Affinity Chromatography of Tryptases: Design, Synthesis and Characterization of a Novel Matrix-Bound Bivalent Inhibitor

Norbert Schaschke,*[a] Dusica Gabrijelcic-Geiger,[b] Andreas Dominik,[c] and Christian P. Sommerhoff[b]

β-Tryptases are mast cell-derived serine proteases that are enzymatically active in the form of an oligomer consisting of four subunits each with trypsin-like activity. The active-site clefts, which are directed toward the central pore of the tetramer, form spatial arrays of four negatively charged S1 binding pockets. Therefore, dibasic inhibitors of appropriate geometry can bind in a bivalent fashion to neighboring subunits. We have recently identified a potent bivalent inhibitor ($K_i = 18$ nM), based on the bifunctional scaffold cyclo-(-D-Asp-L-Asp-) and the arginine mimetic DL-3-aminomethyl-phenylalanine methyl ester as a ligand for S1 pockets that takes advantage of the this unique tetrameric geometry. To generate an affinity matrix, the bivalent ligand was modified and immobilized on a Sepharose matrix by use of the PEG derivative Jeffamine ED 900 as spacer. This matrix selectively recognizes and binds β-tryptase from crude protein mixtures and thus is useful as a geometry-driven means of isolating and purifying human mast cell tryptases.

Introduction

Affinity chromatography, as it is known today, was introduced in the late 1960s by Cuatrecasas,[1,2] and has evolved into a powerful and efficient technique broadly applied in the life sciences and biotechnology for the isolation and purification of proteins from complex mixtures. Central to this technique is an immobilized ligand that selectively recognizes and binds its target protein. A wide variety of useful ligands has been identified by the use of protein structure- and protein function-based designs, as well as by combinatorial approaches.[3] In the particular case of proteases, reversible inhibitors have been the ligands of choice, and a broad spectrum of inhibitors ranging from proteins to small synthetic molecules has been successfully applied for affinity-based isolation protocols. In the late 1960s Fritz et al. took advantage of a resin-bound version of the protein trypsin-kallikrein inhibitor for the purification of trypsin-like serine proteases.[4,5] Further representative examples of ligands known to be suitable for affinity chromatography protocols include the 27-mer peptide derived from the exon 1B of the endogenous calpain inhibitor calpastatin,[6] the propeptide of cathepsin D,[7] and the peptide boronic acid Ala-Ala-boroVal directed against human neutrophil elastase.[8]

Human tryptases represent a group of serine proteases (clan PA(S), family S1) with trypsin-like activity that are almost exclusively expressed in mast cells.[9–11] Dominant among them, both by the amounts present in mast cells and by activity, is β-tryptase, which occurs in four, highly homologous isoforms (β1a, β1b, β2, and β3). β-Tryptase is stored in catalytically active form within the secretory granules of mast cells and is released into the surrounding tissue upon mast cell activation, for example, by allergens/IgE.[10,11] Because β-tryptase is thought to play a role in the pathogenesis of allergic and inflammatory disorders[12–14] it has become an interesting target for therapeutic intervention, particularly in asthma and allergic rhinitis.[15] The X-ray structure of human β2-tryptase has revealed a unique tetrameric architecture (see Figure 1 A).[16,17] In particular, the active site clefts of the four quasi-identical subunits (A, B, C, and D in Figure 1 A) are directed towards a central pore, representing a spatial array of four negatively charged S1 pockets each terminated by an aspartic acid residue.

Because of its unique tetrameric architecture, β-tryptase recognizes and binds dibasic compounds of appropriate length. A variety of bivalent inhibitors that utilize this particular feature of the protease has been described.[18–23] By using piperazine-2,5-dione-based bifunctional scaffolds in combination with 3-aminomethyl-phenylalanine as ligand for the S1 pockets, we have recently synthesized a series of structurally related dibasic tryptase inhibitors with systematically increasing distances between the terminal amino methyl groups; 3-aminomethyl-phenylalanine was used in its racemic form to present the S1 ligand in different orientations.[24] These dibasic compounds were utilized to probe the distance between the active sites of the A/D or B/C subunit pairs of the β-tryptase tetramer. Among them, compound **1** (see Scheme 1) was identified as a

[a] *Dr. N. Schaschke*
Max-Planck-Institut für Biochemie
Am Klopferspitz 18a, 82152 Martinsried (Germany)
Fax: (+49) 89-8578-2847
E-mail: schaschk@biochem.mpg.de

[b] *Dr. D. Gabrijelcic-Geiger, Prof. Dr. C. P. Sommerhoff*
Abteilung für Klinische Chemie und Klinische Biochemie
Klinikumsstandort Innenstadt, LMU München
Nußbaumstraße 20, 80336 München (Germany)

[c] *Dr. A. Dominik*
Altana Pharma AG
Byk-Gulden-Straße 2, 78467 Konstanz (Germany)

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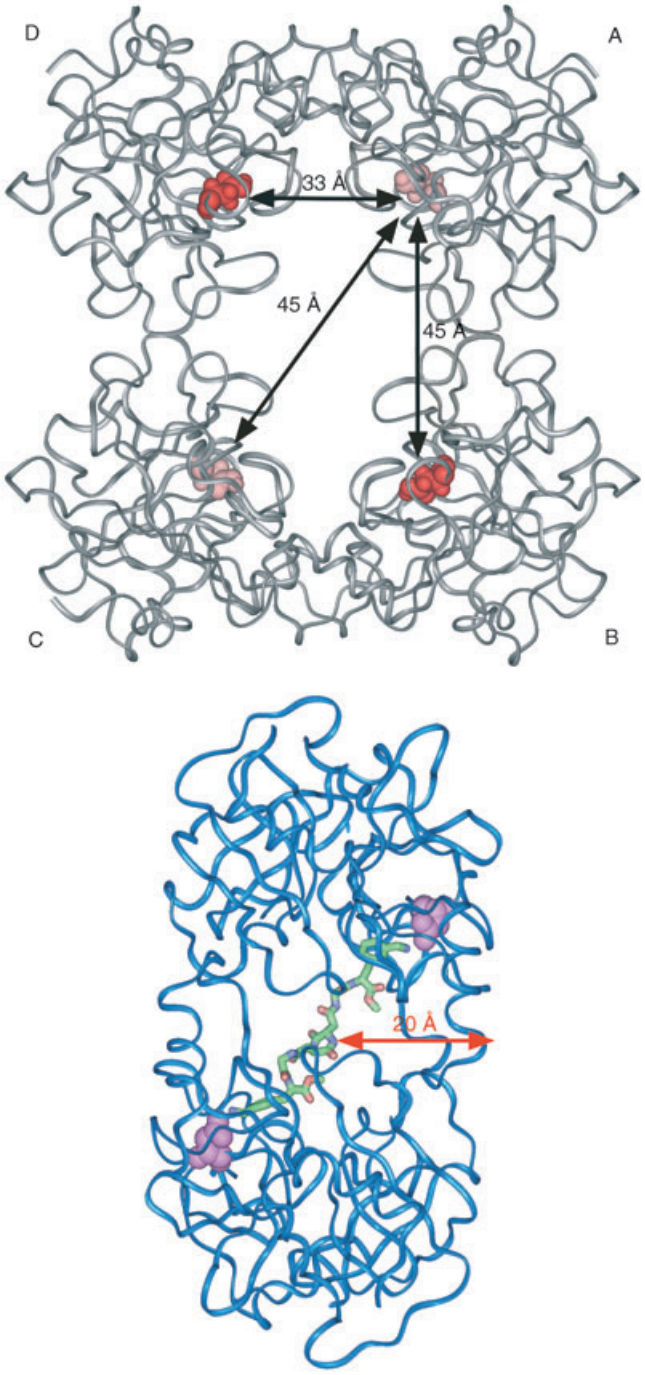
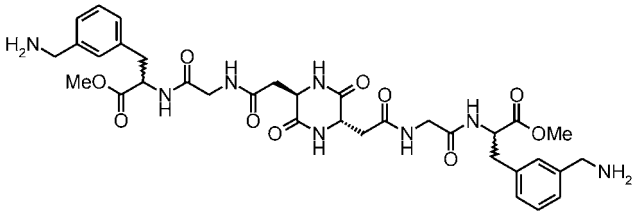


