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Synthesis and characterization of cyclic pseudopeptide libraries containing thiomethylene and thiomethylene-sulfoxide amide bond surrogates

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Summary

We describe the first examples of a series of cyclic pseudopeptide libraries that have been prepared in a systematic approach in order to facilitate both synthesis and subsequent deconvolution attempts. Our synthetic strategy involved the attachment of a trifunctional amino acid (Asp, Asn or Glu) to a polystyrene resin via its side chain, and stepwise chain elongation using either protected amino acids or a pseudodipeptide building block. Head to tail cyclic peptides were formed by removal of the temporary N- and C-terminal protecting groups followed by ring closure by amide formation. Cyclization of the hexa, hepta, and octapseudopeptides on the resin avoided dimer formation, as monitored by mass spectrometry. We utilized a 'psi-scan' approach in which a second fixed position was serially addressed by stepping a dipeptide surrogate, Proψ[CH_2S]Gly around the rings to generate a group of cyclic pseudopeptide sub-libraries. Oxidation of ψ[CH_2S] to ψ[CH_2SO] helped validate the synthesis and also provides a strategy for forming a new set of pseudopeptide libraries (previously described as 'libraries from libraries'). Our results suggest that libraries of cyclic pseudopeptides are an efficient method of preparing and assaying these synthetically more challenging entities as potential drug leads.

Introduction

Peptides are an important class of molecules with potential therapeutic utility in virtually all known human disease categories. However, in spite of their widespread use (insulin, vasopressins, calcitonin, LH-RH analogs and Sandimmune among others) peptides are often discounted as preferred new drug candidates due to their lack of resistance toward enzymatic clearance and poor transfer rates across cell membranes and intestinal mucosa. In order to transform a peptide lead into a useful drug, several approaches including cyclization [1] and peptide backbone modifications [2,3] can be employed. The resulting cyclic peptides and pseudopeptides therefore represent useful intermediates along the drug discovery pathway.

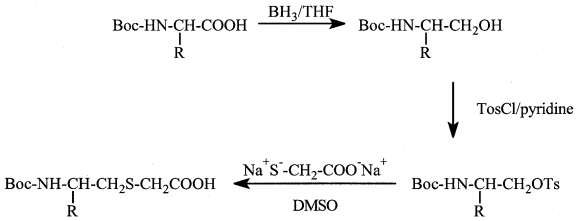


Figure 1. Synthesis route of the preformed pseudodipeptides, Boc-AAψ[CH_2S]Gly-OH.

Intrigued by the potential of combinatorial libraries [4,5], and especially peptide libraries [6], for the identification of potential macromolecular ligands and

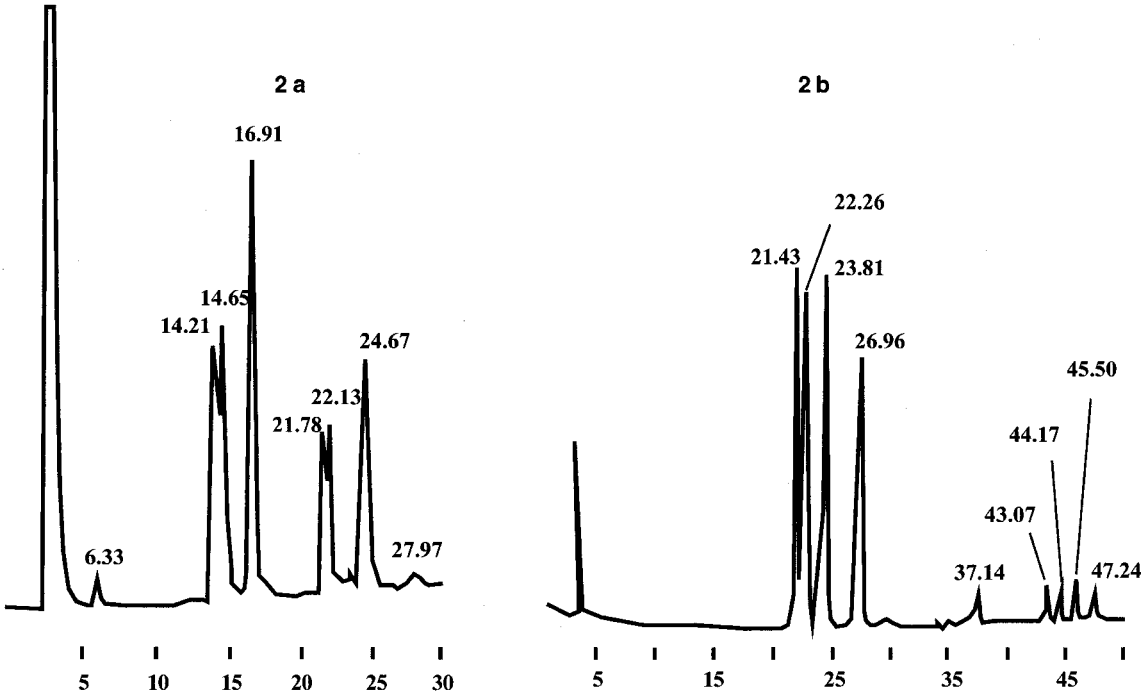


Figure 2. (a) RP-HPLC profile of the pseudopeptide mixture H-Tyrψ[CH_2S]Gly-Phe-Xxx-Leu-OH, where Xxx = (Ala, Glu, Lys, Arg). Gradient: 15–65% buffer B in 28 min, monitored at λ = 214 nm. (b) RP-HPLC profile of the pseudopeptide mixture H-Tyr-D-Alaψ[CH_2NH]Gly-Xxx-Phe-Leu-OH, where Xxx = (Asp, Ser, Leu, Val). Note that the amount of benzyl-modified products in 2b represents a considerably smaller proportion than in 2a suggesting the influence of the ψ[CH_2S] surrogate in enhancing the cationic alkylation side products. Gradient: 15 to 35% buffer B in 48 min, monitoren at λ = 214 nm. Buffer A: 0.05% TFA/H_2O. Buffer B: 0.05% TFA/A_cCN.

drug leads, we reasoned that the combinatorial synthesis of cyclic peptide and pseudopeptide libraries would add a new dimension to the generation of molecular diversity, thus providing a useful molecular tool for facilitating drug lead discovery. In addition, due to the availability of large numbers of cyclic peptides and pseudopeptides (generated from the combinatorial approach), some fundamental questions about these novel ring systems can be perhaps more efficiently addressed. These include the sequence dependence of cyclizations for linear peptides and pseudopeptides, as well as preferential interactions between various metal ions and cyclic peptides and pseudopeptides (host-guest relationships) using a combinatorial synthesis approach.

To assist such studies, we have developed a general strategy for synthesizing head-to-tail lactam cyclic peptide libraries [7,8] using a resin bound cyclization approach [9]. In subsequent reports, we have studied the racemization problem of a C-terminal aspartic acid residue during the synthesis [10] and explored library variations in structure and ring size [11]. More recently, we have reported our studies on metal binding affinities of cyclic peptide mixtures [12] and on the synthesis and characterization of cyclic pseudopeptides containing the ψ[CH_2NH] surrogate [13]. In this report, we extend our study to cyclic pseudopeptide libraries containing the ψ[CH_2S] and ψ[CH_2SO] surrogates. These amide bond replacements were first designed for incorporation within collagen sequences [14] and for backbone modified linear LH-RH analogs as potential contraceptive agents [15]. The facile oxidation of these compounds to sulfoxides, but not sulfones, using hydrogen peroxide treatment was also previously described [16]. The synthesis, characterization, and bioassays of these libraries are described.

