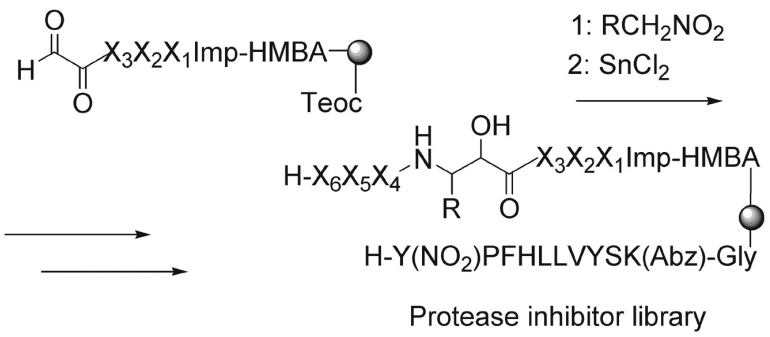
JOURNAL OF combinatorial CHEMISTRY

Article

Solid-Phase Combinatorial Library of Norstatine-Type Isosters by the Nitroaldol Reaction

Marianne Willert, Juan M. Benito, and Morten Meldal

J. Comb. Chem., **2003**, 5 (2), 91-101• DOI: 10.1021/cc0100722 • Publication Date (Web): 14 January 2003



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 1 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

Journal of Combinatorial Chemistry is published by the American Chemical Society.
1155 Sixteenth Street N.W., Washington, DC 20036

Solid-Phase Combinatorial Library of Norstatine-Type Isosters by the Nitroaldol Reaction

Marianne Willert, Juan M. Benito, and Morten Meldal*

Department of Chemistry, Carlsberg Laboratory, Gamle Carlsbergvej 10, DK-2500 Valby, Denmark

Received October 3, 2001

A combinatorial library of norstatine-type peptide isosters as putative inhibitors of aspartic proteases is presented. The library was synthesized using a split-and-mix strategy designed to afford a one-bead-two-compounds library with the isosteric elements positioned centrally in peptide chains. Application of ladder synthesis during library generation enabled structure identification by MALDI-TOF mass spectroscopy. The library was screened against aspartic protease renin, and two types of inhibitors were identified, that is, XXX-ψ{CHRCHOH)-XXX and an aldehyde arising from unreacted starting material. Selected hits were resynthesized and assayed in solution, revealing inhibitors of nanomolar potency.

Introduction

Intra- and extracellular proteolytic enzymes selectively catalyze the hydrolysis of peptide bonds positioned either exo or endo in the peptide chain. Proteases regulate many physiological processes, such as digestion, fertilization, growth, cell signaling/migration, immunological defense, wound healing, and blood pressure.[1,2] During regulation of blood pressure, the renin−angiotensin system plays a crucial role. The aspartic protease renin in the renin−angiotensin system selectively cleaves the protein angiotensiongen to the decapeptide, angiotensin I, which is biologically inactive. Angiotensin I is cleaved by the metallo protease angiotensin converting enzyme (ACE) to yield the biologically active octapeptide, angiotensin II, which constricts blood vessels and stimulates the release of aldosterone.[3,4] Disorders in the renin−angiotensin system may cause hypertension; consequently, inhibitors of renin may act as antihypertensive agents. A large number of renin-inhibitory peptides derived from substrate transition-state analogues, for example, statine and norstatine, have been reported as potent antihypertensive drugs.[5−8]

The conventional methodology for development of protease inhibitors is a rational design based on the knowledge of native substrate and in combination with crystal structures of the active site of the protease. It involves an iterative process in which compounds are synthesized individually and tested for biological activity. Potent inhibitors of proteases have been identified using this methodology. Application of solid-phase combinatorial chemistry offers the advantage of synthesizing many compounds simultaneously. Combinatorial libraries generated by the split-and-mix strategy are of the one-bead-one-compound format in which each bead is viewed as a confined reaction vessel. Previously, it was demonstrated that the hydrophilic resin PEGA is very suitable for on-bead screenings of one-bead-one-compound libraries for the characterization of proteases.[9−12] The one-bead-two-compounds library (Supporting Information Figure 1) is an extension of the one-bead-one-compound format. In this format each bead contains two different compounds, that is, a random library member and an internally quenched substrate which, upon enzymatic incubation, gives rise to competitive inhibition.[13] The enzyme either binds to the substrate with subsequent cleavage of substrate and the bead becomes fluorescent or the enzyme binds to the inhibitor and the bead remains dark.

In the present work, preparation of a combinatorial library with a one-bead-two-compounds format of putative inhibitors containing a centrally positioned norstatine-type fragment and subsequent screening against the aspartic protease renin is described. Ladder synthesis[14] in combination with MALDI-TOF mass spectrometry was applied as structure identification of selected hits. Validation of the library hits was performed by resynthesis and solution-phase analysis of selected compounds.

Results and Discussion

Solid-Phase Synthesis of Norstatine-Type Isosters. Potent inhibitors of renin have been synthesized previously by amino acid substitution in the native substrate with Leu → Phe;[15] with D-amino acids;[16] and by introduction of transition-state analogues, such as statine,[5,6] norstatine,[7,8,17] and hydroxyethylene.[18,19] A vast majority of these inhibitors have the isoster positioned at the C terminal of a peptide derivative. In addition, the importance of both prime and nonprime sites of the substrate for good interaction between renin and substrate has previously been described.[20,21] We therefore decided to synthesize nosrtatine-type isosters that were positioned centrally in a peptide. Initially, model studies (Scheme 1) were performed on amino-functionalized POE-POP$_{1500}$ resin.[22,23] Reaction conditions were established for the generation of norstatine-type isosters using model compounds **1**−**4**, fulfilling the following criteria: (1) the presence of several amide bonds, which enabled UV detection at 215 nm; (2) the absence of side-chain functionalities

* Corresponding author. Fax: +45 3327 4708. E-mail: mpm@crc.dk.

10.1021/cc0100722 CCC: $25.00
Published on Web 01/14/2003

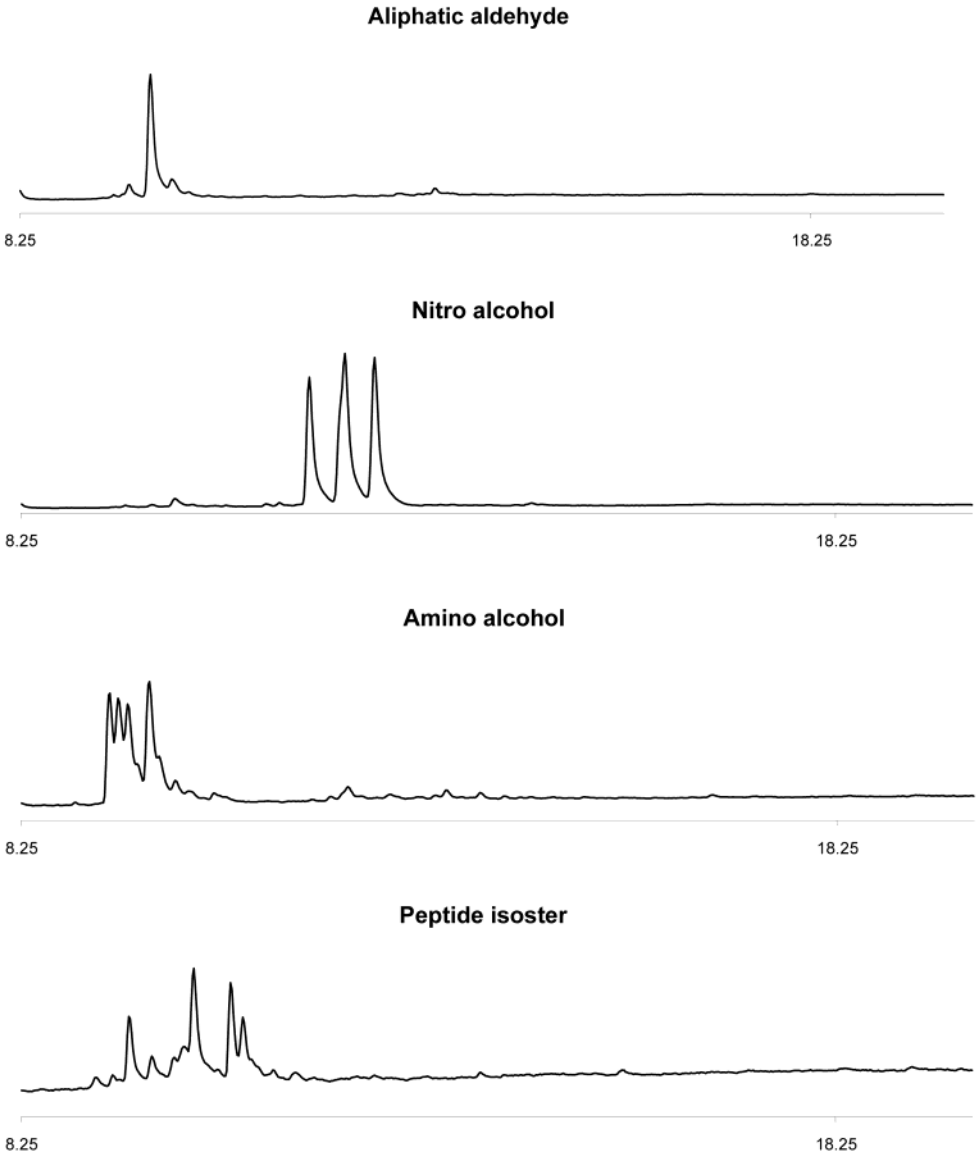


Figure 1. RP-HPLC chromatograms presenting the stepwise solid-phase transformation of aldehydo-peptides into four diastereomeric norstatine isosters: (a) aldehyde, (b) nitro alcohol, (c) amino alcohol, and (d) acylation of amine.

would minimize side reactions; and (3) the easy release of the products from the solid support enabled rapid analysis by ES-MS.