Figure 1. *Design of the affinity ligand. A) X-ray structure of human β-tryptase. The β-tryptase tetramer is shown in gray ribbon representation and Asp189, at the bottom of each S1 pocket, in red CPK representation. The distances between the S1 subsites are indicated. B) Bivalent docking of (S,S)-**1** into the S1 pockets of neighboring subunits of the β-tryptase tetramer. The β-tryptase tetramer (view from the top) is shown in blue ribbon representation, Asp189 of each S1 pocket in magenta CPK representation, and the inhibitor in stick representation (color code: C atoms green, N atoms blue, and O atoms red).*

potent inhibitor ($K_i = 18$ nM). We now present the structure-based design and synthesis of a derivative of inhibitor **1**, its immobilization on a Sepharose matrix, and the use as of this novel affinity matrix for the isolation and purification of human β-tryptases.



Scheme 1. *Structure of the bivalent inhibitor **1**.*

Results and Discussion

Stucture-based design of the affinity ligand

To permit the covalent linkage of the bivalent inhibitor **1** on a Sepharose matrix, several chemical modifications were necessary. In particular, it was essential: i) to increase the chemical stability of **1**, ii) to replace the bifunctional scaffold with a trifunctional one, thus providing an anchoring position for the spacer attachment, and iii) to identify an appropriate spacer.

For the regeneration and cleaning of a Sepharose matrix washing of the material with 0.1 M NaOH is recommended. This procedure, however, is incompatible with the base-labile methyl ester groups present in **1** blocking the terminal carboxylic acid functions. Extensive SAR studies suggest that a free carboxylic acid group at this position is not tolerated in inhibitors of trypsin-like serine proteases. Therefore, the methyl esters were replaced by the corresponding methyl amides.

To identify an anchoring point suitable for the attachment of the spacer, the bivalent binding mode of inhibitor **1** to the β-tryptase tetramer was characterized by use of a novel modeling program for the docking of bivalent ligands. Figure 1B shows that the (*S*,*S*) diastereomer of the inhibitor **1** can adopt a conformation that allows simultaneous interactions of the terminal aminomethyl groups of (*S*,*S*)-**1** with the Asp189 moieties terminating the S1 pockets of the neighboring subunits A and D (or their symmetry equivalents B and C). Docking of the other three diastereomers of **1** gave similar results (not shown). More importantly, these docking studies clearly indicate that the piperazine-2,5-dione scaffold itself is not involved in the binding and thus that one of its amide nitrogens is ideally suited as an anchoring point for the spacer.

In many cases, the nature of the spacer used for the immobilization of a ligand is not crucial for optimal recognition and binding of the target protein. In the particular case of the tryptase tetramer, however, the limited accessibility of the active site clefts made the selection of an appropriate spacer far more difficult, the matrix-bound ligand having to pass through one of the entrances formed by the four subunits to reach the S1 binding pockets located within the water-filled catalytic chamber. From the docking study/crystal structure, a distance of at least approximately 20 Å from the amide nitrogen of the piperazine-2,5-dione scaffold indicated in Figure 1B to the entrance of the pore has to be bridged. Besides an appropriate length, the physicochemical nature of the spacer has to resist hydrophobic collapse under the aqueous conditions required for affinity chromatography. Accordingly, Jeffamine ED 900

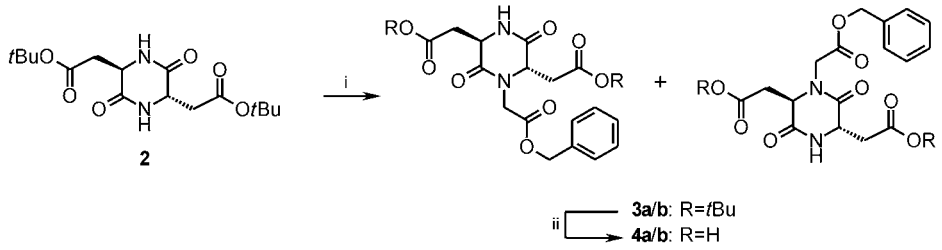
(*O*,*O*′-bis(2-aminopropyl)polyethylene glycol 800; $M_r \sim 900$) was selected as a spacer fulfilling both the length and the solubility requirements.

Synthesis of the affinity ligand

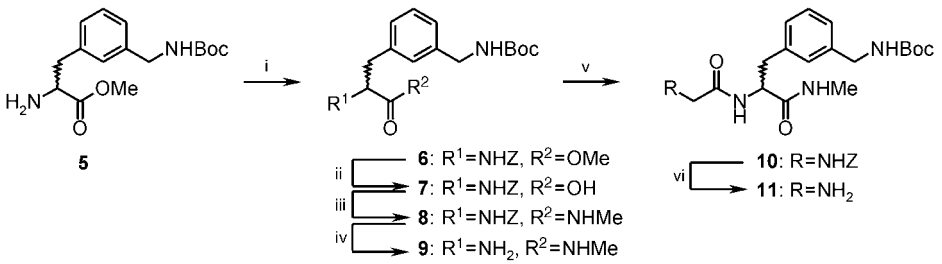
In view of the required modifications described above, the strategy for the synthesis of inhibitor **1**[24] was modified as follows. The trifunctional scaffold **4** was obtained in two steps starting from the piperazine-2,5-dione **2** as outlined in Scheme 2. One of the amide nitrogens was alkylated with benzyl bromoacetate in the presence of silver(I) oxide as base, by a procedure described by Falorni et al. for the bisalkylation of related piperazine-2,5-diones.[25] The *tert*-butyl ester groups were then cleaved by use of TFA/$CHCl_3$ (1:4, *v*/*v*) instead of the commonly applied TFA/H_2O (95:5, *v*/*v*) in order to avoid the hydrolysis of the benzyl ester. The trifunctional scaffold was obtained as a 1:1 mixture of the two possible isomers (**4a**/**4b**), according to the *R*/*S* stereochemistry of **2**.

The synthesis of the modified S1 ligand is summarized in Scheme 3. As starting material, H-DL-Phe(3-BocNH-CH_2)-OMe (DL-3-*tert*-butoxycarbonylaminomethyl-phenylalanine methyl ester; **5**), synthesized as described previously,[24] was selected and converted in six steps by standard procedures into **11** (overall yield 53%). The two binding heads (**11**) were then connected through amide linkages (EDC/HOBt-promoted coupling) with the trifunctional scaffold **4** to give **12** (Scheme 4). After unmasking of the functional groups of **12** in two steps, the terminal aminomethyl functions were Fmoc-protected. This change of protecting groups was required to prepare the ligand for the covalent linkage onto the Sepharose matrix, as the cleavage conditions for the Fmoc group (piperidine/DMF) are compatible with the highly hydrophilic carbohydrate-based matrix in terms of stability and solvent accessibility.

