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Design of Peptidomimetic Ligands for the pp60^{src} SH2 Domain

Mark S. Plummer,[a,*] Elizabeth A. Lunney,[a] Kimberly S. Para,[a] Aurash Shahripour,[a] Charles J. Stankovic,[a] Christine Humblet,[a] James H. Fergus,[b] James S. Marks,[b] Roman Herrera,[c] Susan Hubbell,[c] Alan Saltiel[c] and Tomi K. Sawyer[a]

[a]*Departments of Chemistry,* [b]*Biochemistry and* [c]*Signal Transduction, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, MI 48106, USA*

Abstract—Potent ligands of the Src SH2 domain, discovered through structure-based drug design efforts, with the general structure Ac-pTyr-Glu-NRR′ are disclosed. Copyright © 1997 Elsevier Science Ltd

Introduction

The tyrosine kinase activity of growth factor receptors is necessary for growth factor signalling. Upon growth factor binding, receptor dimerization is induced resulting in tyrosine phosphorylation of the cytoplasmic portion of the receptor by its tyrosine kinase. These phosphorylated tyrosine (pTyr) residues serve as binding sites for the SH2 domains of non-receptor tyrosine kinases such as pp60$^{c\text{-}src}$, and other proteins, which in turn mediate downstream intracellular signal transduction.[1] The Src SH2 domain binds not only to pTyr containing proteins such as middle T antigen,[2] PDGF receptor,[3] EGF receptor,[4] and focal adhesion kinase,[5] but also to pTyr containing peptides in a sequence-dependent manner.[6] Generally, for SH2 ligands, the three residues in a peptide or protein immediately C-terminal to the pTyr residue confer binding specificity. The structure of the v-Src SH2 domain complexed with a high affinity phosphopeptide, which contains a Src SH2 specific peptide sequence Glu - Pro - Gln - pTyr - Glu - Glu - Ile - Pro - Ile - Tyr - Leu, $IC_{50}=0.9\ \mu M$, has been determined by X-ray crystallography to 2.7 Å resolution.[7] Recently, structural information has been disclosed which employs solution NMR to explore a pentapeptide (Ac-pTyr-Glu-Glu-Ile-Glu) complexed with the Src SH2 domain.[8] These studies have shown specific interactions between the Src SH2 domain and the common P−P+3 amino acid residues of the aforementioned peptides.

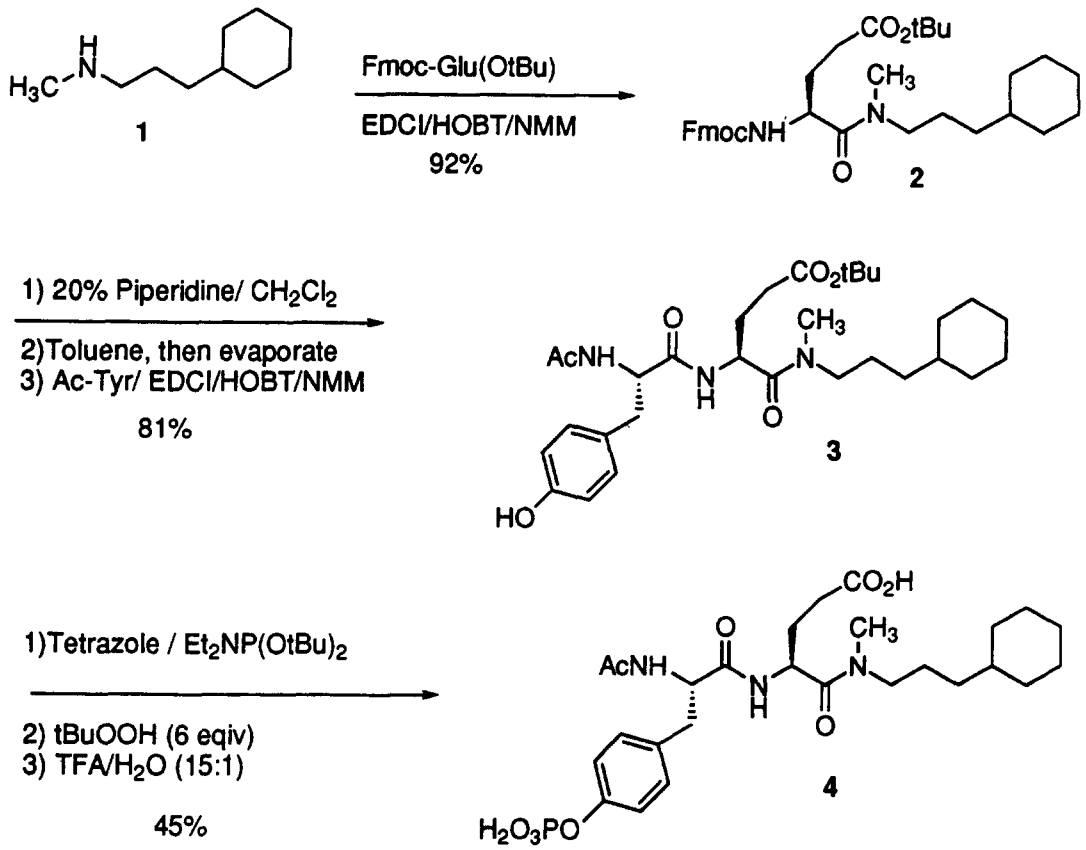
The ubiquitous importance of SH2 domains for intracellular signal transduction, combined with the available 3D structural information prompted us to design and synthesize specific ligands for the Src SH2 domain. Such ligands hold great potential for delineating signaling pathways, and development of rationally designed chemotherapeutic agents which disrupt signal transduction pathways. Disruption of such a pathway by employing peptides or peptidomimetics containing PTPase resistant pTyr analogues has already been demonstrated in the case of ZAP-70 and the T-cell antigen receptor.[9] Recent reports disclosing an extensive structure–activity relationships of pentapeptide ligands[10] have appeared as well as a more recent report of tripeptide ligands of the Src SH2 domain.[11] We have previously disclosed our success in designing tripeptide ligands[12] and report here the discovery of potent and selective dipeptide ligands of the Src SH2 domain.