The individual steps in isoster formation was followed by RP-HPLC, as exemplified in Figure 1 for formation of compound **2**, including initial formation of the aldehyde, nitroaldol reaction, reduction, and acylation. The serine residue of the common solid-phase-bound starting material was oxidized using sodium *meta*-periodate, as illustrated in Scheme 1. This resulted in quantitative conversion to the corresponding glyoxaldehyde (Figure 1a). The aldehyde was reacted with four different commercially available nitro compounds in the base-catalyzed nitroaldol reaction, affording four different nitro alcohols (>95%) (Figure 1b). No selectivity in the formation of any of the diastereomers has been observed. Reductions of aromatic nitro groups with $SnCl_2$ in DMF to furnish aniline derivatives in varying yields and purities have been performed.[24−27] However, reduction of the aliphatic nitro alcohols using $SnCl_2$ in DMF did not result in the desired products. Reduction with $SnCl_2 \cdot 2H_2O$ in DMF produced some of the desired amino alcohols, but by raising the amount of water, quantitative conversion of the nitro alcohols was obtained (Figure 1c). Then, the acylation of the amino alcohols with activated Fmoc-Val, which resulted in four diastereomeric norstatine isosters (Figure 1d), demonstrated the compatibility of the Fmoc chemistry with the applied reaction conditions.

Studies have shown that the $PEGA_{1900}$ resin is also suitable for solid-phase synthesis and enzymatic screenings.[9,11−13,28−32] Therefore, oxidation, nitroaldol reaction, and reduction were successfully repeated with the same model system as described above using $PEGA_{1900}$ as the solid support. Compound **1**−**4** were subsequently resynthesized on a larger scale, purified by preparative RP-HPLC, and fully characterized. The stability of different protecting groups toward the employed reaction conditions was investigated and is presented in Table 1. The conditions for reduction with $SnCl_2$ completely removed the Boc group and partially cleaved the *tert*-butyl and Trt groups as a result of the Lewis acid character, while Pmc, Alloc, and Teoc were completely stable

Scheme 1. Model Reaction for the Formation of Four Different Norstatine-Type Isosters on Solid Support

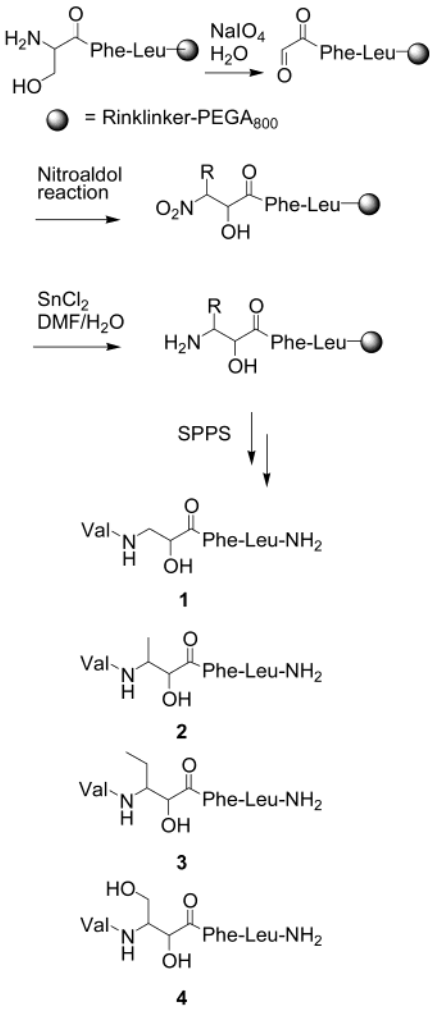


Table 1. Stability Versus Cleavage Conditions for Selection of an Orthogonal Set of Protecting Groups Suitable for the Nitroaldol Reaction and Subsequent Reduction

	Boc	Trt/^{t}Bu	Pmc	Alloc	Teoc
oxidation	+	+	+	+	+
reduction	−	−/+	+	+	+
TBAF, pH 6, 50 °C	+	+	+	+	−

toward the conditions. The results revealed that the generation of norstatine-type isosters could be incorporated in library format; hence, a combinatorial library based on these norstatine-type isosters was prepared and subsequently screened against the aspartic protease renin.

Library Design. A one-bead-two-compounds library format[13] (Supporting Information) was selected with use of the split-and-mix strategy.[33] Generation of the library proceeded through initial derivatization of the individual beads with two orthogonal protecting groups (PG_1 and PG_2). The combinatorial library was generated from the first site (PG_1), and subsequently, an internally quenched substrate was introduced at the second site (PG_2). After deprotection of the first site (PG_1), the ladders of the inhibitor library were synthesized using PG_1 protection in combination with an orthogonal capping agent (PG_3). The inhibitor library was prepared by ladder synthesis[14] to facilitate structure identification by MALDI-TOF MS of the individual library hits. Hence, a three-dimensional protecting strategy compatible with all reaction conditions employed was required in order to generate a library containing two distinct compounds, that is, a putative library inhibitor and the internally quenched substrate.

The studies of the stability of different protecting groups toward peptide synthesis as well as the nitroaldol and reducing conditions (Table 1) concluded that an initial derivatization with Fmoc/Teoc would produce two orthogonally protected sites, since Teoc is stable to common basic reagents such as piperidine.[34] Hence, the Fmoc protection was selected for the library synthesis while the substrate would be introduced at the Teoc protected site. Prior to the introduction of isosters by nitroaldol condensation and reduction, use of Alloc-protected amino acids as the capping reagent in combination with Fmoc-protected amino acids resulted in partial termination in each step for ladder formation, whereas after the isoster generation, Boc- and Fmoc-protected amino acids were conveniently employed. Final removal of the Teoc group allowed the introduction of a preformed internally quenched substrate affording a one-bead-two-compounds library.

To facilitate detachment and analysis of the active compound ladder from the bead, quantitative release and mild conditions were required. At the same time, the selected linker had to be stable to all the applied conditions, that is, library generation and different deprotection conditions. Previous detachments of ladder mixtures have been performed either by introducing a methionine initially in the peptide sequence[14] or by the application of a photolabile linker,[30−32] resulting in either a lactone or an amide, respectively, at the C terminal of the peptides. These strategies were not compatible with the applied reaction conditions because of oxidation of the methionine or reduction of the aromatic nitro group in the photolabile linker, respectively. Consequently, the base labile linker hydroxymethyl benzoic acid (HMBA) was selected, and it was demonstrated that the linker was stable toward all applied reaction conditions, that is, oxidation, nitroaldol, reduction, and fluoride ion treatment (pH 6). Treatment of individual beads with a 10% hydrazine solution (dioxane/MeOH) for 1 h at room temperature cleanly produced the ladder of products as hydrazides. The product mixture was extracted from the bead with 70% MeCN (aq), CHC matrix was added, and MALDI-TOF spectra were acquired. The hydrazides showed good ionization in the spectrometer.

Prior to completion of the one-bead-two-compounds library preparation, a suitable internally quenched substrate for the aspartic protease renin had to be developed. According to studies of renin substrate specificity presented by Skeggs et al.,[35,36] eight amino acids were crucial for good interaction between renin and its substrate. This has led to the development of different fluorogenic substrates that are commercially available. On the basis of these, the internally quenched substrate Boc-Y(NO_2)ProPheHis(Trt)LeuLeuVal-Tyr(^{t}Bu)Ser(^{t}Bu)Lys(Abz)Gly-OH **5** was synthesized on $PEGA_{800}$ using solid-phase peptide synthesis and the HMBA linker. The influence of the FRET pair on substrate hydrolysis was estimated in a solution-phase assay. A small portion