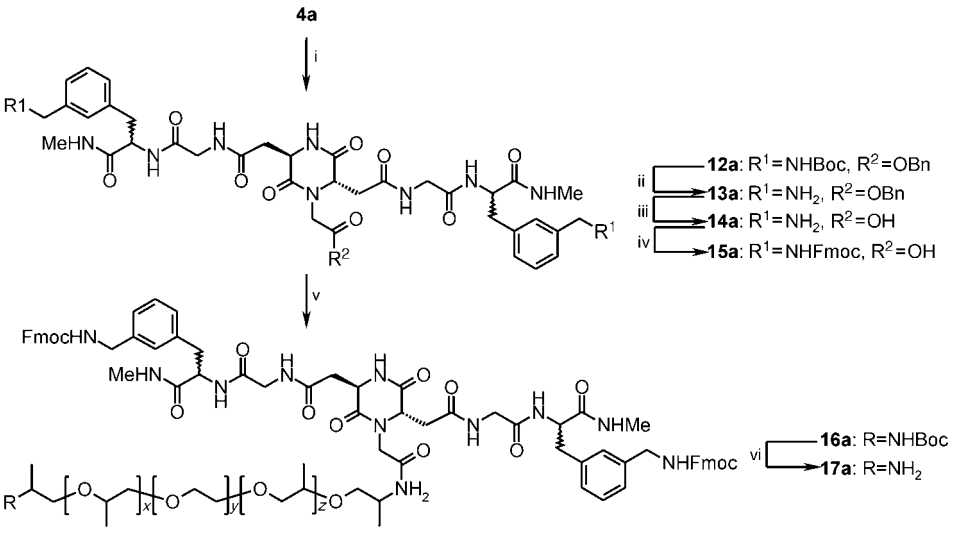
To immobilize the bivalent inhibitor on a NHS-activated Sepharose it was necessary to protect the spacer—Jeffamine ED 900—at one of its amino functions with the acid-labile Boc group. Because of its hydrophilic character, the isolation and purification of mono-Boc-protected Jeffamine ED 900 from the reaction mixture proved to be difficult; preparative RP-HPLC



Scheme 2. *Synthesis of the trifunctional scaffold **4**: i) $BrCH_2CO_2Bn/Ag_2O$, DMF, 50°C (20%), ii) TFA/$CHCl_3$ (1:4, v/v), 0°C→RT (91%).*

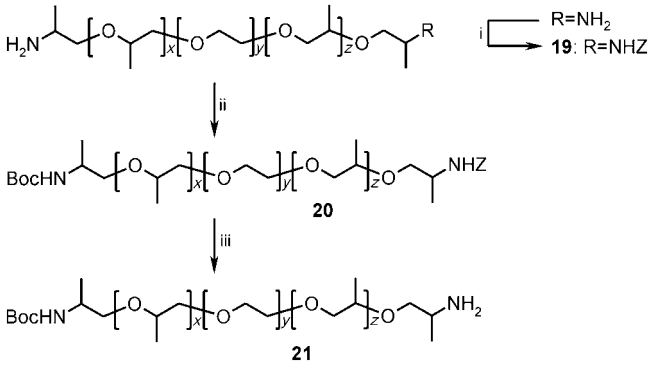


Scheme 3. *Synthesis of the modified S1 ligand **11**: i) Z-OSu/$NaHCO_3$, dioxane/H_2O (1:1, v/v) (90%), ii) 0.1 N NaOH, THF (81%), iii) aq. methylamine/EDC/HOBt, DMF (90%), iv) 10% Pd-C/H_2, MeOH, v) Z-Gly-OH/DIEA/EDC/HOBt, $CHCl_3$ (91% over two steps), vi) 10% Pd-C/H_2, MeOH (90%).*



Scheme 4. *Synthesis of the spacer-functionalized ligand **17** (the reactions were performed with the isomeric mixture **4a/b**; to simplify the scheme, only the isomers derived from **4a** are shown): i) **11**·HCl/DIEA/EDC/HOBt, $CHCl_3$ (74%), ii) 95% aq. TFA, 0°C→RT (88%), iii) 10% Pd-C/H_2, MeOH (82%), iv) Fmoc-OSu/$NaHCO_3$, dioxane/H_2O (2:1) (51%), v) mono-Boc-protected Jeffamine ED 900 (**21**)/EDC/HOBt, $CHCl_3$ (83%), vi) 95% aq. TFA, 0°C→RT (88%).*

was not the method of choice because the usually applied acidic eluent system (MeCN/1% aq. TFA) is only compatible with the Boc group within limits. Therefore, a step-wise liquid–liquid extraction procedure that can also be used at the multi-gram scale was chosen for isolation and purification. The crude monoprotected Jeffamine ED 900 was dissolved in water and neutralized. In a first step, doubly protected Jeffamine ED 900 was removed by washing with AcOEt. In a second step, monoprotected Jeffamine ED 900 was extracted into water-saturated *n*BuOH while unprotected Jeffamine ED 900 remained in the water phase. To increase the solubility of the monoprotected Jeffamine ED 900 in *n*BuOH and thus to facilitate the second extraction step, the mono-Boc-protected Jeffamine ED 900 was synthesized via the mono-Z-protected Jeffamine ED 900 as outlined in Scheme 5.



Scheme 5. *Synthesis of the mono-Boc-protected Jeffamine ED 900 (**21**): i) Z-OSu, dioxane/H_2O (2:1, v/v) (57%), ii) Boc_2O/1 N NaOH, dioxane/H_2O (2:1, v/v), iii) 10% Pd-C/H_2, AcOEt (45% over two steps).*

The mono-Boc-protected Jeffamine ED 900 (**21**) was coupled with **15** to yield **16** by the EDC/HOBt method (Scheme 4). For this step, the use of chloroform stabilized with amylene as solvent proved to be important: when MeOH-stabilized chloroform was used not only the desired Jeffamine ED 900 functionalized ligand but predominately the methyl ester was obtained, due to the slow coupling rate of the amine nucleophile. After cleavage of the Boc group, the Jeffamine ED 900 functionalized ligand **17** was analyzed by LC-MS. Figure 2 shows the $[M+2H]^{2+}$ and $[M+3H]^{3+}$ cluster of species corresponding to Jeffamine ED 900 containing no further ($OCH_2CH(CH_3)$) units ($x + z = 0$). Within the $[M+2H]^{2+}$ cluster, the mass signals could be clearly assigned to ligands with 7–19 (OCH_2CH_2) units ($y = 7$–19). A closer look at the $[M+2H]^{2+}$ cluster reveals further series of