Chemistry

The synthesis of one dipeptide ligand will be illustrated (Scheme 1) and is representative. The protected amino acid Fmoc-Glu(OtBu) was coupled with *N*-methyl-3-cyclohexylpropyl amine (**1**) providing **2**. The Fmoc group was removed employing 20% piperidine in dichloromethane. After 30 min, toluene was added to double the volume and the reaction was evaporated to dryness providing the amine. The amine was immediately coupled with *N*-acetyl tyrosine giving the dipeptide **3** in 81% yield after column chromatography. The tyrosine residue in **3** was phosphorylated by employing di-tert-butyl diethylphosphoramidite and tetrazole in dichloromethane. The phosphitylated tyrosine residue was oxidized in situ with tert-butyl hydroperoxide to the protected phosphate which was isolated. The phosphate group was deprotected with 90:9.5:0.5 trifluoroacetic acid:dichloromethane:water. The crude peptide was precipitated with dry either providing **4** in 85% purity; purification of crude **4** was the accomplished by reversed-phase HPLC.

Results

In exploring the structure–activity relationships of our previously reported tripeptides,[12] exemplified by **5** (Table 1), which employed a D-homophenylalanine amide derived hydrophobic side chain to occupy the P+3 Ile binding site in the Src SH2 domain, we discovered that the carboxamide group could be removed, as in **6**, without compromising binding affinity. This carboxamide was previously suggested to be potentially important for pre-organization of the



Scheme 1. Synthesis of dipeptide ligand **4**.

binding conformation of **5** by forming an intramolecular hydrogen bond between the carboxamide NH_2 group and the pTyr carbonyl oxygen atom. Much to our satisfaction, **7**, an analogue of **6**, also bound with good affinity, confirming that a pTyr containing dipeptide amide is a sufficient ligand for the Src SH2 domain (Table 1).

Efforts now focused on modifying the aromatic ring and constraining the alkyl linker in **6** and **7** with the goal of increasing potency. Substitution of a methyl group *para* on the phenyl group of the amide provides **8** and results in a small increase in binding affinity. Substitution with a *p*-hydroxyacetic acid group as in **9** also results in modestly increased potency (Table 1). When the hydroxyacetic acid substituent is in the *meta* position as in **10**, however, a fourfold increase in potency is observed. Computer modeling suggests that this acidic moiety is interacting with Arg-217. A similar potency enhancing interaction is suggested to occur with the P+4 Glu residue in the Glaxo pentapeptides.[10] When the acidic group in **10** is replaced by a neutral amide, **11**, diminished potency results, indicating that an ionic interaction occurs with **10**.

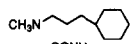
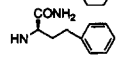
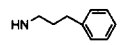
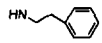
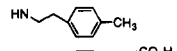
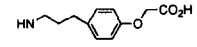
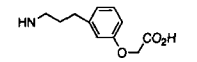
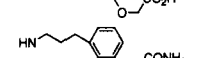
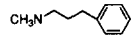
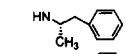
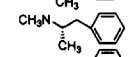
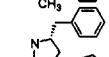
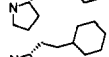
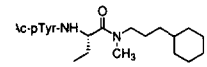
Though incorporation of an acidic unit results in increased potency, the additional charged group may be detrimental to cellular penetration, thus non-ionizable modifications were sought. The first attempt at such a modification was reducing the rotational freedom of the alkyl linker found in the C-terminal amide of **6** by judicious introduction of methyl groups. N-Methylation of the C-terminal amide in **6** provides **12**, which produced nearly a threefold increase in potency. Stereospecific incorporation of a methyl group alpha to the amino group in **7** gives **13** and also provides a threefold increase in affinity (Table 1). Noteworthy is that this methyl group effectively replaces the carboxamide unit in the tripeptide series. Combining these two potency-enhancing modifications provides ligand **14** with an IC_{50} of 2.5 μM, which suggests that these two features are not additive.