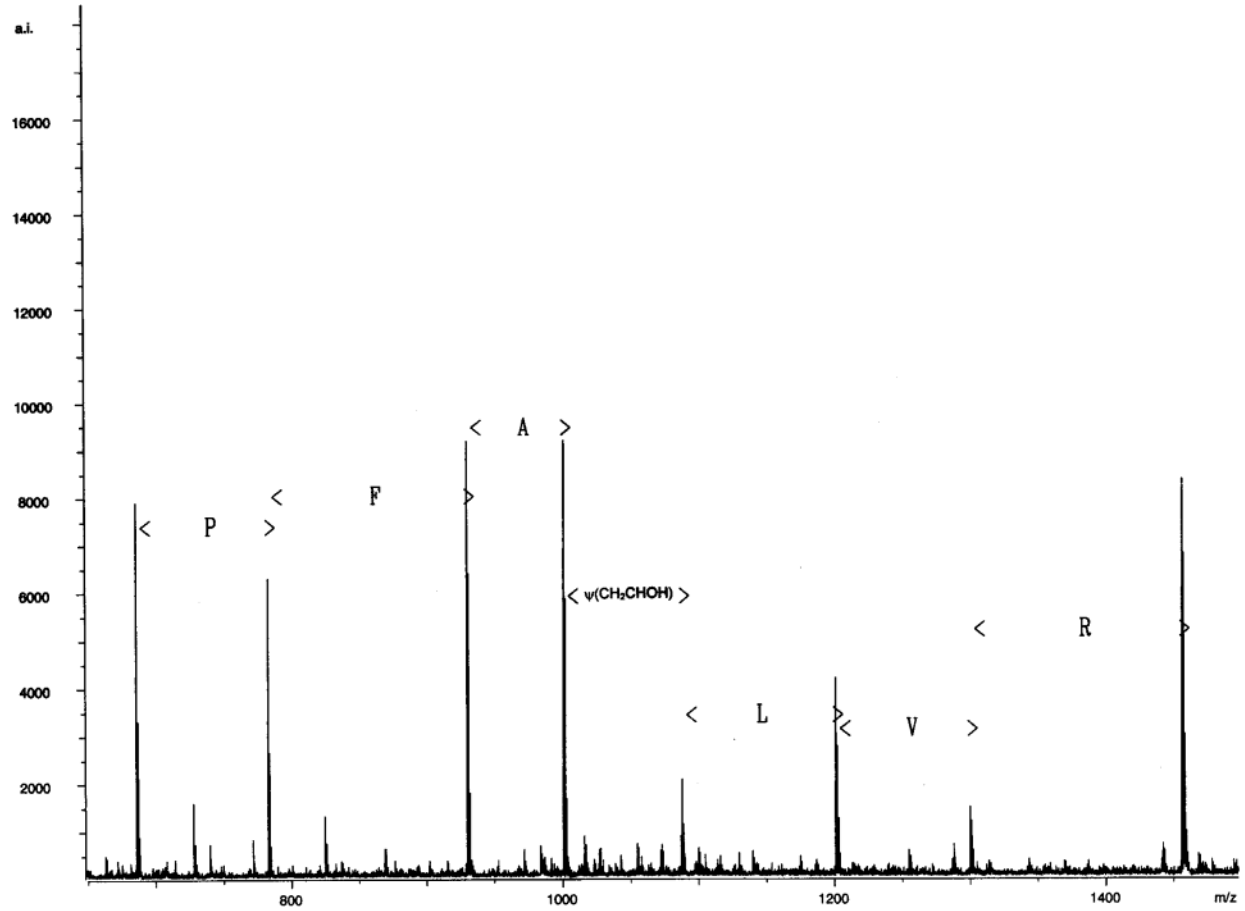
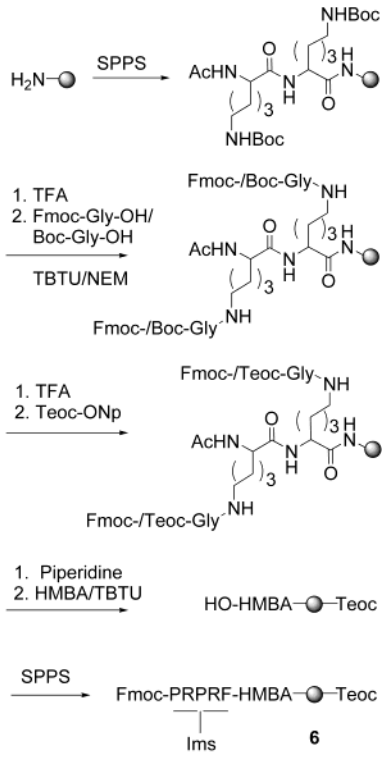


Figure 2. Formation of inhibitors by ladder synthesis using a fotolabile linker to facilitate structure determination. The MALDI-TOF spectrum presents the ladder obtained from a single bead isolated during the library screening upon UV-irradiation.

of the resulting peptide was completely deprotected, detached from the resin with 0.1 N NaOH, neutralized, and purified on preparative RP-HPLC. The internally quenched substrate was subsequently assayed with porcine kidney renin in pyrophosphate buffer (pH 6.5), determining the binding constant $K_m = 2\ \mu M$, while verification of the actual cleavage site (Leu-Leu) was confirmed by MALDI-TOF mass spectrometry (Supporting Information Figure 2). The fully protected peptide was purified using VLC, and a small portion of $PEGA_{1900}$ resin was acylated with the substrate and subsequently deprotected. Porcine kidney renin (0.1 units) dissolved in pyrophosphate buffer was added, and the resin containing the internally quenched substrate was incubated at 37 °C. The increase in fluorescence was periodically monitored, and after 3 days fluorescent beads with complete substrate hydrolysis were obtained. Renin was able to penetrate and retain activity in the resin, and the system was therefore suitable for on-bead inhibitor screening.

Library Synthesis. The synthesis of the library was performed in a library generator and is illustrated in Schemes 2 and 3. $PEGA_{1900}$ resin (0.8 g, 320 000 beads, loading 0.2 mmol/g) was employed as a solid support. The number of beads did not cover the total number of combinations ($5^3 \cdot 4 \cdot 17^3 = 2\ 456\ 500$). All library members had a C-terminal ionization mass spacer (IMS) in common.[30] This fragment served the purpose of a proteolytically stable spacer and a desorption promoter. It had been demonstrated elsewhere that the ratio between Fmoc and the capping Alloc or Boc protected amino acids should be ~9:1.[30] Two ajacent Fmoc-Lys(Boc) residues were introduced, the Fmoc group was removed, and the N-terminal amine was acetylated. The ϵ-amino group of the lysines were Boc-deprotected with aqueous TFA and acylated with mixture of Fmoc-Gly-OH/Boc-Gly-OH (Fmoc loading 0.28 mmol/g). The Boc groups were removed with aqueous TFA, and subsequently, the free amines were reacted with Teoc-ONp. Fmoc groups were removed, and the free amines were acylated with hydroxymethylbenzoic acid. A Phe residue was attached to the hydroxy group through a MSNT coupling and elongated to form the common IMS (Pro-Arg-Pro-Arg-Phe) using Fmoc-amino acids-OPfp esters. The resin was transferred to a multiple column synthesizer block[9] suitable for generation of libraries using the split-and-mix strategy. The library was elongated by three randomized residues (X_1', X_2', X_3') using 5 different Fmoc-Aa-OH/Alloc-Aa-OH (9:1) mixtures in 4 columns each and TBTU activation (Table 2). Then, Fmoc-Ser-OH was introduced, Fmoc group was removed, and serine was oxidized using aqueous sodium *meta*-periodate. The resin containing aldehydes was transferred to four syringes, and the nitroaldol reaction was performed with four different nitro compounds (Table 2). The resulting nitro alcohols were reduced using a molar solution of $SnCl_2 \cdot H_2O$ in H_2O/DMF (9:1), and transferred back to the synthesizer block. The amino alcohols were acylated with another three amino acids (X_1, X_2, X_3) using a mixture of Fmoc-Aa-OH/Boc-Aa-OH (9:1) (Table 2). Seventeen different amino acids

Scheme 2. Preparation of the Orthogonally Protected Central Core of the One-Bead-Two-Compound Library



were employed, while Cys, Met, Ile, and Gln were omitted because of the sensitivity of Cys and Met and the isobaric mass of Ile and Gln with Leu and Lys, respectively. Instead, to bias the library toward the lipophilic character of the native substrate, cyclohexylalanine (Cha), Phe, and Leu were introduced in duplicates. The ultimate acylation (X_3) was performed with Boc-Aa-OH.

Evaluation of the synthesized library was performed by complete deprotection of a small portion of beads using first $Pd(PPh_3)_4$/AcOH/NEM to remove Alloc followed by TFA/H_2O/triisopropylsilane to remove acid-labile protecting groups. The beads were placed on MALDI-TOF targets and individually treated with a hydrazine solution. The resulting hydrazides were extracted from the beads, and MALDI-TOF spectra were acquired. All major peaks were assigned a mass and mass differences were assigned with a software program using an edited list of mass differences. The spectra obtained were of a good quality (Figure 2), and sequencing was fast. Sixty beads were analyzed, and the results are presented in Supporting Information Figure 3. From ~10% of the analyzed beads, no structural information could be obtained, but 14% of the beads gave a partial sequence lacking one or two residues, probably as a result of side reactions of functional amino acid side chains. For the remaining 76% of the beads, the complete structure of the compounds could be established. Some of the 24% contained unreacted aldehyde or nitro compound. The presence of aldehyde was surprising, since preliminary investigations had revealed that the nitroaldol reaction was quantitative, whereas traces of unreduced nitro alcohols had already been detected.

Screening of the Library. Resin **7** (100 mg, 40 000 beads) was treated for 4 h with TBAF in hot THF/AcOH at pH 6. The completely protected, internally quenched substrate **5** was coupled to the free amine using TBTU as coupling reagent to yield resin **8**. The library was deprotected as described above with $Pd(PPh_3)_4$ followed by aqueous TFA treatment. The resin was washed with water, with $NaHCO_3$ solution, and three times with pyrophosphate buffer (pH 6.5). The library was incubated with renin (0.125 units, 1 unit of enzyme liberate 1 μmol/min of angiotensin I from angiotensinogen at pH 6.0 and 37 °C) in 275 μL of pyrophosphate buffer. Competitive inhibition could be observed as dark beads on the background of fluorescent inactive beads. Prolongation of the incubation time resulted in fewer hits and improved inhibitor selectivity. Library beads, which had not been incubated, were used as a reference for selection.