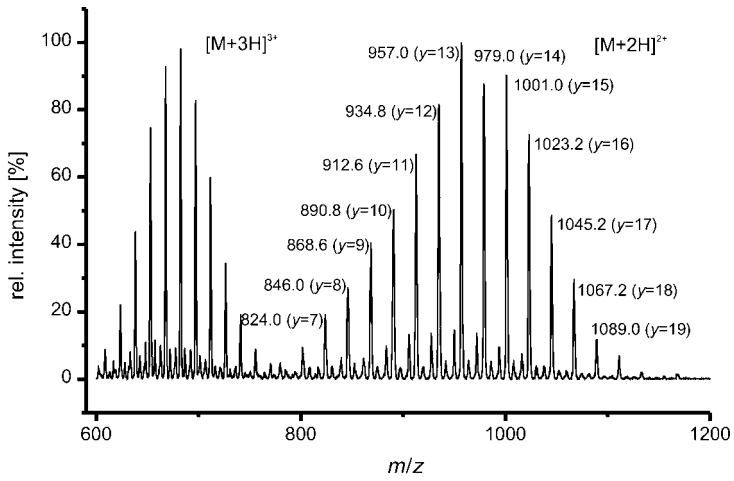


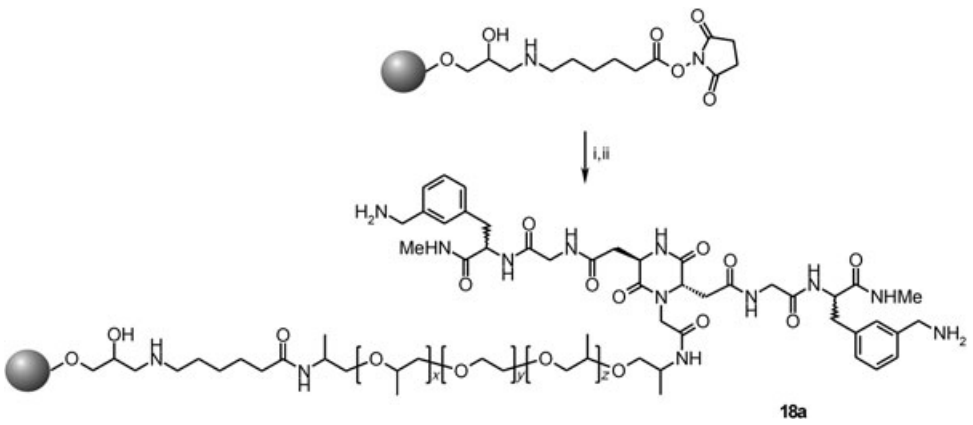
Figure 2. *ESI-MS of **17** ($x + z = 0$).*

$[M+2H]^{2+}$ clusters corresponding to species containing additional ($OCH_2CH(CH_3)$) units ($x + z \geq 1$).

Finally, the NHS-activated Sepharose was loaded with **17** as summarized in Scheme 6. The coupling reaction with this carbohydrate matrix was performed in *i*PrOH/H_2O/0.2 M $NaHCO_3$ (1.5:3.5:20, *v/v/v*) and reached completeness as monitored by the disappearance of **17** in the reaction solution by RP-HPLC. After capping of the remaining *N*-hydroxysuccimimide-activated carboxylic acid functions with ethanolamine, the Fmoc protecting groups were cleaved with piperidine/DMF (5:95, *v/v*) to give the affinity matrix **18**.

Characterization of the affinity matrix

The chemical and/or structural modifications of the bivalent inhibitor **1** required for its immobilization had only a minor effect on the inhibitory potency: both **1** and **13** inhibit human β-tryptase with similar affinities (equilibrium dissociation constants for the complexes $K_i = 18$ nM and 50 nM, respectively). After coupling with the Sepharose matrix, the amount of im-



Scheme 6. *Immobilization of the ligand **17** on the Sepharose matrix (the reactions were performed with the isomeric mixture **17a**/**b**; to simplify the scheme, only the isomers derived form **17a** are shown): i) inhibitor, iPrOH/H_2O/0.2 M $NaHCO_3$ solution (1.5:3.5:20, v/v/v), ii) 0.5 M ethanolamine, iPrOH, iii) piperidine/DMF (5:95, v/v).*

mobilized inhibitor was quantified by quantitative amino acid analysis, advantage being taken of the presence of the amino acids glycine and aspartic acid in the scaffold **4**. Approximately 0.54 μmol ligand were immobilized per mL of swollen Sepharose. This amount is sufficient for efficient affinity purification of tryptase because it allows the binding of $\geq$1.5 mg tryptase per mL of the matrix even if only 10% of the ligand is available for binding. Loading of the matrix with larger amounts was avoided because, while overloading might increase its capacity for purification, it is also likely to hamper the elution of the bound protease and thus to decrease recoveries.

To determine the usefulness of the matrix for affinity purification of β-tryptase its ability to recognize tryptase and to bind it reversibly was investigated. A crude extract from human lung tissue was clarified by hydrophobic interaction chromatography to remove lipids and other hydrophobic components and subsequently applied to a column (8×2 cm) containing the affinity matrix. Analysis of the fractions by SDS-PAGE and Western blot shows that impurities cross the matrix material in the flow-through (Figure 3A, lane 3), whereas tryptase that is detectable in the lung tissue extract by immunoreactivity is completely bound (Figure 3B, lanes 2 and 3). After extensive washing of the column an apparent highly homogenous protein fraction was eluted with a buffer containing 2 M NaCl (lanes 4 and 5 in Figure 3A and B). From measurements of enzymatic activity, >99.5% of the applied tryptase was bound to the column and 66% was recovered by elution with NaCl. SDS-PAGE and Western blot show the characteristic diffuse band of the glycosylated tryptase monomer with a mass of approximately 33 kDa and suggest that the protease is virtually homogeneous.

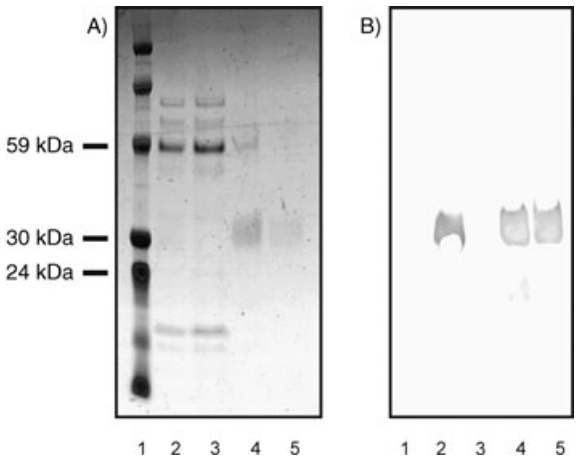


Figure 3. *Analysis of the affinity chromatography of human β-tryptase by: A) SDS-PAGE, and B) Western blot. Lane 1: molecular mass standards, lane 2: extract from human lung tissue after hydrophobic interaction chromatography, lane 3: flow-through of the affinity matrix, lanes 4 and 5: eluted fractions.*

Conclusion

By exploiting the interaction of a dibasic inhibitor with β-tryptase we have developed a novel affinity matrix for this unique tetrameric serine protease. Our approach to immobilizing bivalent ligands with trifunctional scaffolds based on piperazine-2,5-diones in combination with the concept of using linkers of different length to optimize the presentation of the head groups to neighboring subunits[24] can easily be adopted to address other tryptases and even other oligomeric proteases. In particular, the affinity matrix should be rapidly optimizable for animal tryptases, which show marked species differences probably reflecting variations in the architecture of the non-covalently linked active tetramer.[11] Furthermore, this approach may help to identify additional oligomeric human tryptases derived from the $\geq$four genes located within the recently identified serine protease cluster on chromosome 16p13.3.[26–28] Finally, an affinity matrix loaded with an array of bivalent ligands addressing different oligomeric architectures in a parallel fashion provides an geometry-driven combinatorial approach for affinity purification.