Materials and Methods

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Specific optical rotations were determined on a Jasco Model 700 at the sodium D line. Proton NMR spectra were recorded on a Bruker AMX-500 Fourier Transform NMR spectrometer. TLC was performed

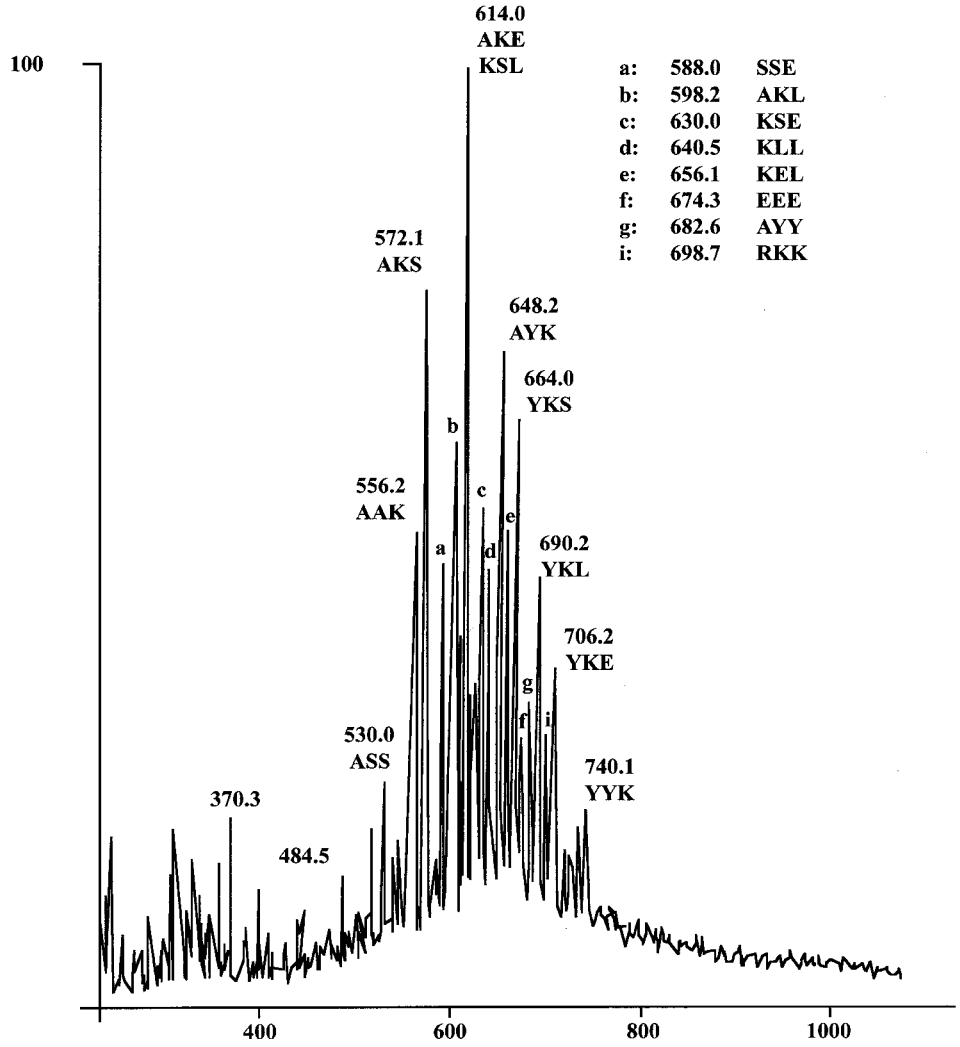


Figure 3. Negative ESMS of L4 or cyclo(Proψ[CH_2S]Gly-Yyy-Yyy-Yyy-Glu) where Yyy is a nearly equimolar mixture of lysine, leucine, arginine, glutamic acid, serine, alanine and tyrosine. The theoretical distribution of this mixture consisted of 343 pseudohexapeptides with 84 unique masses ranging from 499 to 775.

on silica gel (Merck 254 plates) in the following systems (v/v): (A) $CHCl_3$/MeOH, 95:5; (B) ethyl acetate/hexane, 1:1; and (C) $CHCl_3$/MeOH/HOAc, 85:10:5; (D) acetone/hexane, 3:7. Solid phase reactions were manually performed in 2 mL syringes using a Teflon 42-vessel multiple peptide synthesis block from CSPS (San Diego, CA). Reaction products were visualized by UV fluorescence (254 nm) or 1% ninhydrin in ethanol. Amino acid analyses were performed on a Dionex D-300 amino acid analyzer and a Dionex CP-3 programmer at the Health Sciences Center of the University of Louisville as follows: the pseudopeptide mixtures were first hydrolyzed in 6 N HCl, containing 1 mg of phenol, at 110 °C for 20–24 h in sealed, evacuated hydrolysis tubes, then derivatized using the PICO TAG method developed by Waters-Millipore. Molecular weight determinations were made using electrospray mass spectrometry (ES–MS) at the University of Michigan on a Micromass (VG) Platform quadrupole mass spectrometer (0–3000 *m/z)* in positive and negative ionization modes. Peptides for mass analysis were dissolved in 50% CH_3CN:50% H_2O.

Technical-grade dichloromethane and crude dimethylformamide solvents were purchased from EM Science, and were distilled prior to use. Protected amino acids were purchased from Bachem Bioscience Inc. (King of Prussia, PA) and Chem-Impex International (IL). Benzotriazol-1-yl-oxy-tris(diethyl-

amino)-phosphoniumhexafluorophosphate (BOP) was purchased from Richelieu Biotechnologies. 1-Hydroxybenzotriazole (HOBt), N,N′-diisopropylethylamine (DIEA), 9-fluorenylmethanol (HOFm), benzylchloroformate (Z-Cl), and lithium aluminum hydride ($LiAlH_4$) were products of Aldrich Chemical Co. (Milwaukee).

Synthesis of Boc-Tyr(Bzl)-CH_2OH (1)

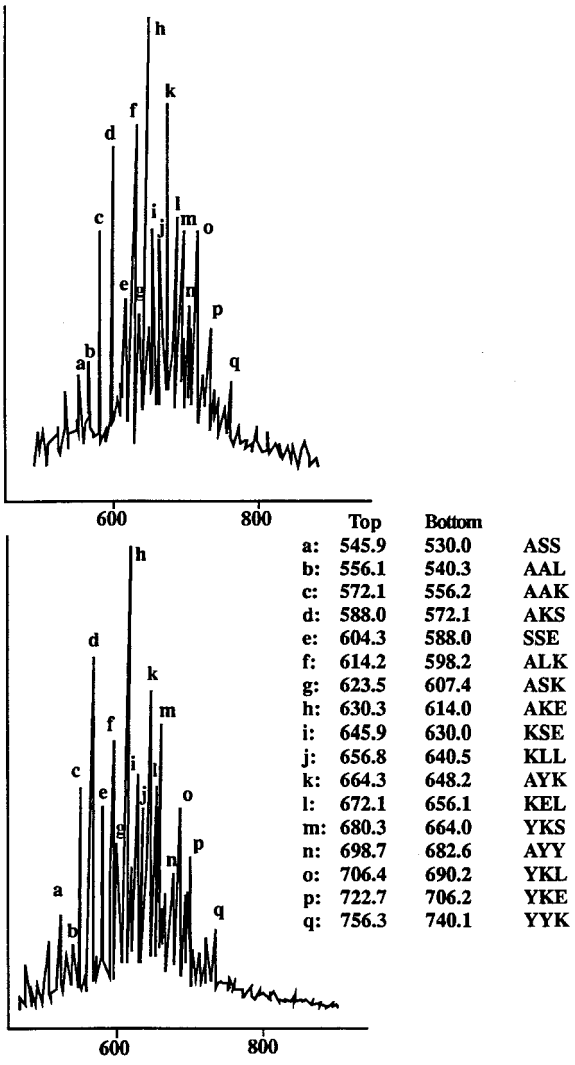
BH_3/THF (30 mL, 30 mmol) was transferred from a sealed bottle to a round bottom flask maintained in an acetone-dry ice bath. To the solution was added 3.71 g Boc-Tyr(Bzl)-OH (10 mmol) in THF dropwise for 30 min. The reaction mixture was stirred at –40 °C for 1 h, then at room temperature for 2 h. The reaction was quenched by slowly adding ice cooled water to the reaction mixture. The solution was evaporated and extracted with EtOAc. The combined extracts were washed with water, saturated $NaHCO_3$, and saturated NaCl solution. The EtOAc layer was dried with anhydrous $MgSO_4$, and evaporated to afford 2.96 g product (82.8%) as a white solid: mp 100–103 °C; $[\alpha]_D$: –0.424 (C1, EtOH); R_f (B) = 0.73; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.39 (s, 9H, Boc CH_3), 2.75 (d, 2H, β-CH_2), 3.49 (m, 1H, α-H), 3.54 (m, 2H, CH_2-O), 3.63 (s, 1H, OH), 4.67 (s, 1H, CONH), 5.02 (s, 2H, CH_2 of Bzl), 6.90 (d, 2H, 3,5-aromatic H of Tyr), 7.10 (d, 2H, 2,6-aromatic H of Tyr), 7.30–7.42 (m, 5H, Ph of Bzl).

Synthesis of Boc-Pro-CH_2OH (2)

The procedure used for this synthesis was virtually identical to the preparation of Boc-Tyr(Bzl)-CH_2OH: 85.9% yield; mp 57–59 °C; R_f(C) = 0.68; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.44 (s, 9H, Boc); 1.75 (m, 2H, γ-CH_2); 1.98 (m, 2H, β-CH_2); 3.29–3.42 (mm, 2H, δ-CH_2); 3.55–3.62 (mm, 2H, CH_2-O); 3.93 (d, 1H, α-H); 4.73 (d,1H, -OH).

