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LIBRARIES OF ANGIOTENSIN CONVERTING ENZYME INHIBITORS: SOLID-PHASE SYNTHESIS AND AFFINITY SELECTION

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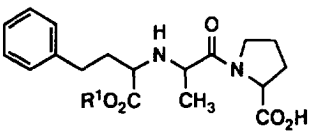
Abstract: Libraries of ACE inhibitors were prepared by solid-phase methods involving reductive alkylation of resin-bound dipeptides, H-X-Pro-DHPP-PEG-PS or H-X-X-PAL-PEG-PS. Affinity selection of the library components identified hydrophilic *N*-alkylated dipeptides as potential inhibitors. © 1997 Elsevier Science Ltd.

Solid-phase synthesis of libraries of potential therapeutic compounds has attracted considerable interest in recent years due to high throughput in both construction and screening using combinatorial strategies.[1] Affinity selection by multiple-column chromatographic techniques[2] is of particular interest because rapid, automated library screening can be accomplished by interfacing mass spectrometry to the liquid chromatography system for identification of active compounds. As an extension of the method for solid-phase synthesis of peptides,[3] we report herein the assembly of libraries of angiotensin converting enzyme (ACE) inhibitors. The libraries were designed to test construction on polyethylene glycol-polystyrene (PEG-PS)[4] graft supports followed by automated chromatographic screening of enzyme binding with identification of active compounds by electrospray ionization (ESI) mass spectrometry.

There have been many reports[5] concerning the design and synthesis of ACE inhibitors of general structure, R-X_1-X_2-OH (where X was an α-amino acid).[6] Structure-activity studies have shown that highest affinity for ACE was conferred when group R was 1-carboxy-3-phenylpropyl, X_2 was proline, X_1 was Ala or Lys and the stereochemistry was S,S,S[5c,7] such as the clinical antihypertensive agent enalapril **1** and its active metabolite enalaprilat **2a**.[8]



1, R^1 = Et; **enalapril** ≡ R^2(COOEt)-Ala-Pro-OH **2**, R^2(COOH)-X-Pro-OH
2a, R^1 = H; **enalaprilat** ≡ R^2(COOH)-Ala-Pro-OH **3**, R^2(COOEt)-X-X-NH_2

To demonstrate the feasibility of our strategy, enalaprilat **2a** was assembled on a solid support, released from the resin and screened by affinity chromatography for binding to ACE. Attempts to prepare the *C*-terminal proline containing *N*-alkylated dipeptide with a *p*-alkoxybenzyl alcohol (PAC) handle attached to PEG-PS were unsuccessful due to the loss of Ala-Pro-OH from the resin as a result of diketopiperazine formation.[9] To overcome this undesired cyclization, 4-(1',1'-dimethyl-1'-hydroxypropyl)phenoxyacetyl (DHPP) handle[10,11] was employed. DHPP linker was coupled to PEG-PS using *N,N'*-diisopropylcarbodiimide (DIPCDI)/1-hydroxy-7-azabenzotriazole (HOAt)[12] in *N,N*-dimethylformamide (DMF) and the hydroxyl group was esterified with 5 equiv of Fmoc-Pro-Cl in a solution of pyridine-CH_2Cl_2 (1:9) for 20 h at 25 °C.[13] Following N^{α}-Fmoc removal with piperidine-DMF (1:4),[14] Fmoc-Ala-OH was added via the acid fluoride generated in situ using 4 equiv of tetramethylfluoroformamidinium hexafluorophosphate[15] (TFFH) in the presence of 8 equiv of *N,N*-diisopropylethylamine (DIEA) in DMF. The resin-bound dipeptide was subjected to N^{α}-Fmoc group deprotection followed by reductive alkylation using 25 equiv of ethyl-2-oxo-4-phenylbutyrate in HOAc–DMF (1:99) in the presence of 40 equiv of $NaBH_3CN$.[16] Release of the *N*-alkylated

dipeptide from the solid support with TFA–H_2O (95:5) for 2 h at 25 °C gave enalapril **1** in excellent purity and yield. Hydrolysis of **1** on the resin was accomplished using 1 N NaOH for 20 h and TFA–H_2O (95:5) cleavage gave enalaprilat **2a** which was used in an affinity chromatography screening assay.[17]

