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Staudinger Ligation of α-Azido Acids Retains Stereochemistry

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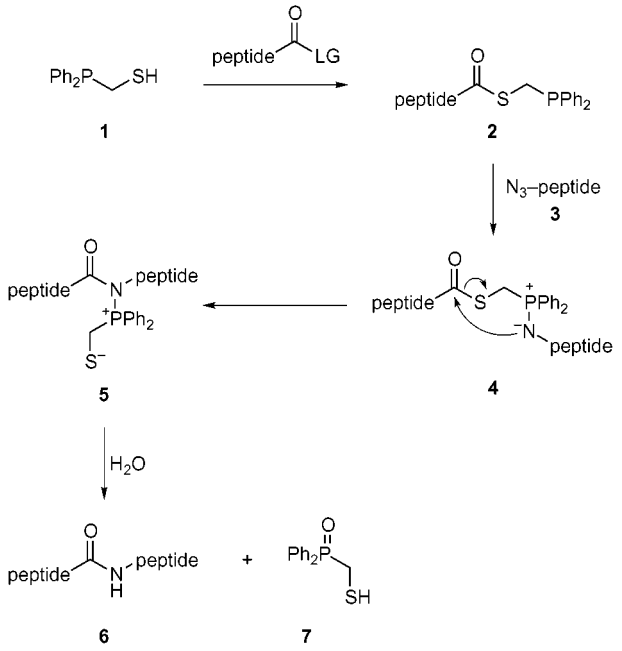
Abstract: The Staudinger ligation of peptides with a C-terminal phosphinothioester and N-terminal azide is an emerging method in protein chemistry. Here, the first Staudinger ligations of nonglycyl azides are reported and shown to proceed both in nearly quantitative yield and with no detectable effect on the stereochemistry at the α-carbon of the azide. These results demonstrate further the potential of the Staudinger ligation as a general method for the total synthesis of proteins from peptide fragments.

The chemoselective ligation of peptides can be used to effect the total chemical synthesis of proteins.[1] The most common ligation method, native chemical ligation, relies on the presence of a cysteine residue at the *N*-terminus of each ligation junction.[2,3] We have identified the "Staudinger ligation" as a peptide ligation method that has the potential to be universal—independent of the presence of any particular side chain.[4] This method is based on the Staudinger reaction, wherein a phosphine reduces an azide via a stable iminophosphorane intermediate.[5] Acylation of this iminophosphorane yields an amide.[6,7]

In our version of the Staudinger ligation (Scheme 1),[4] a peptide fragment having a *C*-terminal phosphinothioester (**2**) reacts with another peptide fragment having an *N*-terminal azide (**3**). The resulting iminophosphorane (**4**) leads, after an S− to N-acyl shift, to an amidophosphonium salt (**5**). The P−N bond of the amidophosphonium salt is hydrolyzed readily to produce the amide product (**6**) and a phosphine oxide (**7**). Importantly, no residual atoms remain in the amide product.[4,6b] Previously, we showed that the ligation of glycyl and phenylalanyl thioesters of phosphinothiol **1** with glycyl azides proceeds in high yield.[4b]

SCHEME 1



All natural α-amino acids except glycine have a stereogenic center at their α-carbon.[8] To be an effective tool for the total chemical synthesis of proteins, a peptide ligation reaction must proceed without epimerization.

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(3) Cysteine is uncommon, comprising only 1.7% of the residues in proteins (McCaldon, P.; Argos, P. *Proteins* **1988**, *4*, 99−122). Modern peptide synthesis is typically limited to peptides of ≤50 residues (ref 1). Hence, most proteins cannot be prepared by any method that allows for the coupling of peptides only at cysteine residues.

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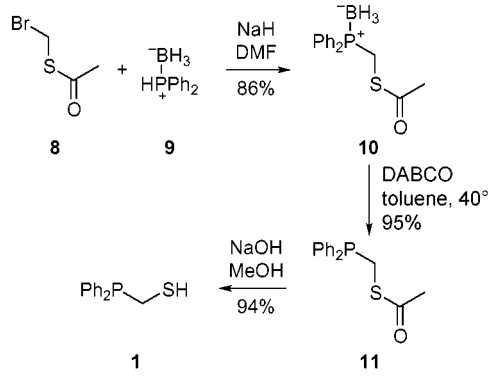
SCHEME 2

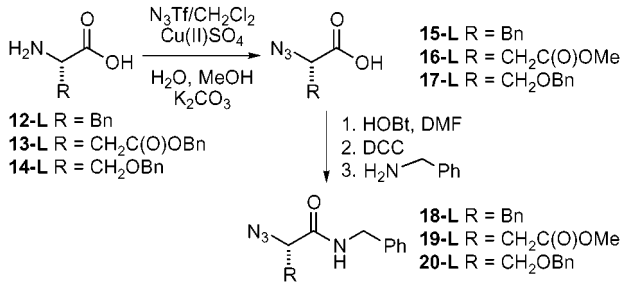


The coupling of thioesters in native chemical ligation, which like the Staudinger ligation (Scheme 1) involves transthioesterification followed by an *S*- to *N*-acyl shift,[2,3] is known to proceed without detectable racemization.[9] Because the Staudinger ligation has been demonstrated previously only with glycyl azides, the propensity for epimerization of the α-azido acid has not been assessed. This issue is of concern because an α-carbanion could be stabilized by inductive or resonance effects in azide **3** and iminophosphorane **4**.[10]