Experimental Section

General: All reagents and solvents used in the synthesis were of the highest quality commercially available. NHS-activated Sepharose 4 Fast Flow was purchased from Pharamacia Biotech, and Jeffamine ED 900 from Fluka. TLC was carried out on silica gel 60 plates (Merck AG, Darmstadt), and compounds were visualized with chlorine/*o*-tolidine. Analytical HPLC was carried out with Waters equipment (Eschborn, Germany) on XTerra TM C8, 5 μm (Waters, Eschborn, Germany) with use of a linear gradient of MeCN/2% H_3PO_4 (5:95 to 90:10 in 15 min). ESI-MS spectra were recorded on a PE SICEX API 165 instrument and ^{1}H NMR spectra were recorded on Bruker AMX 400 or AMX 500 spectrometers.

Synthesis of the affinity ligand

***tert*-Butyl ((2*S*,5*R*)-1/4-benzyloxycarbonylmethyl-5-*tert*-butoxycarbonylmethyl-3,6-dioxo-piperazin-2-yl)-acetate (3a/b)**: Benzyl bromoacetate (0.46 mL, 2.92 mmol) in DMF (50 mL) and Ag_2O (0.67 g, 2.92 mmol) were added to a solution of **2** (1.00 g, 2.92 mmol). While the black colored suspension was stirred overnight at 50 °C the color gradually turned gray-green. The AgBr formed was removed by centrifugation, the solvent was evaporated, and a mixture of *tert*-butyl methyl ether/petroleum ether (1:1, *v*/*v*; 50 mL) was added. The precipitate (recovered starting material) was filtered off and washed with *tert*-butyl methyl ether/petroleum ether (1:1, *v*/*v*; 3×50 mL). The mother liquor was evaporated, and the crude product was purified by flash chromatography (100 g silica gel; eluent *tert*-butyl methyl ether/petroleum ether 1:1 *v*/*v*, followed by neat *tert*-butyl methyl ether); yield: 0.28 g (20%, 1:1 mixture of isomers); TLC (*tert*-butyl methyl ether) R_f 0.68; ^{1}H NMR (400 MHz, [D_6]DMSO): δ=1.38 (s, 18H; $OC(CH_3)_3$), 2.57 (dd, J=16.88, 6.23 Hz, 1H; βCH_2 Asp), 2.69 (dd, J=16.88, 4.22 Hz, 1H; βCH_2 Asp), 2.78 (dd, J=17.43, 4.59 Hz, 1H; βCH_2 Asp), 2.87 (dd, J=17.43, 4.77 Hz, 1H; βCH_2 Asp), 4.14 (s, 2H; NCH_2CO), 4.16 (t, J=4.59 Hz, 1H; αCH Asp), 4.42 (t, J=4.95 Hz, 1H; αCH Asp), 5.13 (s, 2H; CH_2–C_6H_5), 7.36 (m, 5H; CH_2–C_6H_5), 8.32 (s, 1H; αNH Asp); HRMS (ESI) calcd for $C_{25}H_{34}N_2O_8 + H^+$ $[M+H]^+$; *m*/*z*: 491.23879, found 491.23781.

((2*S*,5*R*)-1/4-Benzyloxycarbonylmethyl-5-carboxymethyl-3,6-dioxopiperazin-2-yl)acetic acid (4a/b): Compound **3** (0.26 g, 0.46 mmol) was dissolved in $CHCl_3$/TFA (4:1, *v*/*v*; 50 mL). After 24 h, the solution was concentrated in vacuo and the resulting oil was evaporated from toluene (3×). Upon treatment with AcOEt, the

title compound was obtained as colorless crystals; yield: 0.16 g (91%, 1:1 mixture of isomers); TLC ($CHCl_3$/MeOH/AcOH 8:8:1, *v/v/v*) R_f 0.51; ^{1}H NMR (400 MHz, [D_6]DMSO): $\delta = 2.61$ (dd, $J = 17.00$, 5.20 Hz, 1H; βCH_2 Asp), 2.66 (dd, $J = 17.00$, 5.77 Hz, 1H; βCH_2 Asp), 2.81 (dd, $J = 17.50$, 4.54 Hz, 1H; βCH_2 Asp), 2.87 (dd, $J = 17.50$, 4.60 Hz, 1H; βCH_2 Asp), 4.11 (d, $J = 17.30$ Hz, 1H; NCH_2CO), 4.16 (t, $J = 4.60$ Hz, 1H; αCH Asp), 4.18 (d, $J = 17.30$ Hz, 1H; NCH_2CO), 4.45 (t, $J = 5.42$ Hz, 1H; αCH Asp), 5.12 (s, 2H; CH_2–C_6H_5), 7.36 (m, 5H; CH_2–C_6H_5), 8.23 (s, 1H; αNH Asp), 12.39 (br s, 2H; COOH); HRMS (ESI) calcd for $C_{17}H_{18}N_2O_8$ + H^+ $[M+H]^+$; *m/z*: 379.11359; found 379.11370.

***Z*-DL-Phe(3-BocHN-CH_2)-OH (7)**: A solution of $NaHCO_3$ (0.49 g, 5.79 mmol) in water (100 mL) was added to a suspension of **5**·HCl (2.00 g, 5.79 mmol) in dioxane (100 mL), followed by Z-OSu (1.44 g, 5.79 mmol), and the resultant solution was stirred at room temperature. After 3 h, the solvent was evaporated and the residual oil was partitioned between AcOEt (100 mL) and water (100 mL). The organic phase was washed with aq. $KHSO_4$ (5%, 3×50 mL) and brine (1×50 mL) and dried (Na_2SO_4), and the solvent was evaporated to give Z-DL-Phe(3-BocHN-CH_2)-OMe (**6**) as a colorless oil (TLC (AcOEt/petroleum ether, 1:2, *v/v*) R_f 0.50; ESI-MS: *m/z* = 443.2 $[M+H]^+$; calcd for $C_{24}H_{30}N_2O_6$: 442.2). The oil (2.24 g, 5.06 mmol) was dissolved in THF (100 mL), and aq. NaOH (0.1 N, 51 mL) was added to the stirred solution from a dropping funnel. Stirring was continued overnight before evaporation of the solvent. The obtained oil was dissolved in water (100 mL), acidified by addition of $KHSO_4$, and extracted with AcOEt (3×100 mL), and the combined organic phases were washed with brine (1×50 mL) and dried (Na_2SO_4). The product was isolated by precipitation from *tert*-butyl methyl ether/petroleum ether to give a colorless powder; yield: 1.74 g (70% over two steps); TLC (cyclohexane/$CHCl_3$/AcOH 45:45:10, *v/v/v*) R_f 0.33; ^{1}H NMR (500 MHz, [D_6]DMSO): $\delta = 1.39$ (s, 9H; $C(CH_3)_3$), 2.82 (dd, $J = 13.73$, 10.53 Hz, 1H; β_2CH_2), 3.03 (dd, $J = 13.73$, 4.27 Hz, 1H; β_1CH_2), 4.09 (d, $J = 6.10$ Hz, 2H; CH_2NHBoc), 4.15 (m, 1H; αCH), 4.97 (m, 2H; CH_2–C_6H_5), 7.02–7.15, 7.21 (2×m, 4H; C_6H_4), 7.24–7.36 (m, 6H; CH_2NHBoc, CH_2–C_6H_5), 7.61 (d, $J = 7.47$ Hz, 1H; αNH); HRMS (ESI) calcd for $C_{23}H_{28}N_2O_6$ + H^+ $[M+H]^+$; *m/z*: 429.20201; found 429.20112.