Synthesis of Boc-Tyr(Bzl)-CH_2OTs (3)

To a solution of 2.9 g Boc-Tyr(Bzl)-CH_2OH (8.1 mmol) in 20 mL $CHCl_3$ and 6.56 mL pyridine (81 mmol) maintained at 0 °C was added 4.63 g tosyl chloride (24.3 mmol) in $CHCl_3$ over 15 min. Upon the addition of tosyl chloride, the color of the solution changed to yellow, and eventually to red. The reaction mixture was stirred at room temperature for 1.5 h. The solution was evaporated to dryness under reduced pressure, the residue was extracted with a mixture of



	Top	Bottom	
a:	545.9	530.0	ASS
b:	556.1	540.3	AAL
c:	572.1	556.2	AAK
d:	588.0	572.1	AKS
e:	604.3	588.0	SSE
f:	614.2	598.2	ALK
g:	623.5	607.4	ASK
h:	630.3	614.0	AKE
i:	645.9	630.0	KSE
j:	656.8	640.5	KLL
k:	664.3	648.2	AYK
l:	672.1	656.1	KEL
m:	680.3	664.0	YKS
n:	698.7	682.6	AYY
o:	706.4	690.2	YKL
p:	722.7	706.2	YKE
q:	756.3	740.1	YYK

Figure 4. Negative ESMS OF L4 (bottom spectrum) of cyclo(Proψ[CH_2S]Gly-Yyy-Yyy-Yyy-Glu and L35 (top spectrum) or cyclo(Proψ[CH_2SO]Gly-Yyy-Yyy-Yyy-Glu) where Yyy is a nearly equimolar mixture of lysine, leucine, arginine, glutamic acid, serine, alanine and tyrosine. Note the molecular weight shift of 16 due to the additional oxygen atom in L35.

EtOAc and water. The combined organic extracts were washed with cold water, 1 N HCl solution, water, 5% $NaHCO_3$ solution, water, and saturated NaCl solution. The EtOAc layer was dried with anhydrous $MgSO_4$, and evaporated to afford 4.15 g crude product. Column chromatography on silica gel (solvent gradients starting from 100% hexane to 90% hexane/acetone, 80% hexane/acetone, and 50% hexane/acetone) gave 3.10 g (74.8%) purified product as a white solid. mp 155–158 °C; R_f (A) = 0.41; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.37 (s, 9H, Boc CH_3), 2.43 (s, 3H, CH_3 of Tos),

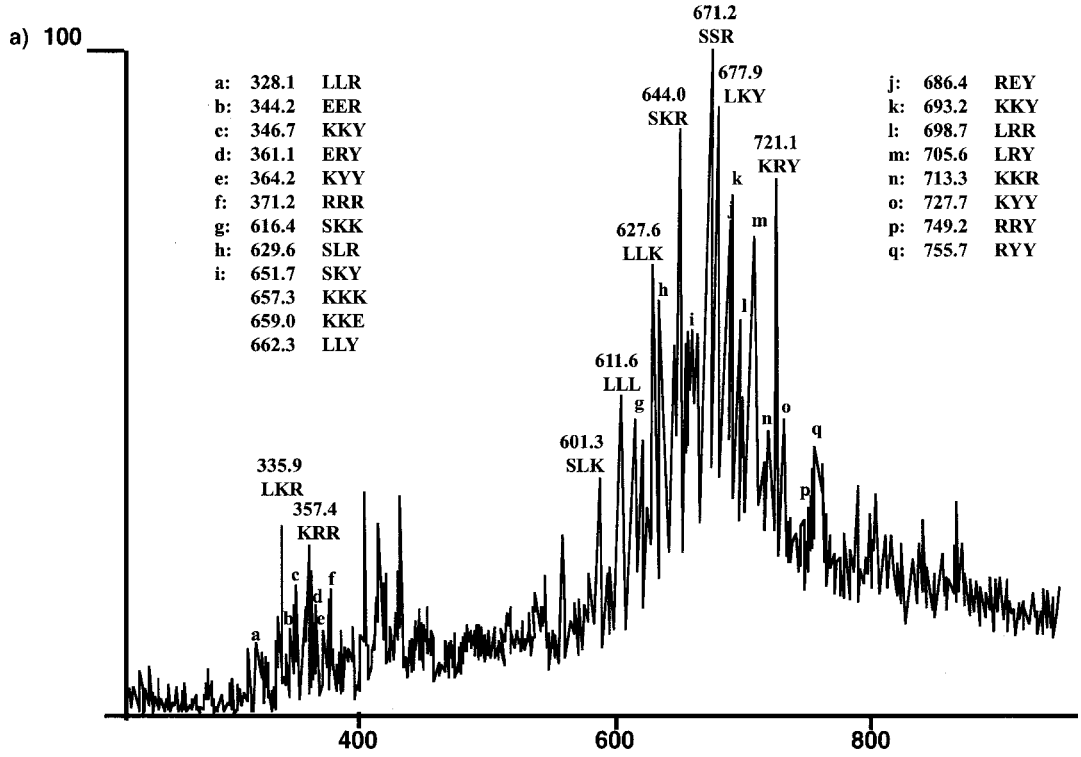


Figure 5a. Negative ESMS of L7 or cyclo(Xxx-Proψ[CH_2S]Gly-Xxx-Xxx-Asp) where Xxx is a nearly equimolar mixture of lysine, leucine, arginine, glutamic acid, serine, and tyrosine. The theoretical distribution of this mixture consisted of 216 pseudohexapeptides with 56 unique masses ranging from 533 to 761.

2.70 (dd, 1H, β-CH_2), 2.78 (dd, 1H, β-CH_2), 3.87 (m, 1H, α-H), 3.98 (d, 2H, CH_2O), 4.69 (d, 1H, CONH), 5.00 (s, 2H, CH_2 of Bzl), 6.82 (d, 2H, 3,5-aromatic H of Tyr), 6.96 (d, 2H, 2,6-aromatic H of Tyr), 7.33 (m, 5H, Ph of Bzl). 7.39 (m, 2H, 3,5-aromatic H of Tos), 7.75 (d, 2H, 2,6-aromatic H of Tos).

Synthesis of Boc-Pro-CH_2OTs (4)

The procedure is almost identical to the preparation of Boc-Tyr(Bzl)-CH_2OTs: white solid; yield 62.4%; mp: 72–74 °C; R_f(C) = 0.77; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.38 (d, 9H, Boc); 1.79 (m, 2H, γ-CH_2); 1.90 (m, 2H, β-CH_2); 2.42 (s, 3H, CH_3 of Tos); 3.26 (m, 2H, δ-CH_2); 3.86 (dd, 2H, CH_2-OTs); 4.06 (m, 1H, α-H); 7.33 (m, 2H, 3,5-aromatic H of Tos); 7.76 (d, 2H, 2,6-aromatic H of Tos).

Synthesis of Boc-Tyr(Bzl)ψ[CH_2S]Gly-OH (5)

Freshly shaved sodium metal (207 mg, 9 mmol) was dissolved in 10 mL anhydrous ethanol solvent. To this solution was added 0.313 mL freshly distilled $HSCH_2COOH$ (4.5 mmol). Upon the addition of $HSCH_2COOH$, white solid immediately precipitated out of solution. The suspension was stirred for 10 min, then evaporated under reduced pressure. The residue was dissolved in benzene and evaporated. This process was repeated several times until a white solid was obtained. The white solid was further dried and dissolved in 2–3 mL DMSO under nitrogen. To the solution was added 2.04 g Boc-Tyr(Bzl)-CH_2OTs (4 mmol). The reaction mixture was stirred at RT under nitrogen for 4 h, and then 15 mL cold water was added to the reaction mixture. The aqueous solution was washed with ether once, and brought to pH 3 by addition of 1 N HCl. The aqueous solution was extracted with EtOAc (3×50 mL). The combined organic extracts were washed with 1 N HCl solution, water, and saturated NaCl solution. The EtOAc layer was dried with $MgSO_4$, and evaporated to afford 1.43 g pure product (82.5%) as a white solid: mp 54–56 °C; R_f(D) = 0.28; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.39 (s, 9H, Boc CH_3), 2.71 (m, 2H, β-CH_2), 2.79 (m, 2H, CH_2-S), 3.27 (m, 2H, S-CH_2), 4.00 (br s, 1H, α-H), 4.70 (br s, 1H, CONH), 5.01 (s, 2H, CH_2 of Bzl), 6.88 (d, 2H, 3,5-