In our tripeptide series, modification of the aromatic ring, such as reducing the D-homophenylalanine amide in **5** to a D-homocyclohexylalanine amide provided about a fourfold increase in binding affinity.[12] Similar modification of dipeptide **12** by incorporation of a *N*-methyl-3-cyclohexylpropyl group to yield **4**, also resulted in fourfold increased potency. This suggests that the hydrophobic groups in the tripeptide and dipeptide series interact with the hydrophobic P+3 pocket in a similar manner. Thus, the P+3 binding pocket of the Src SH2 domain is capable of recognizing both aromatic (e.g. phenyl), hydrophobic cyclic alkyl groups (e.g. cyclohexyl), and branched alkyl such as the isobutyl side chain of the isoleucine.

We speculated that improved potency might be obtained by further reduction of rotational freedom in **14** by incorporating a pyrrolidine ring. Such a modification was invoked in **15** but unfortunately, binding

affinity did not improve relative to **14**. However, when the phenyl substituent was extended by one methylene group, as in **16**, potency was restored. Modification of the aromatic ring in **16** to a more hydrophobic cyclohexyl ring provides **17**, a highly potent ($IC_{50}=0.56\ \mu M$), ligand for the Src SH2 domain. By modification of the rotational freedom and hydrophobicity of the amide side chain in **6** and **7** we have been able to increase binding affinity nearly 20-fold.

Table 1. Structure–activity relationships of dipeptides

Compound number	Ac-pTyr-Glu-R_1 R_1	IC_{50} (μM)
4		0.79
5		8.5
6		10.0
7		9.8
8		6.3
9		6.4
10		2.5
11		8.1
12		3.5
13		3.4
14		2.5
15		5.4
16		1.3
17		0.56
18		5.4[a]

[a]The P+1 amino acid is L-α-amino butyric acid.

Briefly, we explored substitution of the acidic P+1 Glu residue by non-charged amino acids to discern the importance of this residue for binding and to reduce the overall charge of the ligand to −2. For example, modification of **4** by substituting the Glu residue with an α-aminobutyric acid (Abu) residue gives **18**. This change in the P+1 residue results in approximately a sevenfold reduction in potency. This same magnitude decrease in potency was observed when a similar substitution was made by Glaxo researchers in a published pentapeptide series.[10]

Dipeptide **4** was studied to determine whether compounds which do not have the pTyr-Glu-Glu-Ile sequence, preferred for binding by the Src SH2 domain, remain selective. Compound **4** was assayed for competitive binding to ^{35}S-labeled SH2 protein constructs of Src, Abl, Grb2, Syp(N), PLCγ1(C) versus the phosphorylated cytoplasmic tail of the PDGF receptor. In this assay **4** bound to Src SH2 with an IC_{50} of 9.3 μM and shows approximately a twofold preferential binding to the Src SH2 over the Abl SH2 (Table 2). Compound **4** does bind with at least 10-fold selectivity to Src versus Grb-2, Syp(N), and PLCγ1(C) SH2 domains, implying that these dipeptide ligands or other small molecules may be capable of selective binding to specific SH2 domains.

Conclusion

The pivotal importance of SH2 domains in intracellular signal transduction, coupled with available 3D structural information prompted us to design and synthesize ligands specific for the Src SH2 domain. These efforts which were based primarily on structural information derived from the co-crystallographic X-ray structure[7] of the Src SH2 domain with Glu-Pro-Gln-pTyr-Glu-Glu-Ile-Pro-Ile-Tyr-Leu ($IC_{50}=0.9\ \mu M$) resulted in the discovery of a dipeptide peptidomimetic ligand **17** that is nearly twofold more potent than the parent phosphopeptide employed in the co-crystal structure.