Z-DL-Phe(3-BocHN-CH_2)-NHMe (8): Aqueous methylamine (11.85 M, 0.22 mL, 2.63 mmol), HOBt (0.24 g, 1.75 mmol), and EDC (0.50 g, 2.63 mmol) were successively added to an ice-cold (0 °C), stirred solution of **7** (0.75 g, 1.75 mmol) in DMF (30 mL). After 1 h the ice bath was removed, and stirring was continued overnight. The solvent was evaporated, and the residual oil was partitioned between AcOEt (100 mL) and water (100 mL). The organic phase was washed with aq. $KHSO_4$ (5%, 3×50 mL), aq. $NaHCO_3$ (5%, 3×50 mL), and brine (1×50 mL), and dried (Na_2SO_4). The solvent was evaporated to give a colorless powder; yield: 0.70 g (91%); TLC (AcOEt/petroleum ether, 4:1, *v/v*) R_f 0.57; ^{1}H NMR (500 MHz, [D_6]DMSO): $\delta = 1.39$ (s, 9H; $C(CH_3)_3$), 2.58 (d, $J = 4.59$ Hz, 3H; $NHCH_3$) 2.73 (dd, $J = 13.57$, 10.09 Hz, 1H; β_2CH_2), 2.93 (dd, $J = 13.57$, 4.59 Hz, 1H; β_1CH_2), 4.08 (d, $J = 6.06$ Hz, 2H; CH_2NHBoc), 4.15 (m, 1H; αCH), 4.95 (s, 2H; CH_2–C_6H_5), 7.03–7.13, 7.19 (2×m, 4H; C_6H_4), 7.23–7.36 (m, 6H; CH_2NHBoc, CH_2–C_6H_5), 7.45 (d, $J = 8.44$ Hz, 1H; αNH), 7.88 (m, 1H; $NHCH_3$); HRMS (ESI) calcd for $C_{24}H_{31}N_3O_5$ + H^+ $[M+H]^+$; *m/z*: 442.23365; found 442.23297.

Z-Gly-DL-Phe(3-BocHN-CH_2)-NHMe (10): Compound **8** (0.70 g, 1.59 mmol), dissolved in MeOH/H_2O (95:5, *v/v*; 100 mL), was hydrogenated over 10% Pd-C at atmospheric pressure. The reaction was monitored by TLC (*n*BuOH/AcOH/H_2O/AcOEt 3:1:1:5, *v/v/v/v*; R_f 0.28) and was complete within 4 h. The catalyst was removed by filtration, the solution was concentrated in vacuo, and the resultant oil was evaporated from toluene (3×). The obtained H-DL-Phe(3-BocHN-CH_2)-NHMe (**9**) was dissolved in $CHCl_3$ (30 mL), and Z-Gly-OSu (0.63 g, 2.06 mmol) was added. After 2 h, DIEA (0.36 mL, 2.06 mmol) and an additional portion of Z-Gly-OSu (0.24 g, 0.79 mmol) was added, and stirring was continued overnight. The solvent was evaporated, and the residual oil was partitioned between AcOEt (100 mL) and water (100 mL). The organic phase was mixed with 1-(2-aminoethyl)piperazine (0.37 mL, 2.85 mmol), washed with aq. $KHSO_4$ (5%, 3×50 mL), aq. $NaHCO_3$ (5%, 3×50 mL), and brine (1×50 mL), and dried (Na_2SO_4). The solvent was evaporated to give a colorless powder; yield: 0.72 g (91%); TLC ($CHCl_3$/MeOH, 9:1, *v/v*) R_f 0.50; ^{1}H NMR (500 MHz, [D_6]DMSO): $\delta =$ 1.39 (s, 9H; $C(CH_3)_3$), 2.55 (d, $J = 4.22$ Hz, 3H; $NHCH_3$), 2.75 (dd, $J =$ 13.57, 8.80 Hz, 1H; β_2CH_2 Phe(3-H_2NCH_2)), 2.94 (dd, $J = 13.57$, 5.31 Hz, 1H; β_1CH_2 Phe(3-H_2NCH_2)), 3.54 (dd, $J = 16.69$, 5.87 Hz, 1H; αCH_2 Gly), 3.66 (dd, $J = 16.69$, 5.87 Hz, 1H; αCH_2 Gly), 4.09 (d, $J =$ 5.68 Hz, 2H; CH_2NHBoc), 4.40 (m, 1H; αCH Phe(3-H_2NCH_2)), 5.02 (s, 2H; CH_2–C_6H_5), 7.05, 7.18 (2×m, 4H; C_6H_4), 7.24–7.40 (m, 7H; CH_2NHBoc, CH_2–C_6H_5, αNH Gly), 7.85 (m, 1H; $NHCH_3$), 8.05 (d, $J =$ 8.25 Hz, 1H; αNH Phe(3-H_2NCH_2)); HRMS (ESI) calcd for $C_{26}H_{34}N_4O_6$ + H^+ $[M+H]^+$; *m/z*: 499.25511; found 499.25435.

H-Gly-DL-Phe(3-BocHN-CH_2)-NHMe·HCl (11): Compound **10** (0.72 g, 1.44 mmol), dissolved in MeOH (100 mL), was hydrogenated over 10% Pd-C at atmospheric pressure. The reaction was monitored by TLC (*n*BuOH/AcOH/H_2O/AcOEt 3:1:1:5, *v/v/v/v*; R_f 0.28) and was complete within 2 h. The catalyst was removed by filtration, aq. HCl (1 N, 1.4 mL) was added, the solution was concentrated in vacuo, and the resultant oil was evaporated from toluene (3×). The hydrochloride was precipitated from *i*PrOH/*tert*-butyl methyl ether/petroleum ether to give a colorless powder; yield: 0.52 g (90%); TLC (*n*BuOH/AcOH/H_2O/AcOEt 3:1:1:5, *v/v/v/v*) R_f 0.18; ^{1}H NMR (500 MHz, [D_6]DMSO): $\delta = 1.39$ (s, 9H; $C(CH_3)_3$), 2.56 (d, $J = 4.77$ Hz, 3H; $NHCH_3$), 2.75 (dd, $J = 13.75$, 9.17 Hz, 1H; β_2CH_2 Phe(3-H_2NCH_2)), 2.97 (dd, $J = 13.75$, 5.13 Hz, 1H; β_1CH_2 Phe(3-H_2NCH_2)), 3.37 (d, $J = 16.14$ Hz, 1H; αCH_2 Gly), 3.50 (d, $J =$ 16.14 Hz, 1H; αCH_2 Gly), 4.09 (d, $J = 6.14$ Hz, 2H; CH_2NHBoc), 4.45 (m, 1H; αCH Phe(3-H_2NCH_2)), 7.07, 7.21 (2 m, 4H; C_6H_4), 7.32 (t, $J =$ 6.14 Hz, 1H; CH_2NHBoc), 7.36 (br s, 3H; αNH_3 Gly), 8.04 (m, 1H; $NHCH_3$), 8.62 (d, $J = 8.07$ Hz, 1H; αNH Phe(3-H_2NCH_2)); HRMS (ESI) calcd for $C_{18}H_{28}N_4O_4$ + H^+ $[M+H]^+$; *m/z*: 365.21833; found 365.21526.