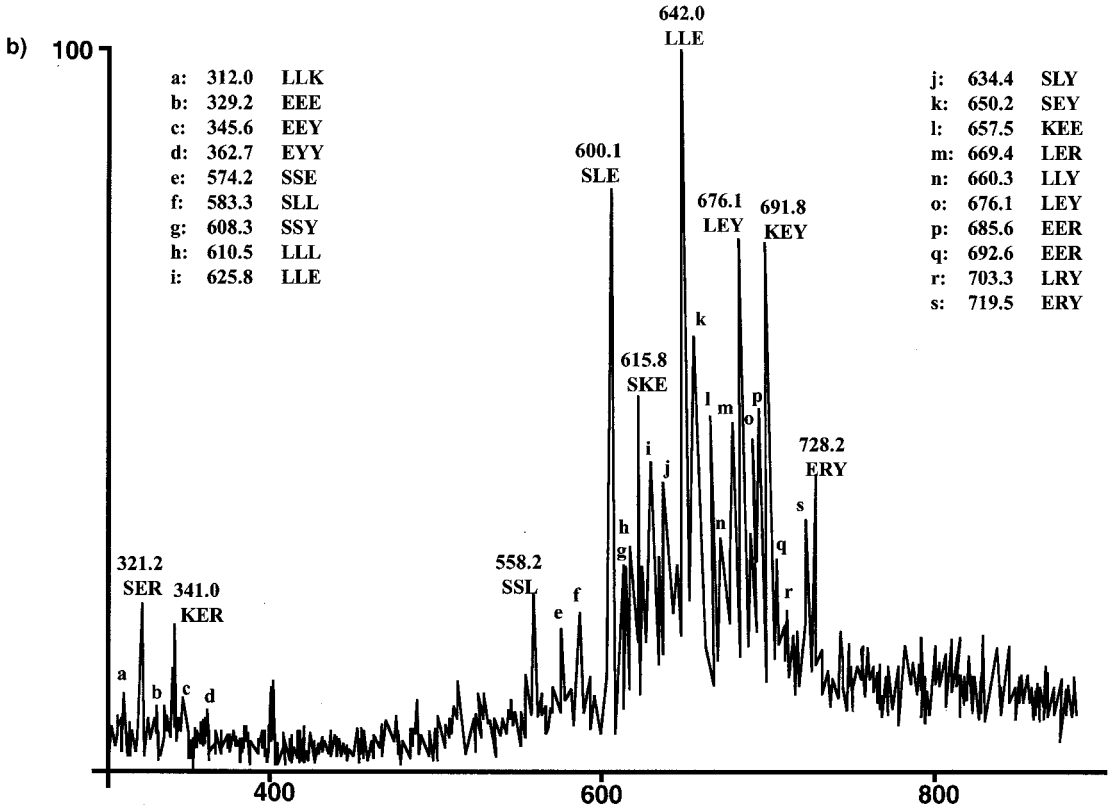


Figure 5b. Positive mode ESMS of L7 or cyclo(Xxx-Proψ[CH_2S]Gly-Xxx-Xxx-Asp) where Xxx is a nearly equimolar mixture of lysine, leucine, arginine, glutamic acid, serine, and tyrosine. Note that the positive mode MS is complementary to the negative mode in that it corresponds to most of the expected base-containing (Lys and Arg) peptide components.

aromatic H of Tyr), 7.07 (d, 2H, 2,6-aromatic H of Tyr), 7.28–7.41 (m, 5H, Ph of Bzl). ES-MS $[M\text{-}H]^-$ = 430.4, found: 430.5.

Synthesis of Boc-Proψ[CH_2S]Gly-OH (6)

A procedure was used similar to the preparation of Boc-Tyr(Bzl)ψ[CH_2S]Gly-OH for synthesizing this title compound. Specifically, 2.5 g Boc-Pro-CH_2OTs (4) (7.1 mmol) was caused to react with the freshly prepared disodium salt of mercaptoacetic acid (7.5 mmol) in 10 mL of DMSO at room temperature for 6 h. Following workup as above, a white solid was obtained: 70% yield; mp: 70–72 °C; R_f(C) = 0.57. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.44 (s, 9H, Boc); 1.82 (m, 3H, γ-CH_2 and β-H); 1.97 (m, 1H, β-H); 2.59 (br s, 1H, CH_2-S-); 2.94 (dd, 1H, CH_2-S); 3.27 (m, 2H, δ-CH_2); 3.34 (m, 2H, S-CH_2); 3.89–3.98 (m, 1H, α-H). ES-MS $[M\text{-}H]^-$ = 274, found: 273.9.

Synthesis of H-Tyrψ[CH_2S]Gly-Phe-Xxx-Leu-OH (7), where: Xxx = Ala, Lys, Glu, and Arg

The synthesis of the title pseudopeptide mixture was initiated with 200 mg of Boc-Leu-Merrifield resin which was prepared following treatment of chloromethylated polystyrene resin (Kodak) with the cesium salt of Boc-leucine (1 equiv, based on an initial chloride content of 1.0 mmol/g). A substitution of 0.72 mmol/g was determined by weight gain. Synthesis was performed using the Boc-SPPS strategy according to the following protocol: (i) CH_2Cl_2, 3 × 1 min; (ii) 47.5% TFA/47.5% CH_2Cl_2/2.5% anisole/2.5% $CH_3SCH_2CH_3$, 2 + 25 min; (iii) CH_2Cl_2, 4 × 1 min; (iv) 10% DIEA/CH_2Cl_2, 2 + 1 min; (v) CH_2Cl_2, 3 × 1 min; (vi) DMF, 3 × 1 min; (vii) Boc-amino acid (3 equiv)/BOP (3 equiv)/HOBt (3 equiv)/NMM (6 equiv), 60 min; (viii) Kaiser test; (ix) DMF, 3 × 1 min; (x) EtOH, 2 × 1 min.

After acidic deprotection of the Boc-protecting group and neutralization of the resulting protonated

amine, the resin was swelled in DMF and divided into four equal portions using the 'portioning-mixing' method [17]. To each portion was coupled one of the following four Boc-protected amino acids (Xxx): Boc-Ala-OH, Boc-Lys(2-ClZ)-OH, Boc-Arg(Tos)-OH, and Boc-Glu(OBzl)-OH, respectively. After the completion of coupling, as judged by the ninhydrin test, the four portions were combined and washed with DMF, methanol, and CH_2Cl_2. Boc-Phe-OH and Boc-Tyr(Bzl)ψ[CH_2S]Gly-OH were then successively coupled to the resin using the protocol described above. Following drying, the cream-colored peptide resin was weighed and yielded 258 mg of the desired peptide resin product, Boc-Tyr(Bzl)ψ[CH_2S]Gly-Phe-Xxx-Leu-Merrifield resin.

Next, 258 mg peptide resin was treated with 5 mL of 90% anhydrous HF/5% anisole/5% $CH_3CH_2SCH_3$ in a Kel-F HF apparatus (Peptide Institute, Osaka, Japan) at 0 °C for 60 min. The HF was evaporated and the resin residue was washed with ethyl ether three times, followed by extraction with 50% aqueous acetic acid. After lyophilization, 97 mg of crude product was obtained. AAA: Glu, 0.22 (0.25); Arg, 0.18 (0.25); Ala, 0.22 (0.25); Lys, 0.20 (0.25); Leu, 1.00 (1.00); Phe, 1.00 (1.00); H-Tyrψ[CH_2S]Gly-OH, nd (1.00). RP-HPLC characterization of the crude product revealed two sets of peaks. The first set of peaks consists of three major peaks at RT = 14.21 min, 14.65 min, and 16.91 min. These were shown by their relative retention times and their molecular weights (MS) to be the desired pseudopeptides, H-Tyrψ[CH_2S]Gly-Phe-Xxx-Leu-OH, where Xxx = Arg, Lys, and Ala, Glu (co-eluting), respectively. ES-MS: $[M+H]^+$: Xxx = Lys, 630.8 (found: 630.9); Xxx = Ala, 573.7 (found: 573.3); $[M-H]^-$; Xxx =Arg, 656.8 (found: 656.7); Xxx = Glu, 629.7 (found: 629.0). Similarly, a minor second set of products consisted of three major peaks at RT = 21.78, 22.13, and 24.67 min. These later eluting, hydrophobic peaks, were identified by ES-MS as the tyrosyl side chain modified benzyl-substituted side products with an increased mass of 90 daltons in each case, relative to the major product components. ES-MS: $[M+H]^+$: Xxx = Lys, 720.8 (found: 721.0); Xxx = Ala, 663.7 (found: 663.4); $[M-H]^-$; Xxx = Arg, 746.8 (found: 746.8); Xxx = Glu, 719.7 (found: 719.2).