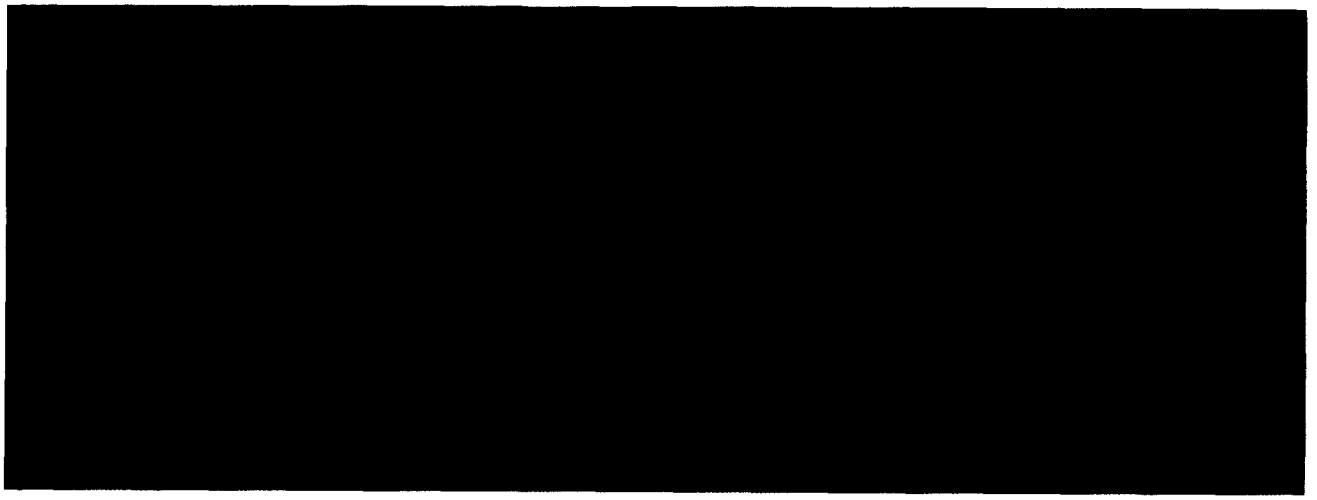


Figure 1. Stereoview: overlay of **4** (yellow) and **5** (magenta) modeled in the Src SH2 domain. The Waksman[7] X-ray crystal complex with the 11mer was used as the starting structure.

Figure 2. Stereoview of **16** (yellow) modeled in the Src SH2 domain. The Waksman[7] X-ray crystal complex with the 11mer was used as the starting structure.

An analogue of **17**, compound **4**, has been tested for specific binding to the Src SH2 domain versus four other SH2 domains and retains at least 10-fold Src SH2 binding selectivity for three of four SH2 domains studied. Therefore, low molecular weight and selective ligands for specific SH2 domains as described in this report will greatly aid our efforts to delineate signal transduction pathways.

Experimental

General

^{1}H NMR spectra were recorded on either a Varian XL300 or XL400 spectrometer in d_6-DMSO (DMSO). ^{31}P NMR spectra were obtained on a Varian Unity-400 spectrometer using a 5 mm Nalorac Quad probe. Spectra were obtained at 293 K, and phosphorus chemical shifts are referenced to external H_3PO_4 in $CDCl_3$ at 0.0 ppm. Chemical ionization mass spectra were recorded on a Fisons (VG Biotech) Trio-2A using 1% ammonia in methane as the reagent gas. Electrospray mass spectra were obtained on a single quadrapole instrument (VG Fisons Trio-2000) in negative ion mode. Flash silica gel chromatography was performed using Keiselgel 230–400 mesh. Analytical HPLC chromatograms were obtained using a C18 analytical column, (Vydac, 4.6 × 250 mm) eluting with a linear gradient of 0–66% acetonitrile containing 0.1% TFA and water containing 0.1% TFA over 22 min unless otherwise indicated and the peaks were detected at 214 nm. Thin layer chromatography employed EM Science silica gel 60 F_{254} glass backed plates. Tetrahydrofuran (THF) was dried over sodium-benzophenone ketyl and distilled. All other commercially available solvents and reagents were used without further purification.

Table 2. SH2 binding specificity of **4**

IC_{50} or % inhibition at 100 μM				
Src	Abl	Grb2	Syp(N)	PLCγ(C)
9.3	16.2	44%	8.7%	57%

Inhibition of ^{125}I-phosphopeptide binding to immobilized Src SH2

The binding affinities of compounds to the Src SH2 domain was determined using a competitive radiolabeled phosphopeptide displacement assay. Specifically, binding of ^{125}I-labeled Glu-Pro-Gln-pTyr-Glu-Glu-Ile-Pro-Ile-Tyr-Leu to a glutathione-*S*-transferase (GST)-Src SH2 fusion protein was performed in 20 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 0.1% NP-40. Assay additions to a Millipore filter plate (0.45 mM PVDF) resulted in Src SH2 fusion protein–glutathione Sepharose bead complex, 2.8 nm ^{125}I-phosphopeptide and 2% DMSO + test compound at different concentrations. Binding was performed at room temperature for 20 min while continuously inverting the plate. Unbound ^{125}I-phosphopeptide was separated from SH2-bound radiolabeled peptide by vacuum filtration and washing two times with 100 μL per well of assay buffer. Results are expressed as IC_{50} values and are averages of at least two duplicate determinations.