Scheme 3. Synthesis of a Combinatorial Norstatine-Type Inhibitor Library Containing a Fluorescence-Quenched Enzyme Substrate for Enzyme Inhibitor Screening

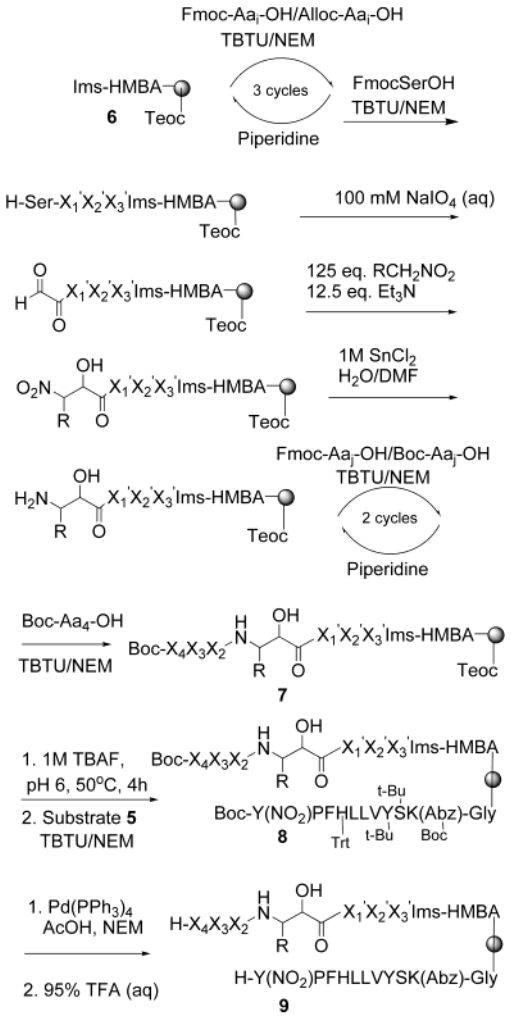


Table 2. Amino Acids and Nitro Compounds Employed for the Generation of the Inhibitor Library[a]

Aa_i[b]	Ala (4), Leu (4), Phe (4), Pro (4), Val (4)
R−CH_2−NO_2	H (5), Me (5), Et (5), EtOTHP (5)
Aa_j[b]	Ala, Arg, Asn, Asp, Cha (2), Glu, Gly, His, Leu (2), Lys, Phe (2), Pro, Ser, Thr, Trp, Tyr, Val

[a] Numbers in brackets indicate when an amino acid was used in more than one column of the library generator (multiplicity). [b] Aa_i indicates randomized positions prior to the isoster formation, and Aa_j are randomized positions after the isoster formation.

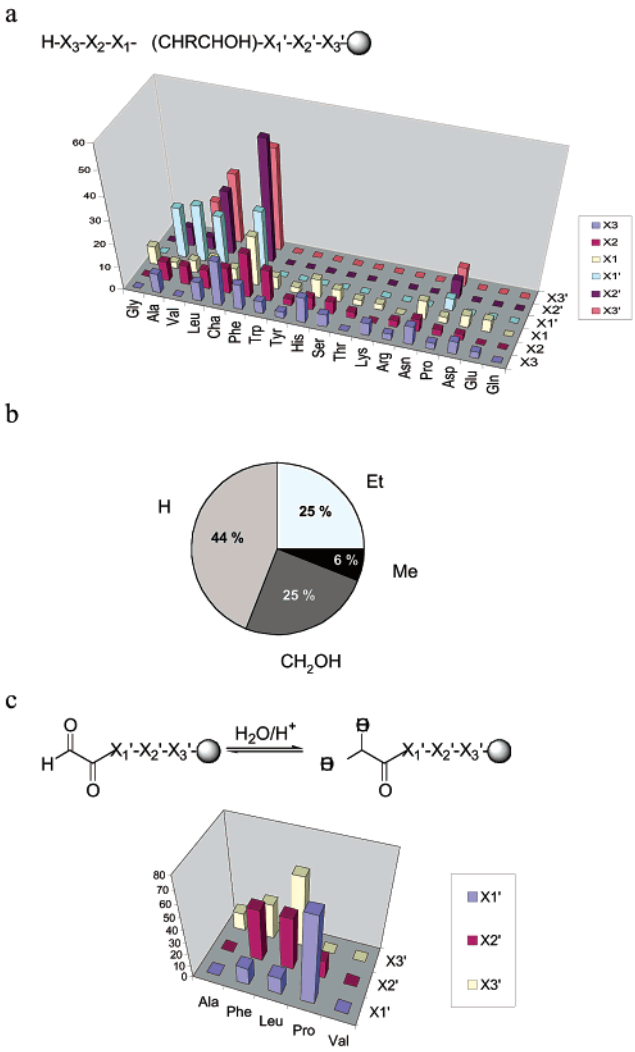


Figure 3. Results of the library screening with aspartic protease, renin: (a) distribution of individual amino acids in inhibitor subsites for active inhibitors with the complete sequence, (b) distribution of the individual norstatine isosters in these active inhibitors, (c) distribution of individual amino acids in subsites for unreacted aldehydes isolated as active inhibitors. Proline predominates in X1′, indicating steric problems with this residue in the nitroaldol condensation.

The development of fluorescence was monitored by periodic inspection of small samples of resin under a fluorescence microscope. After 2 days of incubation, renin was quenched by addition of aqueous TFA. The resin was washed with $NaHCO_3$ solution and several times with water before inspection of the library under a fluorescence microscope. The fluorescence intensity of the beads varied from extremely bright to relatively dark. Collection of dark beads was performed manually under the microscope. The beads were placed on a MALDI-TOF target and treated with hydrazine. Forty-three dark beads were selected from the screened library, corresponding to 0.1%.

Library Hits. Of the analyzed beads, 86% contained the complete sequence, whereas surprisingly, 14% of the selected beads contained mainly the unreacted aldehyde in combination with Pro in X_1'. In aqueous media, the aldehyde exists in equilibrium with the hydrated form and might probably mimic the transition state of the hydrolysis. The frequencies of the individual amino acids at the different positions are presented in Figure 3a, and the occurrence of isoster substitution in Figure 3b. Full structures of active hits are presented in Supporting Information, Table 1. The binding mode and orientation of the identified inhibitors has not been determined; however, it was assumed that the inhibitors bind in analogy with the substrate. As a consequence, the nomenclature of the individual position for the inhibitors (X_3, X_2, X_1, X_1', X_2', X_3') were assigned in analogy to that of the substrate (P_3-P_3').[37] A high preference was found for cyclohexylalanine in X_3, some preference for Phe in X_2, and a significant preference for Phe in X_1. No preference was observed for X_1', but some preference for Phe and Leu was observed for X_2' and X_3'. Previously, norstatine inhibitors of renin have had bulky groups (cyclohexyl, *iso*-butyl) at this position. Surprisingly, a high preference was observed for the unsubstituted isoster. The selected aldehyde inhibitors had Pro in X_1' (Figure 3c); however, the presence of aldehyde in the library was mainly due to the adjacent proline's reducing the reactivity of the aldehyde in the nitroaldol reaction. The same preference for Phe and Leu in X_2' and X_3' was observed for these aldehydes as for the complete sequences.

Thus, a high preference for lipophilic groups was observed for all subsites of these new renin inhibitors. These results are in good agreement with the lipophilic nature of the native substrate.

Evaluation of Hits. The screening results were validated by assaying selected hits, controls, and consensus sequences in solution (Table 3). Four sequences were selected directly from the library hits (**11**−**14**). Compound **11** represented the aldehyde, and compounds **12** and **13** represented general sequences close to consensus with R = Et. Compound **14** was selected to investigate the effect of acidic residues which could be in conflict with the overall negative charge of renin at pH 6.5 (pI 5.2).[4] Compound **15** was selected as a library hit different from **12** and **13** with R = H. Compound **10** was derived from the native substrate by insertion of the most occurring isoster, R = H, thus disregarding any frame shift in the binding inferred by the insertion. All sequences were synthesized as the free acids using SPPS with subsequent preparative purification on RP-HPLC, and the compounds were identified by high-resolution mass spectrometry and amino acid analysis. The compounds were assayed in solution as described by Nicklin et al.[38] using the internally quenched substrate **5**, $K_m = 2\ \mu M$. The results are presented in Table 3. The selected inhibitors were all in nanomolar range. The inhibitors **12** and **13** derived from the consensus sequences were the most potent. Inhibitors **14** and **15** were the second most potent, whereas the aldehyde **11** and the one derived from the native substrate **10** were the less potent, all of them in the nanomolar range.

The results indicated that the limit of discrimination between inhibitor potencies in the solid-phase assay was in the nanomolar range, that is, it was not possible to visually distinguish fluorescence in beads containing inhibitors in this range of activity. However, the discrimination limit of the assay depends on the K_m of the substrate, substrate/inhibitor ratio on the individual bead, and the enzyme concentration. Furthermore, the assay can be performed by repeated,

Table 3. Presentation of Structure, Analytical HR-MS Data, and IC_{50} Values for Compounds 10−15 Prepared By Parallel Synthesis for Kinetic Inhibitor Studies in Solution

	X_3	X_2	X_1	R[a]	X_3'	X_2'	X_3'	mass calc[b]	mass found[b]	yield (%)[c]	IC_{50} (nM)
10	F	H	L	H	L	V	Y	900.4590	900.4610	7	250
11					P	F	L	454.1949	454.1966	55	210
12	F	F	F	Et	F	F	V	990.4736	990.4711	4	50
13	Cha	F	F	Et	V	F	F	996.5205	996.5226	5	53
14	D	L	D	Et	L	L	F	872.4376	872.4391	8	68
15	F	W	F	H	V	L	L	933.4805	933.4789	6	68

[a] −NH−CH**R**−CHOH−CO−. [b] $[M + Na]^+$ by HR-MS. [c] After RP-HPLC purification.

sequential screenings with intermediate isolation of the active beads and, therefore, with increased enzyme concentrations. Evaluation of selected hits indicated that an appropriate assay had been selected as a result of the detection of inhibitors with IC_{50}'s in the nanomolar range.