To explore the utility of this scheme, library **2** was prepared by coupling H-Pro-DHPP-PEG-PS to a mixture of nineteen Fmoc-L-amino acids (Cys omitted) with ^{t}Bu-based side-chain protection[18] using *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide[19] (HATU) (4 equiv)/DIEA (8 equiv) followed by Fmoc deprotection, reductive alkylation and hydrolysis as described above. Release of the alkylated dipeptide and removal of the side-chain protecting groups was effected using TFA–iPr$_3$SiH–H_2O (95:5:5) for 2 h at 25 °C. The ESI mass spectrum of this mixture (Figure 1) strongly suggested that all 19 of the expected components were present in the library.[20] Screening of library **2** using the conditions described above identified enalaprilat **2a** as the most active compound in the mixture.[17]

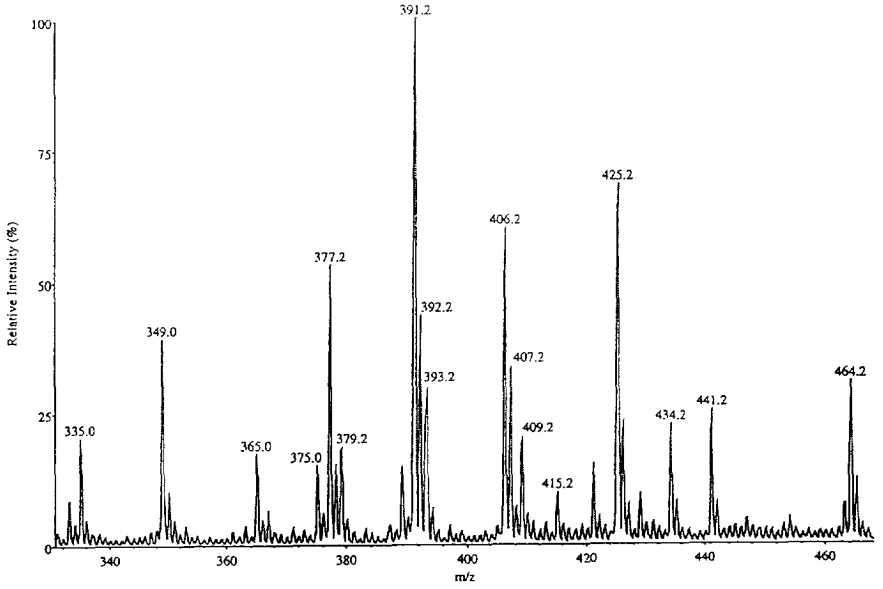


Figure 1. ESI mass spectrum of *N*-alkyl-X-Pro-OH library derived from a mixture of 19 Fmoc-amino acids.

In order to probe the influence of amino acid sequence and *C*-terminal amide substitution on ACE binding, a 2X library of enalapril amides **3** was assembled by coupling a mixture of 19 Fmoc-amino acids to 5-(4-(9-fluorenylmet-hyloxycarbonyl)aminomethyl-3,5-dimethoxy-phenoxy)valeric acid handle PAL-PEG-PS[21] using HATU/DIEA, deprotecting and repeating the process. Resin H-X_1-X_2-PAL-PEG-PS was subjected to reductive alkylation and cleavage as described previously. The 361 component library (722 including stereoisomers) was analyzed by ESI and the spectrum had a close resemblance to a simulated spectrum giving confidence that the expected sequences were well represented. Library **3** was screened for binding to ACE using a size-exclusion/reversed-phase HPLC technique and the data suggested that the members with the highest affinity have a mass of 423 Da corresponding to the following dipeptides with a mass of 232 Da: Asn/Thr, Ser/Gln and Ser/Lys.[22] The six dipeptides were prepared individually on PAL-PEG-PS using HATU-mediated coupling and subjected to reductive alkylation conditions. Release of the *N*-alkylated dipeptides from the solid support was accomplished using TFA–anisole–β-mercaptoethanol (95:3:2) for 2 h at 25 °C. Preliminary results based on inhibition assays of ACE with these targets indicated that Ser-Lys-, Ser-

Gln-, Lys-Ser- and Gln-Ser-containing molecules are most potent, albeit less effective than enalaprilate or enalaprilate-amide.[23] These hydrophilic dipeptides contrast the structure of enalapril and will be the subject of further investigation.

To conclude, an efficient solid-phase synthesis of enalapril and enalaprilat together with their corresponding diastereoisomers was conducted by reductive alkylation of the appropriate dipeptide on DHPP-PEG-PS. The reaction proceeded more cleanly and in higher yield than comparable reactions in solution phase[5d] because a large excess of α-ketoester was employed. Combinatorial libraries were generated in which α-amino acids provided the diversity elements. Analysis by affinity selection and mass spectrometry led to the isolation of four new high affinity ACE binders. The combination of these techniques was a useful method for rapid generatation and screening of potential therapeutic compounds.

