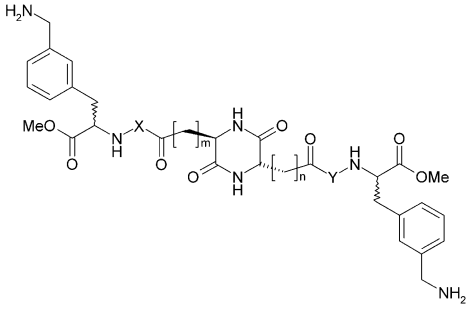



**Scheme 3.** (a) Synthesis of the symmetric dibasic inhibitors. Reaction conditions: (i) $n=1$: **18** or **19** or **20**; $n=2$: **17**/DIEA/EDC/HOBt, DMF; (ii) 95% aq TFA, 0 °C→rt. (b) Synthesis of the asymmetric dibasic inhibitor. Reaction conditions: (i) **18**/DIEA/EDC/HOBt, DMF; (ii) 10% Pd–C/$H_2$, AcOEt; (iii) **19**/DIEA/EDC/HOBt, DMF ; (iv) 95% aq TFA, 0 °C→rt.

affinity of the inhibitors. The dibasic inhibitor **21**, that is that with the shortest distance between the terminal amino groups, is only about as potent as the headgroup (2.4 μM vs 13.7 μM, respectively). An increase of the distance between the amino groups, however, is accompanied by a large increase in affinity, reaching a maximal cooperativity with compound **23** that is 1370-fold more potent than the binding head.

A novel modeling program[17] that allows the docking of bivalent inhibitors was applied on this set of dibasic distance probes. For each inhibitor two of its possible diastereomers (i.e., the *S*,*S*- and *R*,*R*-compounds; the absolute stereochemistry refers to the head groups, respectively) were docked. The result obtained for the *S*,*S*-diastereomer of inhibitor **23** is shown in Figure 2. The inhibitor can adopt a conformation that allows the simultaneous interaction of its both positively charged headgroups with the Asp-189 residues within the S1 pockets of the neighboring subunits A and D (or their equivalents B and C). According to the docking experiments, the main contribution to the affinity of the dibasic inhibitors appears to originate from interactions of the headgroups within the S1 subsites, whereas contacts between the central spacer region and the enzyme were not detectable. Furthermore, such modeling confirmed that there is sufficient space for two dibasic ligands within one tetramer and thus, allowing a stoichiometry β-tryptase tetramer/dibasic ligand of 1:2. The predictions whether the inhibitors bind in a mono- or bivalent manner are in full agreement with the SAR data derived from inhibition kinetics. Both diastereomers of inhibitor **21** as well as the *R*,*R*-diastereomer of inhibitor **28** were

**Table 1.** Inhibition of the β-tryptase tetramer by the set of dibasic distance probes



| Compd | *m* | *n* | X | Y | Number of bonds between the amino groups | $K_i$ (μM) |
|---|---|---|---|---|---|---|
| **21** | 2 | 2 | — | — | 25 | 2.4 |
| **28** | 1 | 2 | Gly | — | 27 | 0.18 |
| **22** | 1 | 1 | Gly | Gly | 29 | 0.018 |
| **23** | 1 | 1 | β-Ala | β-Ala | 31 | 0.010 |
| **24** | 1 | 1 | 4-Abu | 4-Abu | 33 | 0.35 |

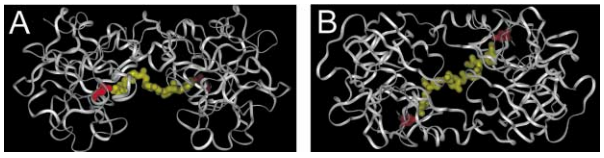



**Figure 2.** Bivalent docking of the *S,S*-diastereomer of inhibitor **23**. Subunits A and D of the β-tryptase tetramer are shown in ribbon representation, the inhibitor (colored in yellow) as well as the amino acid residue Asp-189 (colored in red) of subunits A and D as stick models: (A) side view, (B) top view.

classified as monovalent binders, whereas all other diastereomers are able to bind in a bivalent manner.

Thus, based on both inhibition kinetics and docking studies the distance probe **21** is too short to interact with the tryptase tetramer in a bivalent manner. The gain in affinity observed with **22** and **23** in comparison to the binding head together with the results from the docking unambiguously classify them as bivalent binders. The decrease in inhibitory potency of **24**, a distance probe still able to interact with β-tryptase in a bivalent manner (bivalent docking), probably reflects the loss of conformational entropy upon binding that partially consumes the entropy effect. In summary, the data obtained clearly show that the β-tryptase tetramer recognizes and binds dibasic inhibitors of appropriate length.

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16. $K_i$-values for the interaction of the inhibitors with the tryptase tetramer were determined essentially as described previously.[14]
17. Briefly, docking of the ligand is performed as follows: The bivalent inhibitor is cut into three parts, that is headgroup A, headgroup D, and spacer fragment. The headgroups are docked into the S1 pockets of subunit A and D, respectively, using the program FlexX.[18] If the headgroups show a binding mode inside the S1 pockets, all fragments of the inhibitor are reconnected. Tripos force field[19] is used to adjust all bond lengths and bond angles to reasonable values. Subsequently, two additional geometry optimization steps are applied to the resulting raw ligand–enzyme complex using the force field MMFF94[20] as implemented in the software package Sybyl.[21] First, an optimization step is performed with fixed tryptase structure but without any constraints on the ligand. For the final geometry optimization also, parts of the tryptase structure are allowed to be flexible. Ligands that pass all steps of the docking algorithm are considered to be bivalent binding inhibitors.
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