Conclusion

The preparation of a combinatorial library of norstatine isosters is presented. This type of isoster has been previously synthesized as building blocks with subsequent incorporation in peptides. Hence, solid-phase generation of norstatine isosters offered the possibility for greater diversity. Structure elucidation of the library was performed by application of ladder synthesis during library generation in combination with acquirement of MALDI-TOF spectra.

A one-bead-two-compounds library format was used in which an internally quenched substrate was incorporated in the resin after the library synthesis. Thus, each bead contained a random library member and the substrate. The library was screened against aspartic protease renin. Two distinct types of inhibitors were identified, the expected isosteric sequence for which the consensus matched well with the substrate preference of renin and the truncated aldehyde due to incomplete nitroaldol reactions when the penultimate amino acid was proline.

Validation of the screening assay was performed with the selected hits. Evaluation of the hits was performed in solution revealing inhibitors in the nanomolar range.

Experimental Section

$PEGA_{800}$ and $PEGA_{1900}$ resin were purchased from Polymer Laboratories, England. Solid-phase peptide chemistry and solid-phase organic chemistry were performed in plastic syringes equipped with sintered Teflon filters (50-μm pores). Fmoc amino acids, their pentafluorophenyl (Pfp) esters, MSNT, MeIm, $NaIO_4$, $SnCl_2$, $SnCl_2 \cdot 2H_2O$, TBAF, TFA, triisopropylsilane, Piperidine, TBTU, and porcine kidney renin (lot no. 99H7415) were purchased from Sigma and used without further purification.

All solvents were HPLC grade and were used as received without further purification. Solution-phase NMR spectra (^1H NMR, COSY, HSQC, HMBC) were acquired on either a Bruker 250 MHz Avance DRX 250 spectrometer or a Varian Unity Innova 500 MHz spectrometer at 25 °C (reference $CHCl_3$ $\delta = 7.24$, ^1H, HDO $\delta = 4.75$, ^1H).

Analytical RP-HPLC was performed on a Waters system (490E detector, two 510 pumps with gradient controller and Ø 8 mm RCM C_{18} column), and preparative RP-HPLC purification of norstatine-type peptides was carried out on a Water system (991 photodiode array detector and 600 E system controller) connected to a Waters Ø 25 mm RCM C18 column. All RP-HPLC procedures were carried out with a linear gradient. Buffers were A, 0.1% TFA in H_2O, and B, 0.1% TFA in MeCN:H_2O 9:1. Vacuum liquid chromatography was performed using a tightly packed column of Merck silica gel 60 H. ES-MS results were obtained on a Fisons VG Quattro 5098 mass spectrometer (mobile phase, 50% aq MeCN, 8 μL/min; sample, 10 μL, ∼20 pmol/L). MALDI-TOF mass spectra using α-cyano-4-hydroxycinnamic acid were acquired on a Bruker Reflex III high-resolution MALDI-TOF mass spectrometer, and sequencing was facilitated using the Aura macro LabelDelta. Library beads were examined using an Optical Star fluorescence microscope with a 320-nm band (20 nm)-pass filter for excitation and a 410-nm low-pass filter for detection. The loading of the resins was determined by spectrophotometric analysis at 290 nm of the dibenzofulvene−piperidine adduct formed upon deprotection of the amino terminal using a Perkin-Elmer Lambda 7 UV/vis spectrometer. Enzyme kinetics was performed using a temperature-controlled Perkin-Elmer luminescence spectrometer (LS 50B).

Standard Fmoc deprotection was performed with 20% piperidine in DMF for 4 + 16 min. Preactivation of amino acids was performed for 5 min. The resin was washed with 1−2 times the volume necessary to swell the resin, and washings were generally repeated six times unless otherwise specified. The Kaiser test was performed according to literature.[39]

General Procedure for Synthesis of Norstatine Isosters 1−4. A 1.69-g portion of $PEGA_{800}$ resin (loading 0.35 mmol/g) was swelled in DMF. FmocGlyOH (3 equiv) was dissolved in DMF, and NEM (4.5 equiv) and TBTU (2.8 equiv). The mixture was preactivated for 5 min before addition to the resin. Complete acylation of free amines was verified by Kaiser test. Fmoc was removed as described above, and the resin was washed with DMF (×6). A preactivated mixture of Rink amide (3 equiv), NEM (4.5 equiv), and TBTU (2.8 equiv) in DMF was added. A dipeptide containing Leu and Phe was synthesized from the linker using mixtures of either Fmoc-Leu-OPfp (3 equiv) and Dbht-OH (1.1 equiv) or Fmoc-Phe-OPfp (3 equiv). Fmoc was removed, and a preactivated mixture of Fmoc-Ser-OH (3 equiv), NEM (4.5 equiv), and TBTU (2.8 equiv) was added. Fmoc group was removed, and the resin was washed twice with H_2O. A 100-mM solution of aqueous sodium *meta*-periodate was added to the resin and left overnight at

room temperature. The resin was washed with with H_2O (×2), MeCN (×2), THF (×2), and CH_2Cl_2 (×2). The resin was divided equally among four syringes and reacted with a mixture of nitroalkane (125 equiv) and Et_3N (12.5 equiv) dissolved in MeCN/EtOH (2:1), resulting in a total volume of 1500 μL/syringe. The nitroaldol reactions were left for 4 h. The resin was drained and washed with H_2O (×2), MeCN (×2), and CH_2Cl_2 (×2). A 1 M solution of $SnCl_2 \cdot H_2O$ (4.5 g, 11 mmol) in 10 mL H_2O/DMF (9:1) was added to each syringe and left overnight. The resin was washed with DMF (×10), 5% DIPEA in DMF (×2), DMF (×5), and CH_2Cl_2 (×4). The four amino alcohols were subsequently acylated with a mixture of Fmoc-Val-OPfp (3 equiv) and Dbht-OH (1.1 equiv). The resins were washed with DMF, the Fmoc group was removed, and the resin was washed with DMF (×6) and CH_2Cl_2 (×6). The resins were dried under reduced pressure. The resins were swelled in 95% aqueous TFA and left for 2 h, during which time additional TFA was added to avoid realkylation. The solvent was concentrated. The detached peptides were purified by preparative RP-HPLC.

***N*-L-Valyl-*N*-(3-amino-2-hydroxypropionyl)-L-phenylalanyl-L-leucylamide (1).** Yield: 13.5 mg (44%). ES-MS: *m*/*z* calc., 463.3; found, 464.3 ($M + H^+$). ^{1}H NMR (250 MHz, D_2O/CD_3CN, 25 °C) δ = 7.07 (2H, m, H-ϵ(Phe)), 7.04 (1H, m, H-ζ(Phe)), 6.97 (2H, m, H-δ(Phe)), 4.37 (1H, m, H-α(Phe)), 4.01 (1H, m, H-α(Leu)), 3.95 (1H, t, J = 7.9, *H*2), 3.44 (1H, dd, J = 6.0, J = 7.9, H-α(Val)), 3.26 (1H, d, J = 6.0, *H*3), 3.12 (1H, d, J = 7.9, *H*2), 2.83 (2H, d, J = 7.2, H-β(Phe)), 1.86 (1H, m, J = 6.9, H-β(Val), 1.30 (3H, m, H-β(Leu), H-γ(Leu)), 0.69 (6H, d, J = 6.9, H-γ-(Val)), 0.62 (3H, m, H-δ(Leu)), 0.57 (3H, m, H-δ(Leu)). ^{13}C NMR (250 MHz, D_2O/CD_3CN, 25 °C) δ = 177.0 (*C*O (Leu)), 173.7 (*C*1), 172.7 (*C*O (Phe)), 169.7 (*C*O (Val)), 136.4 (C-γ(Phe)), 129.8 (C-δ(Phe)), 129.3 (C-ϵ(Phe)), 127.8 (C-ζ(Phe)), 70.4 (*C*2), 59.3 (C-α(Val)), 54.8 (C-α(Phe)), 52.5 (C-α(Leu)), 43.1 (*C*3), 40.6 (C-β(Leu)), 37.8 (C-β(Phe)), 30.3 (C-β(Val)), 24.6 (C-γ(Leu)), 22.7 (C-δ (Leu)), 21.2 (C-δ (Leu)), 17.5 (C-γ(Val)).