Compound 12: Compound **4** (0.196 g, 0.518 mmol), HOBt (0.140 g, 1.036 mmol), and EDC (0.238 g, 1.243 mmol) were added successively to an ice-cold (0 °C), stirred solution of **11**·HCl (0.498 g, 1.243 mmol) and DIEA (0.220 mL, 1.243 mmol) in DMF (30 mL). After 1 h, the ice bath was removed and stirring was continued overnight. The solvent was evaporated, and the residual oil was subjected to flash chromatography (100 g silica gel, eluent $CHCl_3$/MeOH 4:1, *v/v*). The homogenous fractions were pooled and evaporated, and the residual oil was precipitated from MeOH/*tert*-butyl methyl ether/petroleum ether to give a colorless powder; yield: 0.410 g (74%); TLC ($CHCl_3$/MeOH 4:1, *v/v*) R_f 0.42; HRMS (ESI) calcd for $C_{53}H_{70}N_{10}O_{14}$ + 2×H^+ $[M+2H]^{2+}$; *m/z*: 536.26092; found 536.26061.

Compound 13: Compound **12** (0.308 g, 0.287 mmol) was dissolved in ice-cold TFA/H_2O (95:5, *v/v*; 20 mL). After 2 h, the acid was removed in vacuo, and the residual oil was evaporated from toluene (3×). The title compound was isolated by precipitation from *i*PrOH/*tert*-butyl methyl ether/petroleum ether to give a colorless powder; yield: 0.276 g (88%); TLC ($CHCl_3$/MeOH/25% aq. NH_3 20:20:9, *v/v/v*) $R_f = 0.80$; HRMS (ESI) calcd for $C_{43}H_{54}N_{10}O_{10}$ + H^+ $[M+H]^+$; *m/z*: 871.40971; found 871.41130.

Compound 14: Compound **13** (0.255 g, 0.233 mmol), dissolved in MeOH (100 mL), was hydrogenated over 10% Pd-C at atmospheric pressure. The reaction was monitored by TLC and was complete within 2 h. The catalyst was removed by filtration, and the solution was evaporated. The resultant oil was precipitated from *i*PrOH/*tert*-butyl methyl ether/petroleum ether to give a colorless powder; yield: 0.192 g (82%); TLC ($CHCl_3$/MeOH/25% aq. NH_3 12:9:4, *v/v/v*) $R_f = 0.5$; HRMS (ESI) calcd for $C_{36}H_{48}N_{10}O_{10} + H^+$ $[M+H]^+$; *m/z*: 781.36276; found 781.36245.

Compound 15: A solution of Fmoc-OSu (66.8 mg, 0.198 mmol) in dioxane (5 mL) was added to a stirred solution of **14** (100.0 mg, 0.099 mmol) and $NaHCO_3$ (24.9 mg, 0.297 mmol) in water (5 mL). After 3 h, the solvent was evaporated, the residual material was dissolved in water (50 mL), acidified ($KHSO_4$), and extracted with AcOEt (3×30 mL) and with $CHCl_3$ (3×30 mL). The organic phases were combined, washed with brine (1×30 mL), dried (Na_2SO_4), and evaporated. The obtained material was transferred into a centrifuge tube and extracted with hot AcOEt (3×) to remove unreacted Fmoc-OSu and to yield the title compound as a colorless powder; yield: 62.0 mg (51%); TLC (*n*BuOH/AcOH/H_2O/AcOEt 3:1:1:5, *v/v/v/v*) $R_f = 0.30$; HRMS (ESI) calcd for $C_{66}H_{68}N_{10}O_{14} + H^+$ $[M+H]^+$; *m/z*: 1225.49892; found 1225.49831.

Compound 17: Compound **21** (65.2 mg, 0.065 mmol) was dissolved in amylene-stabilized $CHCl_3$ (5 mL) and the pH was adjusted to approximately 8 by addition of DIEA (2 drops). Compound **15** (40.0 mg, 0.032 mmol), HOBt (4.3 mg, 0.032 mmol), and EDC (12.5 mg, 0.065 mmol) were added to the stirred solution at 0 °C (ice bath). After 1 h the ice bath was removed and stirring was continued. The reaction was monitored by HPLC and went to completion overnight. The solvent was removed in vacuo, and the resultant oil was partitioned between AcOEt (100 mL) and water (100 mL). The organic phase was washed with aq. $KHSO_4$ (5%, 3×30 mL), aq. $NaHCO_3$ (5%, 3×30 mL), and brine (1×30 mL), and dried (Na_2SO_4). The solvent was evaporated to give a waxy colorless solid (**16**, yield: 57.0 mg (80%); HPLC t_R 10.9 min). The obtained material was dissolved in ice-cold TFA/H_2O (95:5, *v/v*; 20 mL). After 2 h, the acid was removed in vacuo and the residual oil was evaporated from toluene (3×) to give a colorless oil; yield: 45.0 mg (80%); HPLC t_R 8.2 min; ESI-MS: $m/z = 957.0$, 1008.2, 1015.2, 1066.2 $[M+2H]^{2+}$; calcd for $C_{98}H_{134}N_{12}O_{27}$: 1910.9 ($y = 13$, $x + z = 0$), $C_{103}H_{144}N_{12}O_{29}$: 2013.0 ($y = 14$, $x + z = 1$), $C_{104}H_{146}N_{12}O_{29}$: 2027.0 ($y = 13$, $x + z = 2$), $C_{109}H_{156}N_{12}O_{31}$: 2129.0 ($y = 14$, $x + z = 3$) (the most abundant species of each cluster is given)

Mono Z-protection of Jeffamine ED 900 (19): A solution of Z-OSu (1.00 g, 4.01 mmol) in dioxane (0.5 L) was added by dropping funnel over 2.5 h to a vigorously stirred solution of Jeffamine ED 900 (3.60 g, 4.01 mmol) in dioxane/H_2O (1:1, *v/v*; 1 L). The solvent was evaporated, the residual oil was dissolved in water (25 mL), and the solution was acidified (1 N aq. HCl), and extracted with AcOEt (3×25 mL) to remove doubly Z-protected material, followed by water-saturated *n*BuOH (10×25 mL). The combined *n*BuOH phases were evaporated to give the title compound as a colorless oil; yield: 2.44 g (57%); TLC ($CHCl_3$/MeOH/25% aq. NH_3 14:2:0.5, *v/v/v*) R_f 0.30. ESI-MS: $m/z = 971.6$, 985.6, 999.6 $[M+H]^+$; calcd for $C_{46}H_{86}N_2O_{19}$: 970.6 ($y = 16$, $x + z = 0$), $C_{47}H_{88}N_2O_{19}$: 984.6 ($y = 15$, $x + z = 1$), $C_{48}H_{90}N_2O_{19}$: 998.6 ($y = 14$, $x + z = 2$) (the most abundant species of each cluster is given).