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6. Abbreviations: Amino acids and peptides are abbreviated and designated following the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **247**, 977-983 (1972). Additionally, the following abbreviations are used: ACE, angiotensin converting enzyme; ^{t}Bu, *tert.*-butyl; DIPCDI, *N,N'*-diisopropylcarbodiimide; DHPP, 4-(1',1'-dimethyl-1'-hydroxy-propyl)phenoxyacetyl; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; DMSO, dimethylsulfoxide; ESI, electrospray ionization; Fmoc, 9-fluorenylmethyloxycarbonyl; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethan-aminium hexa-fluorophosphate *N*-oxide; HEPES, *N*-(2-hydoxyethyl)piperazine-*N*-(2-ethanesulfonic acid; HOAt, 1-hydroxy-7-azabenzotriazole; HPLC, high performance liquid chromatography; PAC, *p*-alkoxybenzyl alcohol handle; PAL, 5-(4-(9-fluorenylmethyloxycarbonyl)aminomethyl-3,5-dimethoxy-phenoxy)valeric acid handle; PEG-PS, polyethylene glycol-polystyrene graft supports; TFA, trifluoroacetic acid; TFFH, tetramethylfluoroformamidinium hexafluorophosphate. Amino acid symbols denote the L-configuration unless indicated otherwise.
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17. Enalaprilat and the R^2(COOH)-X-Pro-OH library were independently dissolved in DMSO–H_2O (1:99) and incubated with 30 μL of ACE (1 mg/mL, Cortex Biochem, CA) in 50 mM HEPES containing 0.3 M NaCl, pH 8.35 at 37 °C for 1 h. Chromatography based screening was performed on the INTEGRAL™ Micro-Analytical Workstation (PerSeptive Biosystems, Inc., Framingham, MA). The complex of enzyme with bound ligands was separated from unbound compounds by size exclusion and the peak corresponding to the protein was collected and analyzed by a PE-Sciex API III plus atmospheric pressure ionization (API) triple quadrapole mass spectrometer (Thornill, ON, Canada). The sample was dissolved in CH_3CN–H_2O (1:1) containing 0.1% HOAc and introduced by direct infusion at 2 μL/min with a syringe pump (Harvard Apparatus, Natick, MA).
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20. The product obtained on evaporation of the cleavage cocktail was washed with hexane and the residue was redissolved in CH_3CN–H_2O (1:1) and analyzed by ESI mass spectrometry. The alkylation reaction introduces a new stereocenter into the molecule and thus each of the nineteen products will be a mixture of two diastereoisomers. Peak intensities are a consequence of relative molar response of the components and do not necessarily reflect concentrations. Arg, Pro, and His containing peptides, which give weak signals in the ESI spectrum, gave intense signals in a matrix assisted laser desorption/time-of-flight mass spectrum of this mixture.
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22. The R(CO_2Et)-X-X-NH_2 library (20 μL, 90 mg/mL) was dissolved in DMSO–H_2O (1:49) and incubated with 100 μL of ACE (1 mg/mL, Sigmal Chemicals, St. Louis) in 50 mM HEPES containing 0.3 M NaCl, pH 8.35 and 100 μL of 10 mM NH_4CO_3, pH 8.8 at 37 °C for 18 h. Chromatography based screening was performed on the INTEGRAL™ microanalytical workstation configured in tandem column mode with the Progel TSK G3000 SWxl column (7.8 x 300 mL, The Nest Group, Inc., Southborough, MA) plumbed in-line with a reversed-phase POROS R2H column (2.1 x 100 mL, PerSeptive Biosystems, Inc.). The enzyme with any bound ligands was eluted from the size exclusion column directly onto the POROS R2H column. The acid conditions of the reversed-phase HPLC column (linear gradient over 11 min of 20% 2-propanol/79.9% CH_3CN–H_2O (85:15)/0.1% TFA and H_2O/0.1% TFA from 0:1 to 1:0, flow rate 1.0 mL/min) dissociated the noncovalently bound ligands from the enzyme and the eluted fractions were analyzed by a ESI-MS.
23. The enzyme activity assays were carried out using a commercially available chromogenic substrate reagent kit *N*(3-[2-furyl]acroyl)phenylalanylglyclglycine, FAPGG, Sigma Chemical Co., St. Louis, MO). Enzyme activity was measured from the decrease in A_{340nm} due to hydrolysis of the FAPGG substrate in the presence and absence of the dipeptides.

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