Inhibition of activated PDGF receptor binding to ^{35}S-SH2 domains

The binding of the dipeptide **4** was determined using ^{35}S-labeled GST SH2 protein constructs and their binding to an immobilized PDGF receptor kinase domain. Binding of ^{35}S-X-SH2-GST (X = Src, Abl, Grb2, Syp-N, and PLCγ(C) domains) to immobilized PDGF receptor kinase domain was performed in a Millipore filter plate (0.45 mM PDVF) in 20 mM Tris buffer (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, and 0.1% Triton. The assay was conducted at room temperature for 30 min while continuously inverting the plate. The resultant SH2–GST–PDGF receptor

kinase complex was separated from excess ^{35}S-SH2-GST protein by vacuum filtration and the amount of bound SH2–GST was determined by scintillation counting. Results are expressed as IC_{50} values and/or % inhibition at specified concentrations and are averages of at least two duplicate determinations.

Fmoc-L-Glu(OtBu)-N-(methyl) (3-cyclohexylpropyl) (2). To methyl (3-cyclohexylpropyl)amine hydrochloride, **1**, (5.0 mmol, 960 mg) in tetrahydrofuran (20 mL) was added Fmoc-L-Glu(OtBu) (5.5 mmol, 2.34 g) followed by sequential addition of HOBT (6.25 mmol, 845 mg), *N*-methylmorpholine (12.5 mmol, 1.37 mL), and EDCI (6.25 mmol, 1.2 g). After stirring 16 h at room temperature, diethyl ether was added and the remaining residue was dissolved in water. The mixture was separated, washed with 10% H_2SO_4, water, then satd Na_2CO_3 and then brine to provide the product as a colorless foam (2.59 g, 92%). ^{1}H NMR ($CDCl_3$, 300 MHz) 0.87 (m, 2H), 1.17 (m, 6H), 1.46 (s, 9H), 1.50–1.80 (m, 8H), 2.05 (m, 1H), 2.33 (m, 2H), 2.95, 3.11 (s, 3H, rotational isomers), 3.18–3.58 (m, 2H), 4.21 (t, 1H), 4.36 (m, 2H), 4.70 (m, 1H), 5.80 (dd, 1H), 7.27–7.45 (m, 4H), 7.60 (m, 2H), 7.77 (d, 2H); IR ($CHCl_3$) 1719, 1642 cm^{-1}; MS (CI, 1% NH_3 in CH_4) *m/z* 563 ($M+H^+$).

Ac-L-Tyr-L-Glu(OtBu)-*N*-(methyl) (3-cyclohexylpropyl) (3). To Fmoc-L-Glu(OtBu)-*N*-(methyl)(3-cyclohexylpropyl), **2**, (2.0 mmol, 1.12 g) in CH_2Cl_2 (20 mL) was added piperidine (4 mL). After 20 min toluene (20 mL) was added and the solvent removed under reduced pressure. Toluene (20 mL) was again added and the solvent evapd. The resulting residue was dissolved in tetrahydrofuran (15 mL) and coupled with Ac-(L)-Tyr (2.2 mmol, 491 mg) in the manner described above to give a solid residue upon work up. Chromatography of the residue (3:7, tetrahydrofuran:dichloromethane) gave the product a colorless foam (880 mg, 81%). ^{1}H NMR (DMSO, 300 MHz) 0.85 (m, 2H), 1.03–1.30 (m, 6H), 1.40 (s, 9H), 1.40–1.90 (m, 10H), 1.75 (s, 3H), 2.20 (t, 3H), 2.60 (dd, 1H), 2.78, 2.95 (s, 3H, rotational isomers), 3.10–3.30 (m, 2H), 4.44 (m, 1H), 4.70 (m, 1H), 6.61 (d, 2H), 7.00 (d, 2H), 7.69 (d, 1H), 8.01 (t, 3H), 9.15 (s, 1H); IR ($CHCl_3$) 1720, 1639 cm^{-1}; MS (CI, 1% NH_3 in CH_4) *m/z* 546 ($M+H^+$).

Ac-(*O*-phosphono)-L-Tyr-L-Glu-*N* (methyl) (3-cyclohexylpropyl) (4). To Ac-L-Tyr-L-Glu(OtBu)-*N*-(methyl) (3-cyclohexylpropyl), **3**, (0.40 mmol, 218 mg) in CH_2Cl_2 (5 mL) was added tetrazole (0.80 mmol, 56 mg) and di-tert-butyl diethylphosphoramidate (0.60 mmol, 0.17 mL). After 3 h thin layer analysis indicated the reaction was complete and tert-butylhydroperoxide (2.4 mmol, 0.22 mL) was added. After 2 h, ethyl acetate was added and the organic phase was washed with 10% sulfuric acid, water, 2% NaOH and then brine. The solvent was removed under reduced pressure to give an oil (432 mg). This oil was treated with trifluoroacetic acid (15 mL) and water (1 mL) for 1 h. The solvent was removed under reduced pressure and the residue precipitated with Et_2O to give a colorless solid. Preparative HPLC of the crude product (75 mg) employing a Vydac C18 (22 × 250 mm) column eluting with a gradient of 0–30% acetonitrile containing 0.1% trifluoroacetic acid and water containing 0.1% trifluoroacetic acid provided pure product after lypholization (31 mg). Analytical HPLC 100%, rt 17.30 min; ^{1}H NMR (DMSO, 400 MHz) 0.83 (m, 2H), 1.18 (m, 7H), 1.41 (m, 1H), 1.63 (m, 6H), 1.76 (s, 3H), 1.85 (m, 1H), 2.22 (m, 2H), 2.68 (m, 1H), 2.93 (m, 1H), 2.80, 2.97 (s, 3H rotational isomers), 3.00–3.30 (m, 2H), 4.45 (m, 1H), 4.72 (m, 1H), 7.03 (d, 2H), 7.18 (m, 2H), 8.04 (m, 1H), 8.16 (t, 1H); ^{31}P NMR −5.40; electrospray MS (50:50 acetonitrile:water + 0.1% NH_4OH) *m/z* 569.5 ($M-H^-$).