Mono Boc-protected Jeffamine ED 900 (21): Aq. NaOH (1 N, 1.4 mL) and Boc_2O (0.36 g, 1.67 mmol) were added to a stirred solution of **19** (1.44 g, 1.35 mmol) in dioxane/H_2O (2:1, *v/v*; 30 mL). After 1 h, additional portions of aq. NaOH (1 N, 1.4 mL) and Boc_2O (0.36 g, 1.67 mmol) were added. After the system had been stirred for 3 h, the solvent was evaporated and the residual oil was partitioned between AcOEt (100 mL) and water (100 mL). The organic phase was washed with aq. $KHSO_4$ (5%, 3×50 mL) and brine (1×50 mL) and dried (Na_2SO_4). The solvent was evaporated to give the doubly protected Jeffamine ED 900 (**20**) as a colorless oil (TLC ($CHCl_3$/MeOH/25% aq. NH_3 14:2:0.5, *v/v/v*) R_f 0.70). The obtained material was dissolved in AcOEt (150 mL) and hydrogenated over 10% Pd-C at atmospheric pressure. The reaction was monitored by TLC and was complete within 2 h. The catalyst was removed by filtration, and the solution was evaporated to give the title compound as a colorless oil; yield: 0.61 g (45%); TLC ($CHCl_3$/MeOH/25% aq. NH_3 14:2:0.5, *v/v/v*; R_f 0.46); ESI-MS: $m/z = 499.6$, 506.6, 513.6 $[M+2H]^{2+}$; calcd for $C_{46}H_{94}N_2O_{20}$: 994.6 ($y = 16$, $x + z = 1$), $C_{47}H_{96}N_2O_{20}$: 1008.6 ($y = 15$, $x + z = 2$), $C_{47}H_{96}N_2O_{21}$: 1024.6 ($y = 18$, $x + z = 0$) (the most abundant species of each cluster is given)

Immobilization of 17 on the Sepharose matrix: NHS-activated Sepharose™ 4 Fast Flow (25 mL) was placed in a chromatography column (volume: 100 mL) fitted with a glass frit and washed with cold aq. HCl (1 mM, 200 mL). Compound **17** (45 mg, 0.020 mmol) was dissolved in *i*PrOH/H_2O (1.5:3.5, *v/v*; 5 mL), aq. $NaHCO_3$ (0.2 M, 20 mL) was added, and this solution was added to the matrix material. The coupling reaction was monitored by following the disappearance of the free ligand by HPLC. After the column had been shaken for 1 h at room temperature, the reaction was complete. The Sepharose was washed with water (300 mL) and subsequently with *i*PrOH (100 mL). To cap the remaining NHS-activated carboxylic acid functions, ethanolamine in *i*PrOH (0.5 M, 25 mL) was added, the column was shaken for 1 h, and the resin was subsequently washed with *i*PrOH (200 mL). The Fmoc protecting groups were cleaved off by treatment of the matrix with piperidine/DMF (5:95, *v/v*; 25 mL) for 20 min (4×). Finally, the Sepharose was washed with *i*PrOH (300 mL) until the eluting *i*PrOH had a pH value of 7. The affinity matrix (**18**) was stored as a suspension in *i*PrOH at 4 °C.

Quantification of the immobilized ligand: The swollen Sepharose (0.50 mL) was washed with *tert*-butyl methyl ether (5 mL) and then with petroleum ether (5 mL) and dried. After addition of aq. HCl (6 N, 1 mL), an aliquot (9.6 mg, ≅0.24 mL swollen Sepharose) was hydrolyzed at 110 °C for 24 h in an evacuated glass flask. The hydrolysate was subjected to quantitative amino acid analysis with a Biotronic amino acid analyzer; loading: 0.54 μmol ligand per mL swollen Sepharose.

Bivalent docking: A stepwise optimization algorithm was used to dock the bivalent inhibitors into a tryptase model comprising subunits A and D of β-tryptase. Atom coordinates and relative orientation of the subunits were taken from the X-ray structure with PDB ID 1AOL,[29] published by Pereira et al.[16] The algorithm is implemented as a SPL script in the Sybyl software package.[30]

As the first step, the sites of breakage are defined for splitting the ligand into three fragments: two head groups and the linker fragment. In a second step, the head groups are docked into the S1 pockets of subunits A and D, respectively, by use of the FlexX docking software.[31]

The previously defined breakage points are now used for automatic successive reassembly of the ligand. Reasonable conformations for the docked bivalent ligand are found by a series of force field calculations, by use of the MMFF94[32] force field as implemented in Sybyl. In a first step of geometry optimization, the ligand fragments are connected by weak constraints between atoms on both sides of the breakage points. This first force field calculation is per-

formed with all atoms of the enzyme and all atoms of the head groups fixed, in order to find possible geometries of the linker fragment suitable to bridge between the two docked head groups. If corresponding atoms at linker and head groups converge to a distance smaller than twice a bond length, the chemical bonds between head groups and linker are added and a second geometry optimization is performed to find reasonable conformations of the reassembled ligand.

For the next optimization step, parts of the head groups are allowed to be flexible and only the hydrogen bond pattern is retained without changes. For the last two steps of force field geometry optimization, more flexibility is introduced. Firstly the entire ligand is allowed to move, and finally the side groups of the enzyme are also allowed to be flexible.

The success of every single step of this algorithm is checked so that ligands can be classified into several classes. Class 0: head groups are not docked successfully into S1 pockets (i.e., inactive head groups). Class B0: head groups are docked, but no geometry allowing reconnection of head groups and linker is found (i.e., linker too short). Class B1: reassembly of the ligand is successful, but one or both head groups slip out of the S1 pocket in the final geometry optimization (i.e., linker slightly too short or disadvantageous geometry). Class B2: successfully bivalent docked ligand.

The quality of a docking result for the resulting structures of class B2 is assessed by calculation of the following parameters: force field energy of the ligand (as difference between the energies of the conformations of a docked and a fully relaxed ligand), force field energy of the enzyme (as difference between the energies of the geometries of an enzyme with bound inhibitor and a fully relaxed enzyme), binding energy (as difference between the energy of an enzyme-fixed complex and the sum of energies of separated molecules), length of the terminal hydrogen bonds to Asp189 in the S1 pocket (too long hydrogen bonds may indicate a high tension of the ligand, bent between the two active sites), and contact surface between ligand and enzyme (missing contacts also indicate a high tension because of an unfavorable linker). Any one of these parameters can reflect problems of a predicted binding mode. Only ligands of class B2 and with all parameters within reasonable ranges are marked as successfully docked bivalent inhibitors.

Inhibition kinetics: The inhibitory activity of the compounds was accessed by determining their effects on the enzymatic activity of human β-tryptase essentially as described previously.[33] Briefly, β-tryptase was incubated either in the absence or in the presence of the inhibitors at RT in assay buffer (50 mM Tris, 150 mM NaCl, 0.1 $\mu g\,mL^{-1}$ heparin, 0.01% Triton X-100, 0.01% sodium azide, pH 7.6). The residual enzymatic activity was quantified after 30 min preincubation by monitoring of the hydrolysis of the substrate tosyl-Gly-Pro-Arg-7-amino-4-methyl-coumarin for 10 min in a Perkin–Elmer HTS 7000 microtiter plate fluorometer. K_i values were calculated by fitting the steady state velocities to the equation for tight-binding inhibitors.[34]

Affinity purification of human β-tryptase: A crude extract from human lung tissue was clarified by hydrophobic interaction chromatography (octyl-Sepharose), diluted in Tris/HCl (50 mM, pH 7.6) containing heparin (500 $\mu g\,mL^{-1}$), and applied onto a column (8 cm × 2 cm) containing the affinity matrix **18**. The column was washed extensively with the loading buffer to elute loosely bound proteins (flow-through). Subsequently, the bound β-tryptase was eluted with NaCl in the same buffer (2 M). Elution was monitored by measuring the activity of tryptase with tosyl-Gly-Pro-Arg-pNA as substrate. Fractions were analyzed by SDS-PAGE with 4–12% NuPAGE Novex Bis-Tris gels (Invitrogen) according to the instructions of the manufacturer. Proteins were detected by Coomassie Blue staining, and kaleidoscope pre-stained standards (BIO-RAD) were used as marker.

Western blot analysis was performed on nitrocellulose membranes with a mouse anti-tryptase monoclonal antibody (MAB 1222, Chemicon Int.) and DAB as substrate (DAB-substrate kit, Vector Laboratories).

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