***N*-L-Valyl-*N*-(3-amino-2-hydroxybutanoyl)-L-phenylalanyl-L-leucylamide (2).** Yield: 2.67 mg (16%). ES-MS: *m*/*z* calc., 477.3; found, 478.6 ($M + H^+$). ^{1}H NMR (500 MHz, D_2O/CD_3CN, 25 °C) δ = 7.38 (2H, m, H-ϵ(Phe)), 7.32 (1H, m, H-ζ(Phe)), 7.29 (2H, m, H-δ(Phe)), 4.68 (1H, m, H-α(Phe)), 4.29 (1H, m, H-α(Leu)), 4.22 (1H, m, *H*2), 3.73 (1H, d, J = 6.3, H-α(Val)), 3.16 (1H, m, H-β(Phe)), 3.06 (1H, m, H-β(Phe)), 2.17 (1H, m, H-β(Val), 1.61 (1H, m, H-β(Leu)), 1.59 (1H, m, H-β(Leu)), 1.55 (1H, m, H-γ-(Leu)), 1.01 (6H, d, J = 7.2, H-γ(Val)), 0.94 (1H, m, *H*3), 0.90 (3H, d, J = 5.0, H-δ(Leu)), 0.85 (3H, d, J = 6.0, H-δ-(Leu)), 0.82 (3H, d, J = 7.0, *H*4). ^{13}C NMR (250 MHz, D_2O/CD_3CN, 25 °C) δ = 177.2 (*C*O (Leu)), 174.1 (*C*1), 173.2 (*C*O (Phe)), 168.7 (*C*O (Val)), 136.5 (C-γ(Phe)), 129.8 (C-δ(Phe)), 129.5 (C-ϵ(Phe)), 127.9 (C-ζ(Phe)), 70.2 (*C*2), 59.4 (C-α(Val)), 55.2 (C-α(Phe)), 52.8 (C-α(Leu)), 48.6 (*C*3), 40.4 (C-β(Leu)), 37.5 (C-β(Phe)), 30.3 (C-β(Val)), 25.0 (C-γ(Leu)), 22.9 (C-δ(Leu)), 21.3 (C-δ(Leu)), 18.3 (C-γ-(Val)), 17.6 (C-γ(Val)), 12.9 (*C*4).

***N*-L-valyl-*N*-(3-amino-2-hydroxypentanoyl)-L-phenylalanyl-L-leucylamide (3).** Yield: 5.36 mg (10%). ES-MS *m*/*z* calc., 491.3; found, 491.3 ($M + H^+$). ^{1}H NMR (500 MHz, D_2O/CD_3CN, 25 °C) δ = 7.33 (2H, m, H-ϵ(Phe)), 7.28 (1H, m, H-ζ(Phe)), 7.24 (2H, m, H-δ(Phe)), 4.64 (1H, m, H-α(Phe)), 4.59 (1H, m, *H*2), 4.25 (1H, m, H-α(Leu)), 3.92 (1H, m, *H*3), 3.70 (1H, d, J = 5.8, H-α(Val)), 3.12 (1H, m, H-β(Phe)), 3.02 (1H, m, H-β(Phe)), 2.18 (1H, m, H-β(Val), 1.57 (2H, m, H-β(Leu)), 1.52 (1H, m, H-γ(Leu)), 1.37 (1H, m, *H*4), 1.19 (1H, m, *H*4), 0.99 (3H, d, J = 6.9, H-γ(Val)), 0.97 (3H, d, J = 6.9, H-γ(Val)), 0.87 (3H, d, J = 5.6, H-δ(Leu)), 0.81 (3H, J = 5.8, H-δ(Leu)), 0.77 (3H, t, J = 7.5, *H*5). ^{13}C NMR (250 MHz, D_2O/CD_3CN, 25 °C) δ = 177.3 (*C*O (Leu)), 174.0 (*C*1), 173.3 (*C*O (Phe)), 169.9 (*C*O (Val)), 137.0 (C-γ(Phe)), 129.9 (C-δ(Phe)), 129.6 (C-ϵ(Phe)), 128.0 (C-ζ(Phe)), 70.2 (*C*2), 59.6 (C-α(Val)), 55.3 (*C*3), 55.1 (C-α(Phe)), 52.7 (C-α(Leu)), 40.6 (C-β-(Leu)), 37.7 (C-β(Phe)), 30.6 (C-β(Val)), 25.0 (C-γ(Leu)), 23.1 (C-δ(Leu)), 21.3 (*C*4), 21.2 (*C*4), 18.6 (C-γ(Val)), 17.4 (C-γ(Val)), 11.1 (*C*5).

***N*-L-Valyl-*N*-(3-amino-2,4-dihydroxybutanoyl)-L-phenylalanyl-L-leucylamide (4).** Yield: 3.34 mg (16%). ES-MS: *m*/*z* calc., 494.3; found, 494.6 ($M + H^+$). ^{1}H NMR (500 MHz, D_2O/CD_3CN, 25 °C) δ = 7.43 (2H, m, H-ϵ(Phe)), 7.37 (1H, m, H-ζ(Phe)), 7.34 (2H, m, H-δ(Phe)), 4.65 (1H, m, H-α(Phe)), 4.29 (1H, m, *H*2), 4.34 (1H, m, H-α(Leu)), 4.31 (1H, m, *H*3), 3.80 (1H, d, J = 5.8, H-α(Val)), 3.61 (2H, m, *H*4), 3.21 (1H, m, H-β(Phe)), 3.15 (1H, m, H-β-(Phe)), 2.27 (1H, m, H-β(Val), 1.62 (2H, m, H-β(Leu)), 1.61 (1H, m, H-γ(Leu)), 1.08 (3H, d, J = 6.8, H-γ(Val)), 1.07 (3H, d, J = 6.8, H-γ(Val)), 0.96 (3H, d, J = 6.3, H-δ(Leu)), 0.91 (3H, J = 6.3, H-δ(Leu)). ^{13}C NMR (250 MHz, D_2O/CD_3CN, 25 °C) δ = 177.2 (*C*O (Leu)), 174.3 (*C*O (Phe)), 173.4 (*C*1), 170.9 (*C*O (Val)), 137.8 (C-γ(Phe)), 131.0 (C-δ(Phe)), 130.7 (C-ϵ(Phe)), 129.1 (C-ζ(Phe)), 71.1 (*C*2), 58.8 (C-α(Val)), 59.1 (*C*4), 54.0 (*C*3), 55.4 (C-α(Phe)), 52.2 (C-α(Leu)), 40.0 (C-β(Leu)), 37.0 (C-β(Phe)), 30.7 (C-β-(Val)), 24.4 (C-γ(Leu)), 22.5 (C-δ(Leu)), 20.8 (C-δ(Leu)), 17.9 (C-γ(Val)), 16.9 (C-γ(Val)).

Substrate for Aspartic Protease Renin Boc-Tyr(NO_2)-Pro-Phe-His(Trt)-Leu-Leu-Val-Tyr(^{t}Bu)-Ser(^{t}Bu)-Lys-(Boc-Abz)-Gly-OH (5). A 1.82-g portion of $PEGA_{800}$ (loading 0.4 mmol/g) was acylated with a preactivated mixture of Fmoc-Gly-OH (3 equiv), NEM (4.5 equiv), and TBTU (2.8 equiv) dissolved in DMF. The completeness of the reaction was identified by the Kaiser test (loading 0.38 mmol/g). The Fmoc group was removed by standard deprotection. The base-labile linker HMBA (3 equiv) was dissolved in DMF and NEM (4.5 equiv, TBTU (2.8 equiv) was added, and the mixture was preactivated before addition to the resin. Fmoc-Gly-OH (2.5 equiv) was dissolved in DMF and MeIm (2.5 equiv), MSNT (2 equiv) was added, and the reaction was left for 45 min (loading 0.35 mmol/g). The Fmoc group was removed, and the free amine was reacted with a preactivated mixture of Fmoc-Lys(Boc-Abz)-OH (3 equiv), NEM (4.5 equiv), and TBTU (2.8 equiv). The subsequent eight amino acids were introduced using a preactivated solution of Fmoc-Aa-OPfp (3 equiv) and DhbtOH (1.1 equiv). The Fmoc was removed, and the free amine was reacted with a preactivated mixture of Fmoc-Tyr(NO_2)-OH (3 equiv), NEM (4.5 equiv), and TBTU (2.8 equiv). The

Fmoc was removed, and the free amine was Boc-protected using a mixture of Boc_2O (20 equiv) and NEM (20 equiv) in DMF. The resin was washed DMF (×6) and CH_2Cl_2 (×6) and dried under reduced pressure overnight.

A 50-mg portion of the resin was completely deprotected using 95% TFA (aq) for 2.5 h. The resin was subsequently washed with aqueous 95% AcOH (×2), DMF (×2), 5% DIPEA in DMF (×2), DMF (×4), and CH_2Cl_2 (×6). The resin was dried under reduced pressure overnight. The resin was swelled in 0.1 N NaOH for 2 h, and the resulting solvent was neutralized with 0.2 N HCl. The solvent was reduced, and the product was purified using a semipreparative RP-HPLC. Purity: >95%. ES-MS: *m*/*z* calc., 1486.72; found, 1488.1 ($M + H^+$), 744.6 ($M + 2H^+$), 497.1 ($M + 3H^+$).

The remaining resin containing completely protected substrate was swelled in 0.1 N NaOH for 2 h. The resin was washed with H_2O (×4) and MeCN (×4). The mixture was cooled to 0 °C, neutralized with 0.2 N HCl, and frozen using a CO_2/acetone bath. The solvent was removed under reduced pressure. The resulting yellow powder was purified by vacuum liquid chromatography on silica using MeCN/EtOAc/H_2O as eluent with a gradient from (9:9:1) to (9:9:2). TLC (MeCN/EtOAc/H_2O, 9:9:2) R_f 0.4. Yield: 0.503 g (34%).