Ac-(*O*-phosphono)L-Tyr-L-Glu-D-Hph-NH_2 (5). The synthesis of **5** has been previously communicated.[12] The spectral data are as follows: analytical HPLC 92%, rt = 12.7 min; ^{1}H NMR (DMSO, 400 MHz) 1.77 (s, 3H), 1.83 (m, 2H), 2.01 (m, 2H), 2.36 (m, 2H), 2.58 (m, 2H), 2.71 (m, 1H), 2.95 (m, 1H), 4.14 (m, 1H), 4.25 (m, 1H), 4.45 (m, 1H), 7.15 (m, 11H), 8.12 (d, 1H), 8.17 (d, 1H), 8.28 (d, 1H); ^{31}P NMR −5.28; electrospray MS (50:50 acetonitrile:water + 0.1% NH_4OH) *m/z* 591.2 ($M-H^-$).

Ac-(*O*-phosphono)L-Tyr-L-Glu-NH(3-phenylpropyl) (6). Synthesized in a manner similar to that described for **4**. The product was obtained as a colorless powder (52 mg). Analytical HPLC 100%, rt = 14.0 min; ^{1}H NMR (DMSO, 400 MHz) 1.69 (m, 2H), 1.76 (s, 3H), 1.85 (m, 1H), 2.23 (m, 2H), 2.56 (t, 2H), 2.68 (m, 1H), 3.11 (m, 4H), 4.20 (m, 1H), 4.45 (m, 1H), 7.04 (d, 2H), 7.22 (m, 6H), 7.83 (t, 1H), 8.10 (d, 2H); ^{31}P NMR −5.54; electrospray MS (50:50 acetonitrile:water + 0.1% NH_4OH) *m/z* 548.3 ($M-H^-$).

Ac-(*O*-phosphono)L-Tyr-L-Glu-NH(2-phenylethyl) (7). Synthesized in a manner similar to that described for **4**. The product was obtained as a colorless powder (27 mg). Analytical HPLC 100%, rt = 12.5 min; ^{1}H NMR (DMSO, 400 MHz) 1.70 (m 1H), 1.77 (s, 3H), 1.85 (m, 1H), 2.11 (m, 2H), 2.62 (m, 2H), 2.91 (dd, 1H), 3.27 (m, 3H), 4.14 (m, 1H), 4.44 (m, 1H), 7.03 (d, 2H), 7.21 (m, 7H), 7.83 (t, 1H), 8.0 (d, 1H), 8.09 (d, 1H); ^{31}P NMR −4.82; electrospray MS (50:50 acetonitrile:water + 0.1% NH_4OH) *m/z* 534.5 ($M-H^-$).

Ac-(*O*-phosphono)-L-Tyr-L-Glu-NH[2-(4-methyl-phenyl)-ethyl] (8). Synthesized in a manner similar to that described for **4**. The product was obtained as a colorless powder (109 mg). Analytical HPLC 92%, rt = 13.7 min; ^{1}H NMR (DMSO, 400 MHz) 1.70 (m, 1H), 1.76 (s, 3H), 1.85 (m, 1H), 2.14 (m, 2H), 2.23 (s, 3H), 2.68 (m, 2H), 2.93 (dd, 1H), 3.52 (m, 3H), 4.18 (m, 1H), 4.46 (m, 1H), 7.06 (m, 6H), 7.19 (d, 2H), 7.84 (t, 1H), 8.06 (dd, 2H); ^{31}P NMR −5.48; electrospray MS (50:50 acetonitrile:water + 0.1% NH_4OH *m/z* 548.4 ($M-H^-$).