N^{α}-(t-butyloxycarbonyl)-γ-(benzylester)-L-glutamic acid-β-fluorenylmethyl ester (8)

To a solution of 3.37 g Boc-Glu(OBzl)-OH (10 mmol) and 2.16 g HOFm (11 mmol) in EtOAc was added 0.81 mL pyridine (10 mmol) and 2.62 g Boc_2O (12 mmol). The reaction mixture was stirred at RT for 20 h. The EtOAc layer was washed with water, 1 N HCl solution, water, 5% $NaHCO_3$, saturated NaCl solution, and dried with $MgSO_4$. Evaporation of EtOAc under reduced pressure afforded an oily residue. The residue was dissolved in MeOH, stored at –15 °C, and yielded 3.5 g (67.9%) white pure product. mp 93–95 °C; ^{1}H–NMR (500 MHz, $CDCl_3$) δ 1.41 (s, 9H, Boc); 1.84 (m, 1H, β-H); 2.10 (m, 1H, β-H); 2.31, 2.35 (m, 2H, γ-CH_2); 4.19 (t, 1H, CH of Fm); 4.36 (m, 1H, α-H); 4.44–4.48 (m, 2H, CH_2 of Fm); 5.05 (d, 1H, CONH); 5.10 (s, 2H, CH_2 of Bzl); 7.24–7.73 (m, 13H, aromatic H of Fm and Bzl).

N^{α}-(t-butyloxycarbonyl)-L-glutamic acid-α-fluorenylmethyl ester (9)

A spatula of Pd/C was added to a solution of 5 g (8) in 160 mL MeOH and 27 mL HOAc. The solution was flushed with nitrogen and hydrogen successively, and stirred at RT for 5 h. The reaction mixture was filtered and washed on a fritted funnel. The filtrates were combined and evaporated to an oily residue. The residue was extracted with EtOAc. The combined organic extracts were washed with saturated NaCl solution, and dried with $MgSO_4$. Evaporation of EtOAc afforded 2.18 g crude product. The crude product was further purified using flash chromatography to afford 1.48 g (51.4%) pure white solid. mp: 67–70 °C; R_f(C) = 0.38; ^{1}H–NMR (500 MHz, $CDCl_3$) δ 1.42 (s, 9H, Boc); 1.81 (m, 1H, β-H); 2.08 (m, 1H, β-H); 2.35 (m, 2H, γ-CH_2); 4.20 (t, 1H, CH of Fm); 4.38 (m, 1H, α-H); 4.46–4.52 (m, 2H, CH_2 of Fm); 5.08 (d, 1H, CONH); 7.28–7.75 (m, 8H, aromatic H of Fm).

Anchoring of (9) to hydroxymethyl resin (10)

To a solution of 1.28 g (9) (3.0 mmol) and 418.5 mg HOBt (3.1 mmol) in THF was added 640.8 mg DCC (3.1 mmol) in THF. The solution was stirred at RT for 15 min, followed by the addition of 2 g hydroxymethyl resin (0.75 mmol/g) and 66.2 mg DMAP to the solution. The reaction proceeded at RT for 12 h. The resin was filtered, washed with THF, EtOH, CH_2Cl_2, and dried *in vacuo*. The loading level of resin (10) was determined as follows: 6.6 mg resin sample was treated with 3 mL 20% piperidine/DMF solution for 20 min. After allowing the resin to settle, 0.4 mL of the supernatant was withdrawn and diluted to 4 mL with MeOH and UV absorbance determined to be 0.560 when recorded at 301 nm. The substitution level was

determined to be 0.35 mmol/g, based on a value of 7200 mol^{-1} dm^3 cm^{-1} as the extinction coefficient for the dibenzofulvene-piperidine adduct.

N^{α}-(t-butyloxycarbonyl)-L-aspartic α-fluorenylmethyl ester (11)

DCC (6.2 g, 30.0 mmol) was added portionwise to a stirred solution of N^{α}-(*t*-butyloxycarbonyl)-L-aspartic acid (7.0 g, 30.0 mmol) in 50 mL ethyl acetate at 0 °C. The reaction was kept at 0 °C for 1 h and 1 h at room temperature. The solution was filtered and the solvent evaporated to dryness to obtain N^{α}-(*t*-butyloxycarbonyl)-L-aspartic anhydride. The latter was dissolved in 50 mL of dry THF with 6.2 g (30.0 mmol) of 9-fluorenylmethanol. Next, 5.2 mL (30 mmol) of DIEA were added and the solution was stirred overnight. The solvent was evaporated and the remaining solid partitioned between AcOEt and a 5% citric acid solution. The aqueous solution was acidified to pH 2 with 4 N HCl. The organic phase was washed with a 5% citric acid solution, then water, dried ($MgSO_4$), and concentrated until the appearance of the first crystals. The product (11) was crystallized from ethyl acetate/hexane, filtered, washed with hexane, and dried *in vacuo*. Yield 7.3 g (63%). TLC R_f (C); 0.63; mp 156 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.50 (s, 9H, Me_3C), 2.95 (ddd, 2H, C*H*$_2$COOH), 4.24 (t, 1H, COOCH$_2$C*H*), 4.50 (multiplet, 2H, COOC*H*$_2$CH), 4.68 (t, 1H, NHC*H*), 5.55 (d, 1H, NH), 7.30–7.79 (cluster, 8H, aromatic H of Fm).

Anchorage of (11) to hydroxymethyl resin (12)

The protocol for attachment of (11) to hydroxymethyl resin is identical to the synthesis of the resin (10). Substitution yield 0.53 mmol/g.

Anchorage of (11) to para-methylbenzhydrylamine resin (13)

p-Methylbenzhydrylamine-resin hydrochloride (3.1 g, 0.7 meq/g) was placed in a reaction vessel and successively washed with DCM (2 × 2 min × 10 mL), DIEA-DCM (1:9) (2 × 2 min × 10 mL), DCM (2 × 2 min × 10 mL), and DMF (2 × 2 min × 10 mL). A solution of (11) (1.8 g, 4.4 mmol), BOP (2.0 g, 4.5 mmol), HOBt (0.6 g, 4.4 mmol), and DIEA (0.8 mL, 4.6 mmol) in DMF was added, and the reactor shaken for 2 h. The resin was washed again with DMF (2 × 2 min × 10 mL) and capped with a solution composed of acetic anhydride (1.9 mL, 20.4 mmol), pyridine (1.6 mL, 20.4 mmol) in DCM for 2 h. The resin was washed again with DMF, DCM, and dried *in vacuo*. Yield 3.75 g. Substitution yield 0.47 mmol/g.

General procedure for the synthesis of libraries L1–L4

The cyclic pseudohexapeptide libraries were synthesized analogously to the synthesis of L7. However Boc-Proψ[CH$_2$S]Gly-OH (6) was utilized instead of Boc-Tyr(Bzl)ψ[CH$_2$S]Gly-OH (5).

General procedure for the synthesis of libraries L5–L34

L5–L19 were synthesized on 100 mg of resin (12) (0.062 mmol of aspartic acid) in each reactor. L20–L34 were synthesized in an identical fashion using100 mg (0.070 mmol of asparagine) of resin (13).

Peptide chain elongation

The resin was washed with DCM (3 × 2 min × 1 mL/syringe) and shaken with 50% TFA in DCM (5 min × 1 mL/syringe). The TFA treatment was repeated for 25 min more, the resin washed as before, neutralized with DIEA-DCM (1:9) (2 min × 1 mL/syringe), and washed again with DCM. For each coupling, the protected amino acids were individually weighed, combined, and divided into individual syringes according to the amino acid ratios shown in Table 3. For each syringe, the proportionate values were Boc-L-Leu (9.9 mg, 0.039 mmol), Boc-L-Lys(2-Cl-Z) (26.3 mg, 0.063 mmol), Boc-L-Ser(Bzl) (12.5 mg, 0.042 mmol), Boc-L-Glu(OBzl) (14.2 mg, 0.042 mmol), Boc-L-Arg(Tos) (27.1 mg, 0.063 mmol), Boc-L-Tyr(2,6-Cl-Bzl) (21.8 mg, 0.050 mmol). Coupling reagent amounts used were BOP (132.7 mg, 0.30 mmol), HOBt (40.5 mg, 0.30 mmol), dissolved with the amino acids, and DIEA in 1 mL of DMF per syringe. Each mL of amino acid solution also contained 104 μL of DIEA (0.596 mmol). The mixture was stirred for 1 min prior to transferral to individual syringes. The coupling of the single protected δ-amino acid (pseudodipeptide) was achieved with a four-fold excess of Boc-Proψ[CH$_2$S]Gly (82.4 mg, 0.30 mmol), BOP (132.7 mg, 0.30 mmol), HOBt (40.5 mg, 0.30 mmol), and DIEA 104 μL (0.596 mmol) in 1 mL of DMF. The block was shaken for 1 h and the completion of the

couplings was monitored by testing of four randomly chosen syringes using the Kaiser ninhydrin test.