Ac-Lys(Fmoc-[IMS]-HMBA-Gly)-Lys(Teoc-Gly)-PEGA (6). A 0.8-g portion of $PEGA_{1900}$ (loading 0.2 mmol/g) was acylated with a preactivated mixture of Fmoc-Gly-OH (3 equiv), NEM (4.5 equiv), and TBTU (2.8 equiv) dissolved in DMF. The completeness of the reaction was identified by the Kaiser test (loading 0.18 mmol/g). A solution of Fmoc-Lys(Boc)-OPfp (3 equiv) and Dhbt-OH (1.1 equiv) in DMF was added to the resin. The resin was washed with DMF, the Fmoc was removed, and the resin was washed with DMF. A solution of Fmoc-Lys(Boc)-OPfp (3 equiv) and Dhbt-OH (1.1 equiv) was added to the resin. The resin was washed with DMF, the Fmoc was removed, and the resin was washed with DMF. The N-terminal amino group was acetylated with in situ-prepared Ac_2O/pyridine (1:1) for 30 min. The resin was washed with DMF and CH_2Cl_2 and treated with 95% aqueous TFA for 30 min. The resin was subsequently washed with CH_2Cl_2, 5% DIPEA in DMF, and DMF. Fmoc-Gly-OH (1.5 equiv) and Boc-Gly-OH (1.5 equiv) were dissolved in DMF, NEM (4.5 equiv) and TBTU (2.8 equiv) were added, and the mixture was left for 5 min before addition to the resin. The resin was washed with DMF and CH_2Cl_2 and treated with 95% TFA (aq) for 30 min. The resin was subsequently washed with CH_2Cl_2, 5% DIPEA in DMF, and DMF. Teoc-ONp (4.5 equiv) was dissolved in DMF, and Et_3N (4.5 equiv) was added. The mixture was added to the resin, and the reaction was left overnight. Fmoc was removed, and HMBA was introduced using HMBA (3 equiv), NEM (4.5 equiv), and TBTU (2.8 equiv). The resin was subsequently washed with DMF (×6), CH_2Cl_2 (×6) and dried under reduced pressure overnight. A mixture of Fmoc-Phe-OH (2 equiv), MeIm (2.5 equiv), and MSNT (2.5 equiv) was preactivated and added to the resin. The reaction was left for 45 min (loading 0.28 mmol/g). The sequence Fmoc-Pro-Arg(Pmc)-Pro-Arg(Pmc) was subsequently introduced using Fmoc-amino acid-OPfp esters. After completion, the resin was washed with DMF (×6) and CH_2Cl_2 (×6) and dried under reduced pressure overnight.

Library Synthesis 7. Ac-Lys(Fmoc-[IMS]-HMBA-Gly)-Lys(Teoc-Gly)-PEGA (**6**) was distributed equally in each well of a 20-well synthesizer block,[9] Fmoc was removed, and the resin was washed with DMF (×6). The split-and-mix steps were performed by adding a preactivated mixture of Fmoc and Alloc-protected amino acids (2.7 equiv:0.3 equiv), NEM (4.5 equiv), and TBTU (2.8 equiv) dissolved in DMF (450 μL). All couplings were stirred overnight at room temperature. After completion of a coupling, the resin was washed with DMF (×6). The synthesizer block was positioned upside-down and shaken for 30 min. The Fmoc was removed, and the next amino acids were introduced. After the third position, a preactivated mixture of Fmoc-Ser-OH (3 equiv), NEM (4.5 equiv), and TBTU (2.8 equiv) was added to the resin. The Fmoc group was removed, and the resin was washed twice with H_2O. A 100 mM solution of aqueous sodium *meta*-periodate was added to the resin and left overnight at room temperature. The resin was washed with H_2O (×2), MeCN (×2), THF (×2), and CH_2Cl_2 (×2). The resin was transferred to four syringes and reacted with a mixture of nitroalkane (125 equiv) and Et_3N (12.5 equiv) dissolved in MeCN/EtOH (2:1), resulting in a total volume of 2500 μL/syringe. The nitroaldol reactions were left for 4 h. The resin was drained and washed with H_2O (×2), MeCN (×2), and CH_2Cl_2 (×2). An 1 M solution of $SnCl_2 \cdot 2H_2O$ (4.5 g, 11 mmol) in 10 mL of H_2O/DMF (9:1) was added to each syringe and left overnight. The reduction was performed twice. After the second reduction, the resin was washed with DMF (×10), 5% DIPEA in DMF (×2), DMF (×5), and CH_2Cl_2 (×4). Kaiser tests were deep blue for all four amino alcohols. The resin was transferred back to the synthesizer block. The fourth and fifth amino acids were introduced using a preactivated mixture of Fmoc- and Boc-protected amino acids (2.7 equiv:0.3 equiv), NEM (4.5 equiv), and TBTU (2.8 equiv) dissolved in DMF (450 μL). The sixth amino acid was introduced using Boc-Aa-OH (3 equiv), preactivated with NEM (4.5 equiv), and TBTU (2.8 equiv).

One-Bead-Two-Compounds Library, 9. TBAF (2 mL, 1 M in THF) was adjusted with glacial acetic acid to pH 6. The solution was added to 100 mg of library **7** placed in a round-bottomed bottle. The reaction was left at 50 °C for 4 h. The resin slurry was transferred to a syringe and drained. The resin was subsequently washed with THF (×2), H_2O (×2), MeCN (×2), DMF (×2), 5% DIPEA in DMF (×2), and DMF (×2). A preactivated mixture of fully protected substrate **5** (3 equiv), NEM (4.5 equiv), and TBTU (2.8 equiv) was added to the resin and left overnight. The Kaiser test was negative. The resin was drained, washed with DMF (×6) and CH_2Cl_2 (×6), and dried under reduced pressure for 4 h. The resin was subsequently flushed with argon (×3). A solution of $CHCl_3$/AcOH/NEM (37:2:1) was prepared prior to the start. The solution was deoxygenated for 15 min by a steady stream of argon. $Pd(PPh_3)_4$ (3 equiv) was dissolved in the solvent mixture, resulting in a final concentration of 5 mM. The Pd solution was added under argon to the resin, and the reaction was left at room temperature for 2h. The resin was drained and washed. A solution of TFA/H_2O/

triisopropylsilane (94:5:1) was added to the resin and left for 2.5 h. The resin was washed with 95% aqueous AcOH (×2), 5% DIPEA in DMF (×2), DMF (×2), and CH_2Cl_2 (×6), then dried under reduced pressure.

Detachment Procedure for HMBA Linker. A bead was placed on a MALDI-TOF target and treated with 1 μL of 10% hydrazine solution (dioxane/MeOH 9:1) for 1 h at room temperature. The solvents were subsequently removed by placing the target on a heating block at 50 °C for 10 min. The products were extracted twice with 70% aqueous MeCN where the solvents were removed between by placing the target on a heating block. A 1-μL portion of CHC matrix (10 mg/1 mL) was added, and the spectrum was acquired.

Incubation of Library 9 with Renin, Selection of Beads and Sequencing of Active Inhibitors. Prior to incubation, library **9** was washed twice with 5% $NaHCO_3$ (aq), H_2O (×5), and twice with pyrophosphate buffer (pH 6.5). Incubation of library **9** was performed with 0.125 units of renin in 250 μL of pyrophosphate buffer (pH 6.5). The fluorescence was monitored under a fluorescence microscope, and after 48 h, the incubation was quenched using 5% TFA (aq). The resin was washed with H_2O (×2) and 5% aqueous $NaHCO_3$. The beads were placed under a fluorescence microscope, and dark beads were collected manually. Forty-three dark beads were selected and placed on MALDI-TOF targets. The individual beads were treated with a 10% hydrazine solution (dixoane/MeOH 9:1) and left at room temperature for 1 h. Extraction of the product mixture with 70% MeCN (aq), addition of CHC matrix, and final acquirement of a MALDI-TOF spectrum produced the ladder fragments from which the complete sequence could be identified.

Synthesis of Inhibitor Sequences 10−15. The peptide isosters were synthesized using 150 mg of $PEGA_{800}$ resin (0.3 mmol/g), Fmoc-protected amino Pfp esters, free acids, and HMBA linker as previously described. The purification was performed by preparative RP-HPLC, and the products were characterized by high-resolution mass spectrometry and amino acid analysis.

Kinetic Evaluation of Substrate 5. Substrate **5** was dissolved in DMF to a final stock concentration of 5 mM. Determination of K_m was carried out in a 100-μL flow cell with 0.01 unit of renin. Under pseudo-first-order conditions, the rate of initial hydrolysis was measured continuously for 5 min for seven concentrations ranging from 0.25 to 35 μM. Hydrolysis of the substrate was carried out with 10 mM pyrophosphate buffer (pH 6.5). Hydrolysis was followed by measurement of the increase in intensity of the Abz fluorescence (λ_{EX} = 320 nm; λ_{EM} = 420 nm; slit width, 10 nm). Total hydrolysis was effected by addition of 10 μL of subtilisin, and final fluorescence was measured after 24 h. The slit width was reduced to 2.5 nm to compensate for the higher fluorescence intensities of more concentrated solutions. Substrate concentrations were corrected using a standard curve. The standard curve was obtained from measurement of the fluorescence intensity of AbzGAGAAF-OH derived from the total hydrolysis of AbzGAGAAFFAY-(NO_2)D-OH by subtilisin at different concentrations (20−180 μM). The K_m was determined from a Hanes plot ([*S*]/v vs [*S*]) of the measured values.

Kinetic Evaluation of Inhibitor Sequences 10−15. Inhibitors **10−15** were dissolved in DMF to a final stock concentration of 5 mM. Determinations of IC_{50} were carried out using a 1000-μL cuvette using 0.01 unit of renin and 1 μM substrate **5**. The IC_{50} values were determined from ($v_0/v_i - 1$) vs [*I*].