Ac-(*O*-phosphono)-L-Tyr-L-Glu-NH{3-[4-*O*-aceticacid)-phenyl]propyl} (9). Synthesized in a manner similar

to that described for **4**. The product was obtained as a white solid (60 mg). HPLC 93%, rt = 12.7 min; ^{1}H NMR (DMSO, 400 MHz) 1.76 (m, 1H), 1.79 (s, 3H), 1.82 (m, 1H), 1.92 (m, 1H), 2.22 (m, 2H), 2.48 (m, 1H), 2.98 (m, 2H), 3.10 (m, 2H), 4.20 (m, 1H), 4.46 (m, 1H), 4.61 (s, 2H), 6.80 (d, 2H), 7.04 (d, 2H), 7.10 (d, 2H), 7.21 (d, 2H), 7.82 (t, 1H), 8.08 (d, 1H), 8.10 (d, 1H); ^{31}P NMR −5.56; electrospray MS (50:50 acetonitrile: water + 0.1% NH_4OH) *m/z* ($M-H^-$).

Ac-(*O*-phosphono)-L-Tyr-L-Glu-NH{3-[3-(*O*-acetic acid)-phenyl]propyl} (10). Synthesized in a manner similar to that described for **4**. The product was obtained as a white solid (73 mg). Analytical HPLC 100% rt = 12.3 min; ^{1}H NMR (DMSO, 400 MHz) 1.67 (m, 2H), 1.78 (s, 3H), 1.81 (m, 1H), 1.92 (m, 1H), 2.23 (m, 2H), 2.52 (m, 1H), 2.67 (dd, 1H), 2.97 (dd, 2H), 3.06 (m, 2H), 4.21 (q, 1H), 4.47 (m, 1H), 4.63 (s, 2H), 6.69 (d, 2H), 6.78 (m, 2H), 7.05 (d, 2H), 7.21 (m, 2H), 7.83 (t, 1H), 8.10 (d, 2H); ^{31}P NMR −5.57; electrospray MS (50:50 acetonitrile:water + 0.1% NH_4OH) *m/z* 622.3 ($M-H^-$).

Ac-(*O*-phosphono)-L-Tyr-L-Glu-NH{3-[3-(*O*-acetamido)-phenyl]propyl} (11). Synthesized in a manner similar to that described for **4**. The product was obtained as a white solid (70 mg). Analytical HPLC 93%, rt = 12.2 min; ^{1}H NMR (DMSO, 400 MHz) 1.62 (m, 1H), 1.68 (m, 4H), 1.83 (m, 1H), 2.21 (m, 2H), 2.52 (m, 1H), 2.65 (m, 1H), 2.98 (dd, 2H), 3.06 (m, 2H), 4.19 (m, 1H), 4.38 (s, 2H), 4.42 (m, 2H), 6.73 (d, 2H), 6.80 (d, 2H), 7.04 (d, 2H), 7.17 (m, 2H), 7.38 (s, 1H), 7.50 (s, 1H), 7.80 (m, 1H), 8.11 (m, 2H); ^{31}H NMR −5.47; electrospray MS (50:50 acetonitrile:water + 0.1% NH_4OH) *m/z* 622.4 ($M-H^-$).

Ac-(*O*-phosphono)-L-Tyr-L-Glu-*N*(methyl)(3-phenylpropyl) (12). Synthesized in a manner similar to that described for **4**. Product was obtained as a colorless solid (108 mg). Analytical HPLC 85%, rt = 14.5 min; ^{1}H NMR (DMSO, 400 MHz) 1.60–1.95 (m, 4H), 1.75 (s, 3H), 2.23 (m, 2H), 2.50 (m, 1H), 2.60 (t, 1H), 2.68 (m, 1H), 3.90 (m, 1H), 2.81, 2.99 (s, 3H, rotational isomers), 3.30 (m, 3H), 4.48 (m, 1H), 4.72 (m, 1H), 7.03 (d, 2H), 7.20 (m, 7H), 8.05 (t, 1H), 8.20 (t, 1H); electrospray MS (50:50 acetonitrile:water + 0.1% NH_4OH) *m/z* 562.5 ($M-H^-$).

Ac-(*O*-phosphono)-L-Tyr-L-Glu-*N*((*S*)-1-methyl-2-phenylethyl) (13). Synthesized in a manner similar to that described for **4**. Product was obtained as a colorless solid (251 mg). Analytical HPLC 100%, rt = 13.6 min; ^{1}H NMR (DMSO, 400 MHz) 1.04 (d, 3H, *J* = 6.5 Hz), 1.62 (m, 1H), 1.76 (s, 3H), 1.80 (m, 1H), 2.00 (m, 2H), 2.68 (m, 3H), 3.93 (dd, 1H), 3.98 (m, 1H), 4.18 (dd, 1H), 4.43 (m, 1H), 7.03 (d, 2H), 7.20 (m, 7H), 7.83 (d, 1H), 7.98 (d, 1H), 8.07 (d, 1H); electrospray MS (50:50 acetonitrile:water + 0.1% NH_4OH) *m/z* ($M-H^-$).