Cyclization

The resin in each syringe was washed with DCM, shaken for 20 min with 1 mL 20% piperidine in DMF, filtered, and washed again with DCM. The Boc group was cleaved with TFA and the resin neutralized as described above. Next the condensing agents were preactivated as follows: HATU (3.42 g, 9.0 mmol), HOBt (1.22 g, 9.0 mmol), and DIEA (3.12 mL, 17.9 mmol) in 30 mL of DMF. The mixture was stirred for 1 min, distributed to the 30 syringes, and the synthesis block shaken for 2 h. The resin was washed with DCM and dried *in vacuo* overnight.

Cleavage and purification

The cleavages were achieved in a 24-chamber multiple HF apparatus. L5–L19 and L20–L34 were cleaved separately. For each syringe, the resin was placed in a cleavage vessel with 200 μL of anisole and 5 mL of anhydrous HF. The mixture was stirred at 0 °C for 1 h before removal of HF. For each reaction vessel, the resin was transferred to a fritted glass funnel, and the peptide products extracted with 50 mL of 30% acetic acid each. The acetic acid solution was washed twice with 25 mL diethyl ether and lyophilized to obtain salty flakes. The peptide libraries were obtained after solid phase extraction on reversed-phase Varian Bond Elut C18: the remaining salts were washed out with water and the peptides were extracted with 50 mL of acetonitrile-water (1:1) and lyophilized. The amino acid compositions of mixtures L8, L11, L14, L21, L24 and L30 were checked by amino acid analysis; results are reproduced in Table 2.

Synthesis of L35

To a solution of 2.9 mg of L4 in 300 μL of acetic acid, 10 μL of 30% hydrogen peroxide was added and the mixture stirred for 1 h. The solution was diluted in 2 mL of water and lyophilized to afford 2.8 mg of product. The isomers were not separated. The mixture was subjected to mass spectral analysis to establish the presence of a new set of compounds, virtually identical in profile, but with an increased mass of 16 Da.

Results and Discussion

Rationale for selection of targets

As one of several commonly used amide bond surrogates, the $\psi[CH_2S]$ surrogate is characterized by its flexibility, enzyme resistance, and lipophilicity. Its introduction within a peptide sequence can be achieved using either a resin-bound nucleophilic displacement approach [18] or a segment condensation approach in which a pseudodipeptide is prepared in solution before it is condensed onto the growing peptide chain on the resin [19]. The resin bound approach provides a fast and convenient method for $\psi[CH_2S]$ formation, but our previous studies on the synthesis of a thioether macrocycle indicated that a considerably more heterogeneous product was obtained from the resin bound approach than that obtained using a segment condensation strategy [18]. Most of the side products generated in the resin bound approach were presumably truncated peptide sequences, due to the difficulty of achieving nearly 100% coupling efficiency between the thiol component and the resin bound bromo-substituted peptide. In contrast, a preformed $\psi[CH_2S]$-containing pseudodipeptide could be synthesized in solution and easily coupled to the peptide resin with nearly quantitative yield (>99%), thus improving the purity of the resulting pseudopeptide. Consequently, the segment condensation strategy was used exclusively in the synthesis of our $\psi[CH_2S]$-containing mixtures. Figure 1 illustrates the general procedures employed for the synthesis of preformed pseudodipeptides, Boc-AA$\psi[CH_2S]$Gly-OH, where AA = Pro or Tyr(Bzl).

Our work began with the careful examination of small libraries containing four linear *pseudopentapeptides* (this nomenclature emphasizes that the surrogates are dipeptide mimics, even though the actual synthesis is made up of only four 'amino acid' building blocks.) Further development led to the elaboration of cyclic pseudohexapeptide libraries L1–L4, and eventually to the synthesis of cyclic pseudohexa-, hepta-, and octapeptide libraries L5–L34 (Table 1). Cyclic peptides were chosen as targets for two main reasons, the first being resistance to exopeptidases, and the second being that cyclization can reduce the conformational flexibility of linear peptides, thus making the peptide more stable. Often this can lead to more selective binding to receptors. Similar approaches have led to the discovery of significant leads

Table 1. Structure of pseudopeptide libraries L1–L35

Code	# cpds	Structure
L1	343	cyclo(Yyy-Yyy-Yyy-Proψ[CH_2S]Gly-Glu)
L2	343	cyclo(Yyy-Yyy-Proψ[CH_2S]Gly-Yyy-Glu)
L3	343	cyclo(Yyy-Proψ[CH_2S]Gly-Yyy-Yyy-Glu)
L4	343	cyclo(Proψ[CH_2S]Gly-Yyy-Yyy-Yyy-Glu)
L35	686	cyclo(Proψ[CH_2SO]Gly-Yyy-Yyy-Yyy-Glu)

Code for Asx =			
Asp	Asn	# cpds	Structure
L5	L20	216	cyclo(Xxx-Xxx-Xxx-Proψ[CH_2S]Gly-Asx)
L6	L21	216	cyclo(Xxx-Xxx-Proψ[CH_2S]Gly-Xxx-Asx)
L7	L22	216	cyclo(Xxx-Proψ[CH_2S]Gly-Xxx-Xxx-Asx)
L8	L23	216	cyclo(Proψ[CH_2S]Gly-Xxx-Xxx-Xxx-Asx)
L9	L24	1296	cyclo(Xxx-Xxx-Xxx-Xxx-Proψ[CH_2S]Gly-Asx)
L10	L25	1296	cyclo(Xxx-Xxx-Xxx-Proψ[CH_2S]Gly-Xxx-Asx)
L11	L26	1296	cyclo(Xxx-Xxx-Proψ[CH_2S]Gly-Xxx-Xxx-Asx)
L12	L27	1296	cyclo(Xxx-Proψ[CH_2S]Gly-Xxx-Xxx-Xxx-Asx)
L13	L28	1296	cyclo(Proψ[CH_2S]Gly-Xxx-Xxx-Xxx-Xxx-Asx)
L14	L29	7776	cyclo(Xxx-Xxx-Xxx-Xxx-Xxx-Proψ[CH_2S]Gly-Asx)
L15	L30	7776	cyclo(Xxx-Xxx-Xxx-Xxx-Proψ[CH_2S]Gly-Xxx-Asx)
L16	L31	7776	cyclo(Xxx-Xxx-Xxx-Proψ[CH_2S]Gly-Xxx-Xxx-Asx)
L17	L32	7776	cyclo(Xxx-Xxx-Proψ[CH_2S]Gly-Xxx-Xxx-Xxx-Asx)
L18	L33	7776	cyclo(Xxx-Proψ[CH_2S]Gly-Xxx-Xxx-Xxx-Xxx-Asx)
L19	L34	7776	cyclo(Proψ[CH_2S]Gly-Xxx-Xxx-Xxx-Xxx-Xxx-Asx)

Xxx is a nearly equimolar mixture of lysine, leucine, arginine, glutamic acid, serine, and tyrosine.
Yyy contains the same amino acids as Xxx plus alanine (seven total or 343 (7^3 per mixture).

for either disulfide [20] or amide-based [21,22] cyclic peptide analogs.

Each library was differentiated by the ring size, the type of derivatized resin used, and the position of the pseudodipeptide in the peptide sequence. Three types of resin were utilized with side chain attachment of glutamic acid L1–L4, aspartic acid L5–L19 and asparagine L20–L34. On-resin cyclization was achieved after completion of each linear sequence. The ring size was kept small (below 8 residues) to prevent the involvement of the linear peptide in unproductive secondary structure, and to afford efficient cyclization. We refer to our approach consisting in the migration of a pseudodipeptide unit along the peptide chain as the 'psi-scan' method. By testing various pseudopeptide groupings for their requisite activities, this permits the determination of the best position in the ring for the peptide bond surrogate as well as effecting the separation of a large library into well defined structural groups. In the synthesis of L5–L34, a mixture of 6 amino acids was used in the couplings with protecting groups as follows: Boc-Tyr(2,6-di-Cl-Bzl), Boc-Arg(Tos), Boc-Leu, Boc-Lys(2-Cl-Z), Boc-Glu(OChx), and Boc-Ser(Bzl). These six residues were chosen to reflect the structural diversity of the 20 naturally occurring amino acids based on standard Hansch criteria (steric, electronic, and hydrophobic factors) [23]. Boc-Ala was added to the 6 previously cited amino acids in the elaboration of L1–L4. We also wished to keep the number of analogs per vial in a moderate range as a compromise between single compound assays [24] and the diversity possible in much larger (20^n or 60^n) amino acid libraries [25].