Abbreviations

Abz: 2-aminobenzoyl; Alloc: allyloxycarbonyl; Boc: *tert*-butoxycarbonyl; ^{t}Bu: *tert*-butyl; CHC: α-cyano-cinnamic acid; Dhbt-OH: 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine; DIPEA: diisopropylethylamine; DMF: dimethylformamide; ES-MS: electrospray mass spectroscopy; Fmoc: 9-fluorenylmethoxycarbonyl; FRET: fluorescence resonance electron transfer; HMBA: hydroxymethylbenzoic acid; IMS: ionization mass spacer; MALDI-TOF MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MeIm: *N*-methyl imidazole; MSNT: 1-(mesitylene-2-sulfonyl)-3-nitro-1H-1,2,4-triazole; NEM: 4-ethyl morpholine; norstatine: 3-alkyl-3-amino-2-hydroxypropionic acid; PEGA: poly(ethylene glycol)-polydimethyl acrylamide resin; Pfp: pentafluorophenyl; Pmc: 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Rink amide: *p*-[(*R*,*S*)-a-[1-(9H-fluoren-9-yl)-methoxyforamido]-2,4-dimethoxybenzyl]-phenoxylacetic acid; RP-HPLC: reversed-phase high-pressure liquid chromatography; SPPS: solid-phase peptide synthesis; Statine: 4-alkyl-4-amino-3-hydroxybutanoic acid; TBAF: *N*,*N*,*N*,*N*-tetrabutylammonium fluoride; TBTU: *O*-(benzotriazol-1-yl-*N*,*N*,*N*′*N*′-tetramethyluronium tetrafluoroborate; Teoc: trimethylsilylethoxycarbonyl; TFA: trifluoro acetic acid; Trt: triphenylmethyl; VLC: vacuum liquid chromatography; Y(NO_2): 3-nitrotyrosine.

Three- and one-letter abbreviations for amino acids are used according to the recommendations from IUPAC. See: http://www.chem.qmw.ac.uk/iupac.

Acknowledgment. This work was supported by the Danish National Research Foundation. Dr. David Simpson is thanked for helpful discussions about the enzyme assay. Bent Petersen and Dr. Charlotte Held Gottfredsen are thanked for acquirement of high-resolution NMR spectra.

Supporting Information Available. Experimental details are available as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Leung, D.; Abbenante, G.; Fairlie, D. P. *J. Med. Chem.* **2000**, *43*, 305−341.
(2) Fairlie, D. P.; Tyndall, J. D. A.; Reid, R. C.; Wong, A. K.; Abbenante, G.; Scanlon, M. J.; March, D. R.; Bergman, D. A.; Chai, C. L. L.; Burkett, B. A. *J. Med. Chem.* **2000**, *43*, 1271−1281.
(3) Inagami, T.; Murakami, K. *J. Biol. Chem.* **1977**, *252*, 2978−2983.
(4) Slater, E. E. *Methods Enzymol.* **1981**, *80*, 427−442.
(5) Umezawa, K.; Aoyagi, T.; Morishima, H.; Matsuzaki, M.; Hamada, M.; Takeuchi, T. *J. Antibiot.* **1970**, *23*, 259−262.
(6) Boger, J.; Lohr, N. S.; Ulm, E. H.; Poe, M.; Blaine, E. H.; Fanelli, G. M.; Lin, T.-T.; Payne, L. S.; Schorn, T. W.; LaMont, B. I.; Vassil, T. C.; Stabilito, I. I.; Veber, D. F.; Rich, D. H.; Bopari, A. S. *Nature* **1983**, *303*, 81−84.

(7) Johnson, R. L. *J. Med. Chem.* **1982**, *25*, 605−610.
(8) Iizuka, K.; Kamijo, T.; Akahane, K.; Kubota, T.; Etoh, Y.; Shimaoka, I.; Tsubaki, A.; Murakami, M.; Yamaguchi, T.; Iyobe, A.; Umeyama, H.; Kiso, Y. *Chem. Pharm. Bull.* **1990**, *38*, 2487−2493.
(9) Meldal, M.; Svendsen, I.; Breddam, K.; Auzanneau, F. I. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 3314−3318.
(10) St. Hilaire, P. M.; Willert, M.; Juliano, M. A.; Juliano, L.; Meldal, M. *J. Comb. Chem.* **1999**, *5*, 509−523.
(11) Rossé, G.; Kueng, E.; Page, M. G. P.; Schauer-Vukasinovic, V.; Giller, T.; Lahm, H.-W.; Hunziker, P.; Schlatter, D. *J. Comb. Chem.* **2000**, *2*, 461−466.
(12) St. Hilaire, P. M.; Alves, L. C.; Sanderson, S.; Mottram, J. C.; Juliano, M. A.; Juliano, L.; Coombs, G. H.; Meldal, M. *ChemBioChem* **2000**, *1*, 115−122.
(13) Meldal, M.; Svendsen, I. *J. Chem. Soc. Perkin Trans. 1* **1995**, 1591−1596.
(14) Youngquist, R. S.; Fuentes, G. R.; Lacey, M. P.; Keough, T. *J. Am. Chem. Soc.* **1995**, *117*, 3900−3906.
(15) Burton, J.; Poulsen, K.; Haber, E. *Biochemistry* **1975**, *14*, 3892−3898.
(16) Poulsen, K.; Burton, J.; Haber, E. *Biochemistry* **1973**, *12*, 3877−3882.
(17) Harada, H.; Iizuka, K.; Kamijo, T.; Akahane, K.; Yamamoto, R.; Nakano, Y.; Tsubaki, A.; Kubota, T.; Shimaoka, I.; Umeyama, H.; Kiso, Y. *Chem. Pharm. Bull.* **1990**, *38*, 3042−3047.
(18) Dondoni, A.; Perrone, D.; Semola, M. T. *J. Org. Chem.* **1995**, *60*, 7927−7933.
(19) Badasso, M.; Sibanda, B. L.; Dhanaraj, V.; DeAlwis, C.; Cooper, J. B.; Wood, S. P.; Blundell, T. L.; Murakami, K.; Miyazaki, H.; Hobart, P. M.; Geoghegan, K. F.; Ammirati, M. J.; Lanzetti, A. J.; Danley, D. E.; O'Connor, B. A.; Hoover, D. J.; Sueiras-Diza, J.; Jones, D. M.; Szelke, M. *J. Mol. Biol.* **1992**, *233*, 447−453.
(20) Wang, W.; Liang, C. *Biochemistry* **1994**, *33*, 14636−14641.
(21) Burton, J.; Quinn, T. *Biochim. Biophys. Acta* **1988**, *952*, 8−12.
(22) Renil, M.; Meldal, M. *Tetrahedron Lett.* **1996**, *37*, 6185−6188.
(23) Grøtli, M.; Gotfredsen, C. H.; Rademann, J.; Buchardt, J.; Clark, A. J.; Duus, J. Ø.; Meldal, M. *J. Comb. Chem.* **2000**, *2*, 108−119.
(24) Philips, G. B.; Wei, G. P. *Tetrahedron Lett.* **1996**, *37*, 4887−4890.
(25) Morales, G. A.; Corbett, J. W.; DeGrado, W. F. *J. Org. Chem.* **1998**, *63*, 1172−1177.
(26) Hari, A.; Miller, B. L. *Angew. Chem., Int. Ed. Engl.* **1999**, *38*, 2777−2779.
(27) Lucrezia, R. D.; Gilbert, I. H.; Floyd, C. D. *J. Comb. Chem.* **2000**, *2*, 249−253.
(28) Meldal, M.; Svendsen, I.; Juliano, L.; Juliano, M. A.; Nery, E. D.; Schaarfstein, J. *J. Pept. Science* **1998**, *4*, 83−91.
(29) Spetzler, J. C.; Westphal, V.; Winther, J. R.; Meldal, M. *J. Peptide Sci* **1998**, *4*, 128−137.
(30) St. Hilaire, P. M.; Lowary, T. L.; Meldal, M.; Bock, K. *J. Am. Chem. Soc.* **1998**, *120*, 13312−13320.
(31) Buchardt, J.; Schiødt, C. B.; Krog-Jensen, C.; Delaisse, J. M.; Foged, J. M.; Meldal, M. *J. Comb. Chem.* **2000**, *2*, 624−638.
(32) Graven, A.; St. Hilaire, P. M.; Sanderson, S.; Mottram, J. C.; Coombs, G. H.; Meldal, M. A *J. Comb. Chem.* **2001**, *3*, 441−452.
(33) Furka, Á.; Sebestyén, F.; Asgedom, M.; Dibó, G. *Int. J. Pept. Protein Res.* **1991**, *37*, 487−493.
(34) Carpino, L. A.; Tsao, J.-H. *J. Chem. Soc., Chem. Commun.* **1978**, 358−359.
(35) Skeggs, L. T.; Kahn, J. R.; Lentz, K.; Shumway, N. P. *J. Exp. Med.* **1957**, *106*, 439−453.
(36) Skeggs, L. T.; Lentz, K.; Kahn, J. R.; Hochstrasser, H. *J. Exp. Med.* **1968**, *128*, 13−34.
(37) Schechter, I.; Berger, A. *Biochem. Biophys. Res. Comm.* **1967**, *27*, 157−162.
(38) Nicklin, M. J. H.; Barrett, A. L. *Biochem. J.* **1984**, *223*, 245−253.
(39) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595−598.

CC0100722