Ac-(*O*-phosphono)-L-Tyr-L-Glu-*N*(methyl)((*S*)-1-methyl-2-phenylethyl) (14). Synthesized in a manner similar to that described for **4**. Product was obtained as a colorless solid (176 mg). Analytical HPLC 91%, rt = 13.8 min; ^{1}H NMR (DMSO, 300 MHz) 1.08, 1.15 (d, 3H, rotational isomers), 1.33 (m, 1H), 1.50 (m, 1H), 1.76 (s, 3H), 1.80–2.02 (m, 2H), 2.22 (m, 1H), 2.60–3.00 (m, 4H), 2.72, 2.82 (m, 3H, rotational isomers), 4.43 (m, 1H), 4.90 (m, 1H), 7.00 (d, 2H), 7.10–7.28 (m, 7H), 8.01 (d, 1H); electrospray MS (50:50 acetonitrile:water + 0.1% NH_4OH) *m/z* 563.4 ($M-H^-$).

Ac-(*O*-phosphono)-L-Tyr-L-Glu-L-Pro(2-decarboxy-2-benzyl) (15). Synthesized in a manner similar to that described for **4**. Product was obtained as a colorless solid (167 mg). Analytical HPLC 100%, rt = 15.4 min; ^{1}H NMR (DMSO, 400 MHz) 1.60–2.00 (m, 5H), 1.78 (s, 3H), 2.32 (m, 2H), 2.56 (m, 1H), 2.75 (m, 2H), 3.50 (m, 2H), 4.15, 4.27 (m, 1H, rotational isomers), 4.52 (m, 1H), 7.03 (m, 2H), 7.20 (m, 4H), 7.30 (m, 3H), 8.00, 8.05 (d, 1H, rotational isomers) 8.20, 8.30 (d, 1H, rotational isomers); electrospray MS (50:50 acetonitrile:water + 0.1% NH_4OH) *m/z* 575.5 ($M-H^-$).

Ac-(*O*-phosphono)-L-Tyr-L-Glu-L-Pro(2-decarboxy-2-(2-phenylethyl)) (16). Synthesized in a manner similar to that described for **4**. Product was obtained as a colorless solid (51 mg). Analytical HPLC 100%, rt = 13.3 min employing 10–76% acetonitrile; ^{1}H NMR (DMSO, 400 MHz) 1.50–2.00 (m, 8H), 1.76 (s, 3H), 2.25 (m, 2H), 2.55 (m, 1H), 2.64 (m, 1H), 2.92 (m, 1H), 3.30 (m, 1H), 3.55 (m, 1H), 4.00 (m, 1H), 4.48 (m, 2H), 7.03 (d, 2H), 7.20 (m, 7H), 8.04 (d, 1H), 8.18 (d, 1H); ^{31}P NMR −5.56; electrospray MS (50:50 acetonitrile: water + 0.1% NH_4OH) *m/z* 589.4 ($M-H^-$).

Ac-(*O*-phosphono)-L-Tyr-L-Glu-L-Pro(2-decarboxy-2-(2-cyclhexylethyl)) (17). Synthesized in a manner similar to that described for **4**. Product was obtained a a colorless solid (51 mg). Analytical HPLC 100%, rt = 15.7 min employing 10–76% acetonitrile; ^{1}H NMR (DMSO, 400 MHz) 0.82 (m, 2H), 1.18 (m, 7H), 1.58–1.95 (m, 12H), 1.76 (s, 3H), 2.25 (m, 2H), 2.66 (m, 1H), 2.93 (m, 1H), 3.42 (m, 1H), 3.57 (m, 1H), 3.90 (m, 1H), 4.46 (m, 2H), 7.03 (d, 2H), 7.20 (d, 2H), 8.03 (d, 1H), 8.15 (d, 1H); ^{31}P NMR −5.57; electrospray MS (50:50 acetonitrile:water + 0.1% NH_4OH) *m/z* 595.5 ($M-H^-$).

Ac-(*O*-phosphono)-L-Tyr-L-Abu-*N*(methyl)(3-cyclohexylpropyl) (18). Synthesized in a manner similar to that described for **4**. Product was obtained as a colorless solid (78 mg). Analytical HPLC 100%, rt = 19.2 min; ^{1}H NMR (DMSO, 400 MHz) 0.80–0.89 (m, 5H), 1.06–1.20 (m, 6H), 1.41–1.67 (m, 9H), 1.75 (s, 3H), 2.67 (t, 1H), 2.93 (m, 1H), 2.80, 2.95 (s, 3H, rotational isomers), 3.13–3.36 (m, 2H), 4.49 (m, 1H), 4.58 (m, 1H), 7.03 (d, 2H), 7.19 (t, 2H), 8.04 (m, 1H), 8.13 (m, 1H); ^{31}P NMR −5.56; electrospray MS (50:50 acetonitrile:water + 0.1% NH_4) *m/z* ($M-H^-$).

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