Synthesis of linear peptides containing the ψ[CH_2S] surrogate: Tyr(Bzl)ψ[CH_2S]Gly

In order to identify any possible side products generated during the synthesis and to have better control over the quality of the ψ[CH_2S]-containing cyclic pseudopeptide libraries, we adopted a systematic ap-

proach for the construction of pseudopeptide libraries. Initially, a small linear pseudopeptide mixture, H-Tyrψ[CH_2S]Gly-Phe-Xxx-Leu-OH, where the Xxx position was varied as lysine, alanine, arginine, and glutamic acid, was synthesized to serve as the test case for validation purposes. The synthesis began with standard Boc-Leu-Merrifield resin. After acidic deprotection of the Boc-protecting group, the resin was divided into four equal portions, and each portion was coupled to one of four amino acids (Lys, Arg, Ala, and Glu), individually. The four resin portions were then combined according to Furka's 'split and join' technique, respectively [17], and the desired pseudopeptide mixture was assembled by successively coupling Boc-D-Phe-OH and a preformed pseudodipeptide, Boc-Tyrψ[CH_2S]Gly-OH, to the resin. The resulting linear pseudopeptide mixture was finally released from the resin using anhydrous HF [26] and the mixture components characterized by RP-HPLC (uncorrected for extinction coefficient variations). The first three (major) peaks appeared at retention times of 14.21, 14.65, and 16.91 (two components), respectively and their masses, $[M\text{-}H]^-$, 656.7, 629.0, and $[M+H]^+$ 573.3, 630.8 correspond to the theoretical values for linear pseudopeptides consisting of Arg, Glu, Ala, and Lys in the general structure, Tyrψ[CH_2S]Gly-Phe-Xxx-Leu. Amino acid analysis was consistent with the above assignments.

As seen in Figure 2a, a nearly identical pattern of later eluting peaks appeared at 21.78, 22.13, and 24.67 min and each of these peaks showed molecular masses of 90 units higher than their major counterparts, consistent with their being benzyl-substituted analogs. The proton NMR pattern of the mixture (data not shown) was also consistent with a modified tyrosine residue, as expected for an alkylation product. Since the UV absorbance was carried out at 214 nm (uncorrected for extinction coefficient variations), the semi-quantitative estimation of 30% as representing their bulk presence appears reasonably valid.

This type of side reaction was first systematically studied by Erickson and Merrifield [27], and is typically overcome by the use of more hindered, albeit more expensive, side chain protecting groups. Interestingly, this side reaction was also observed in our early studies on the synthesis of ψ[CH_2NH]-containing pseudopeptide mixtures, but only to a degree of about 10% [13]. Careful comparison of the RP-HPLC profiles between the ψ[CH_2S]-containing mixture and the ψ[CH_2NH]-containing mixture (Figure 2b) suggested that the ψ[CH_2S] surrogate may play a role in the modification of the tyrosine side chain during the strong acid HF cleavage, even though the ψ[CH_2S]-containing pseudopeptides were different from those of the ψ[CH_2NH]-containing pseudopeptide sequences. A possible explanation is that the ψ[CH_2S] surrogate might be attacked by the benzyl cation to form an S-benzyl sulfonium intermediate during the acidic cleavage. If such an intermediate were operative, it is evident that the thioalkylation is not permanent, as evidenced by the generation of ψ[CH_2SO]-containing tyrosine side chain modified pseudopeptides upon the oxidation of crude pseudopeptide mixtures with hydrogen peroxide. This is in contrast to what has been observed by Ramaligan et al. [28] for the S-alkylation of methionine side chains by benzyl cation (S-alkylation) during the synthesis of phosphotyrosine-containing peptides using Boc-Tyr[$PO_3(Bzl)_2$].

This initial result convinced us that ψ[CH_2S]-containing pseudopeptide mixtures could be readily accessed using a combination of solution phase and solid phase synthesis methods. In addition, it is likely that the quality of the pseudopeptide mixture could be improved by the careful selection of side chain protecting groups for tyrosine residues during the synthesis. For example, a sterically hindered 2,6-dichlorobenzyl protecting group for the tyrosine side chain hydroxyl group should minimize any tendency for side chain modification significantly [27]. Based on these promising initial results, we undertook the more ambitious task of synthesizing and characterizing a series of ψ[CH_2S]-containing cyclic pseudopeptide libraries of different ring sizes.

Synthesis of cyclic pseudohexapeptide libraries containing Proψ[CH_2S]Gly L1–L4, using glutamic acid for side chain attachment

The synthesis of ψ[CH_2S]-containing cyclic pseudopeptide mixtures featured a resin bound cyclization strategy. In this strategy, Boc-Glu-OFm was attached by its side chain to hydroxymethyl resin. The Boc-Glu-OFm derivative was prepared via two steps: synthesis of Boc-Glu(OBzl)-OFm and hydrogenation of Boc-Glu(OBzl)-OFm. The synthetic yield was 35%. The major problem with this synthetic approach is the partial premature loss of the OFm ester during hydrogenation as previously reported [29], which reduces overall yields.

In addition to the use of Boc-Glu-OFm during the synthesis, the 'multiple coupling' method instead of

'portioning-mixing' was used for the incorporation of individual amino acids at the variable positions within the peptide sequence. Although the portioning-mixing method affords a nearly equimolar formation of each pseudopeptide component in the mixture, it remains a labor intensive process. On the other hand, the multiple coupling method dramatically simplifies the whole synthetic process, making the mixture synthesis a one-pot (vessel) synthesis. During the synthesis, the appropriate relative concentrations of each amino acid (Leu, Arg, Tyr, Ser, Lys, Ala, and Glu) in the mixture were modulated according to Geysen et al. [30] to ensure an approximately equal coupling proportion for each amino acid.

The quality of the library was evaluated prior to cleavage by amino acid analysis of the on-resin peptide products (Table 2). The AAA results appear to be in good agreement with the theoretical values for each amino acid of the mixture, although the values for glutamic acid and serine are higher than expected. While the library composition appeared acceptable using this criteria, additional characterization was achieved using electrospray mass spectrometry.

A cyclic peptide library in which p positions are coupled with a mixture of n amino acids contains n^p different compounds among which only (n+p–1)!/p!(n–1)! masses are unique because of redundancy. Indeed when one or more positions are fixed, the number of compounds are a simple multiple of the variable positions, as with linear peptide libraries. Nevertheless, many different sequences possess common molecular weights. For example, our pseudohexapeptide libraries contained 343 compounds with only 84 different molecular weights; thus, for purposes of partial characterization of mixtures, mass spectroscopy is complementary to amino acid analysis. Since many cyclic pseudopeptides in the mixture differ only slightly in their molecular masses (one to several mass units), we would expect a fairly symmetric distribution of molecular weights for mixture L4. Further expansion of the molecular weight region (molecular weight envelope) led to the unambiguous assignment of molecular weights for many expected cyclic pseudohexapeptides (Figure 3). Because some of the components of the library contained only a positively charged residue (Arg, Lys) while others only a negatively charged residue (Asp, Glu), it was necessary to run all samples in both negative and positive modes to visualize the maximum number of components. Again, due to the small molecular weight differences between pseudopeptides, the molecular weight peaks in the mass spectrum were broad, and not all molecular masses were clearly revealed in the spectrum. Electrospray mass spectrometry is not as sensitive for compounds without acidic or basic sites, as it is for compounds with easily charged sites. Nevertheless, the overall results from ES-MS strongly suggested that most of the desired products, if not all of them, were present in the mixture. Moreover, very few side product peaks were observed in the spectrum, suggesting the success of the whole synthesis. This conclusion was further supported by the amino acid analysis results of mixture L4, which gave acceptable ratios for each of the amino acids in the mixture. The overall amino acid analysis results also support the approximate equal incorporation of each amino acid at the variable positions within the peptide sequence using the multiple coupling method [30].

The $\psi[CH_2SO]$ (R and S)-containing linear and cyclic pseudohexapeptide mixtures were easily generated by oxidizing $\psi[CH_2S]$-containing mixtures using aqueous acidic hydrogen peroxide. In a validation study, a linear mixture of 4 pseudopeptides was oxidized by H_2O_2 in acetic acid at room temperature. The oxidation was monitored by HPLC, and found to be complete in less than 60 min. The identities of the resulting $\psi[CH_2SO]$ (R and S)-containing pseudopeptides were confirmed by ES-MS. Based on this initial study, the 343-component mixture L4 was subsequently oxidized to generate the corresponding $\psi[CH_2SO]$ (R and S)-containing mixture L35.

The latter was thoroughly characterized by ES-MS. A direct comparison of the mass spectra between mixtures L4 ($\psi[CH_2S]$) and L35 ($\psi[CH_2SO]$) clearly reveals a 16 mass unit difference between two mixtures (Figures 4a, b). This analysis also provides confidence that there are no significant product components lacking the thiomethylene ether surrogate in the parent mixture.

In conclusion, reasonably clean $\psi[CH_2S]$ and $\psi[CH_2SO]$ (R and S)-containing pseudopeptide mixtures can be prepared using a combination of solution phase and solid phase synthesis techniques. Cyclic mixtures were effectively synthesized using the Boc-Glu-OFm resin-bound cyclization strategy and the so-called 'multiple coupling' method.

Table 2. Amino acid analysis L1, L2, L3, and L4. Samples were hydrolyzed at 110 °C for 24 h using 6 N HCl

	L1		L2		L3		L4	
	calc.	found	calc.	found	calc.	found	calc.	found
Glu	1.43	1.82	1.43	1.73	1.43	1.75	1.43	1.62
Leu	0.43	0.40	0.43	0.38	0.43	0.39	0.43	0.35
Lys	0.43	0.44	0.43	0.42	0.43	0.40	0.43	0.36
Arg	0.43	0.43	0.43	0.49	0.43	0.46	0.43	0.44
Ser	0.43	0.58	0.43	0.61	0.43	0.56	0.43	0.53
Ala	0.43	0.43	0.43	0.43	0.43	0.42	0.43	0.44
Tyr	0.43	0.41	0.43	0.40	0.43	0.43	0.43	0.43

Synthesis of cyclic pseudohexa-, hepta-, and octapeptide libraries containing Proψ[CH_2S]Gly L5–L34 with Asp and Asn side chain attachment

The resin preparation consisted in the side chain attachment of Boc-L-Asp-OFm to the solid support, a method developed by Rovero and colleagues [9]. Two types of resin were synthesized by attaching Boc-L-Asp-OFm [31] to either a *para*-hydroxymethyl polystyrene solid support leading to aspartic acid resin or to a *para*-methylbenzhydrylamine polystyrene resin to obtain an asparagine resin. The substitution yields were evaluated by UV quantification of fluorenylmethanol on a known amount of resin [32] and found to be 0.53 mmol/g for asparagine resin (12) and 0.47 mmol/g for asparagine resin (13). Two libraries were generated in parallel fashion using these two resins: L5–L19 (Table 1) represent the aspartic acid based libraries and L20–L34 (Table 1) reflect their asparagine counterparts.

The synthesis of the libraries was performed in a synthesis block consisting of 42 polypropylene syringes equipped with a plastic frit [33]. Each of the syringes was loaded with 100 mg of amino acid substituted resin and acted as an individual reaction vessel. Boc chemistry was used to elongate the peptide and included 50% trifluoroacetic acid cleavage and a phosphonium-based condensing agent [34] BOP/HOBt. The resin was kept in suspension through agitation of the synthesis block with a rotary shaker. Completion of the couplings was monitored by the Kaiser ninhydrin test [35] on 4 syringes chosen at random at each step. In the event of a positive (blue) color, the couplings for all vials were repeated.

A mixture of the 6 previously cited amino acids (Leu, Arg, Tyr, Ser, Lys, and Glu) was used in each of the variable position couplings. As described earlier, attachments were achieved using a four-fold excess of amino acids in ratios determined by Geysen [30] in such a way that nearly equivalent amounts of each single amino acid are produced in the products after coupling.

After completion of the synthesis of the linear peptides, the N-terminal Boc protecting group was cleaved using trifluoroacetic acid, and the C-terminal fluorenylmethyl ester protecting function was removed with 20% piperidine in DMF. On-resin cyclizations were carried out using HATU/HOBt [36] for 2 h. A much faster cyclization kinetic was afforded when using the uronium-based coupling reagent HATU instead of BOP. Cleavage and side chain deprotection were achieved with a 24-chamber HF apparatus [37] using anisole as a scavenger. After removal of HF, the resin was transferred to a fritted funnel, triturated with diethyl ether for removal of remaining scavengers, and the peptide-product extracted with a 30% acetic acid solution. Salt-free peptides were afforded after reversed solid phase extraction using Varian Bond Elut C18. The products were individually lyophilized and obtained with an average percent yield of 50%.

The mixtures were characterized by amino acid analysis (Table 3). Even though serine and tyrosine were damaged in the hydrolysis process, the experimental results were consistent with the theoretical values for the other amino acids (deviations remained below 10%, as commonly observed). Further analysis was carried out using ES-MS (Figures 5a, b) and the spectra appeared to contain all of the expected compounds, with allowances for ion-suppression effects and peak overlap.

Table 3. Amino acid analysis of randomly selected libraries: L8, L11, L14, L21, L24 and L30. Samples were hydrolyzed at 110 °C for 24 h using 6 N HCl

	L8		L11		L14		L21		L24		L30	
	calc.	found	calc.	found	calc.	found	calc.	found	calc.	found	calc.	found
Asp/Asn	1	1.25	1	1.12	1	1.23	1	1.08	1	1.17	1	1.08
Leu	0.50	0.45	0.66	0.67	0.83	0.79	0.50	0.46	0.66	0.59	0.83	0.83
Lys	0.50	0.52	0.66	0.71	0.83	0.83	0.50	0.51	0.66	0.64	0.83	0.78
Glu	0.50	0.49	0.66	0.61	0.83	0.87	0.50	0.53	0.66	0.70	0.83	0.81
Arg	0.50	0.50	0.66	0.61	0.83	0.80	0.50	0.48	0.66	0.65	0.83	0.82
Ser	0.50	0.46	0.66	0.62	0.83	0.76	0.50	0.46	0.66	0.64	0.83	0.82
Tyr	0.50	0.44	0.66	0.61	0.83	0.81	0.50	0.46	0.66	0.62	0.83	0.79

Biological assays

The $\psi[CH_2S]$ pseudopeptide libraries L5–L34, which incorporated the Pro$\psi[CH_2S]$Gly 'psi-scan' motif, were screened in more than 60 biological assays. All mixtures were tested for their *in vitro* anti-HIV activity by the National Cancer Institute (NCI). The compounds were tested using an 8-point assay with concentrations varying from 0.003 μg/mL to 10 μg/mL. Activities were measured after a 6-day incubation period at 37 °C. Thus far none of the tested libraries have provided a positive hit in this system.

Conclusions

The approach undertaken for the synthesis of cyclic pseudopeptides featuring side chain attachment of the first amino acid to the resin, utilization of ratios of amino acids for coupling of mixtures, on-resin cyclization, and utilization of a preformed pseudodipeptide for the introduction of the peptide bond surrogate has been validated by the MS studies, as well as by the amino acid analysis data. Our results confirm that linear and undeprotected compounds, dimers, as well as deletion peptides remain low. While MS can be a very effective tool to study mixtures, it does show some weaknesses due to the intrinsic nature of the products being analyzed. These include:

– Redundant peptides are indistinguishable.
– The peak distribution does not reflect the isobaric composition of the mixture, due to ion suppression effects. These effects are more pronounced for electrospray mass spectrometry of small peptides, where many contain neither basic nor acidic residues. For larger peptides, electrospray can show a variation in the number of charges, causing a different type of complication.
– Low resolution mass spectrometry cannot readily distinguish peptides of similar molecular weight.

Nevertheless, correlations between the scope and diversity of a library, as manifested by the lower and upper molecular weight limits, the qualitative distribution of molecular weights (assisted by the 'quantized' nature of MW distribution), the absence of significant lower molecular weight fractions, and the lack of significant products in the dimer region, combine to validate the quality of the synthesis. AAA can also be a reliable correlate, especially when proper ratios must correspond to a larger dynamic range due to the appearance of fixed amino acids such as Asp or Asn in our samples. Finally, the oxidation of $\psi[CH_2S]$ to $\psi[CH_2SO]$, which generates '(pseudopeptide) libraries from (pseudopeptide) libraries' and corresponds to a net addition of 16 daltons to each member of the precursor library, also adds greatly to the confidence of the synthetic process. Because this modification does not dramatically change the acidity or basicity of the peptide, the ESI spectrum should only shift in mass; the relative intensity of the MS peaks would not be expected to change as they might for a modification adding a charged site: e.g., phosphorylation. Coupled with our previous systematic studies for the racemization/epimerization problem [10], we believe our current products have been produced in their theoretical distributions with a reasonably high level of confidence, especially considering the complex nature of pseudopeptide synthesis in general and cyclic pseudopeptides in particular.

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