Here, we report the first use of the Staudinger ligation to couple a peptide containing a nonglycyl azide. We also search for epimerization[11] during Staudinger ligation reactions. Finally, we report an improved synthesis of phosphinothiol **1** (Scheme 1), which is the most effective known phosphinothiol for effecting the Staudinger ligation of peptides.

The previously reported synthesis of phosphinothiol **1** required four steps, two of which were problematic, with an overall yield of only 39%.[4b] We have developed an improved synthesis that uses air-stable borane protection of the phosphine (Scheme 2).[12] The synthesis is based on the easily prepared alkylating agent **8**[13] and the commercially available borane−diphenylphosphine complex **9**. Compound **9** is deprotonated by sodium hydride in DMF followed by addition of **8** to give borane complex **10** (86% yield).[14] Complex **10** is stable to air and moisture and can be stored on the shelf at room temperature for months without any sign of oxidation or decomposition. The borane complex is disrupted by mild heating with DABCO in toluene for 4 h (95% yield).[15] The protecting group of the resulting acyl phosphinothiol **11** is removed as described previously[4b] to give phosphinothiol **1** (94% yield). The overall yield for this three-step synthesis is 74%.

SCHEME 3



We then prepared several nonglycyl α-azido acids to determine if epimerization occurs during the Staudinger ligation. The azido benzamides of both the D and L enantiomers of phenylalanine, serine, and aspartic acid were prepared (Scheme 3). The azido group was prepared by diazo transfer;[10] the benzamide was prepared by DCC/HOBt coupling with benzylamine. Phenylalanine, aspartic acid, and serine were chosen as being representative of three distinct side chains and moderate (phenylalanine) to high (aspartate and serine) propensity to epimerize during standard peptide couplings.[16]

Each of these azido acids was coupled with phosphinothioester **21**[17] (Table 1). The couplings were carried out in THF/H_2O (3:1) for 12 h at room temperature with a 1:1 stoichiometry of starting materials. The resulting peptides were purified by flash chromatography to give a nearly quantitative yield of each product (Table 1). The high yield of this equimolar reaction of phosphinothiol **1** with nonglycyl azides is consistent with those observed previously with glycyl azides.[4b]

The chirality of the Staudinger ligation products from the reaction of the D and L α-azido acids was analyzed by HPLC using a D-phenylglycine chiral column. The chromatographic conditions enabled the baseline resolution of the two possible enantiomeric products (Figure 1).[18] After reaction of the D epimer, there was no evidence of product containing the L epimer, and vice versa. Thus, the Staudinger ligation proceeds without detectable epimerization of the α-carbon of the azido acid.[19]

We conclude that the Staudinger ligation can be used to couple nonglycl azides in nearly quantitative yield and without detectable epimerization. Thus, the Staudinger ligation holds promise as a general method for the convergent assembly of proteins from peptide fragments.

Experimental Section

Reactions were monitored by thin-layer chromatography with visualization by UV light or staining with ninhydrin or I_2. Silica gel used in flash chromatography had 230−400 mesh and 60 Å

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(11) Because the reaction products herein are enantiomers and not diastereomers, we actually probe "racemization" (Reist, M.; Testa, B.; Carrupt, P.-A.; Jung, M.; Schurig, V. *Chirality* **1995**, *7*, 396−400). We use the term "epimerization" to focus attention on the fate of the chirality at the α-carbon of the azide in couplings of peptides that contain multiple stereogenic centers.

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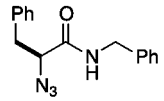
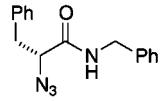
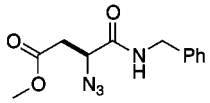
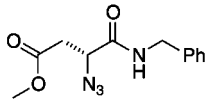
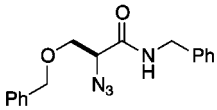
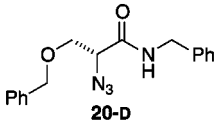
(17) The preparation of phosphinothioester **21** was modified from our previous reports, in which coupling using DCC alone led to lower yields and several undesired products (ref 4a and 4b). Pretreatment of *N*-acetylglycine (**8**) with HOBt and DCC followed by addition of phosphinothiol **1** improved the yield dramatically. See the Experimental Section for details.

(18) Material was injected onto a D-phenylglycine analytical chiral HPLC column and eluted with 30% (v/v) 2-propanol in hexanes (isocratic) for 20 min followed by a shallow gradient to 50% (v/v) 2-propanol for 40 min.

(19) We estimate the detection limit of the HPLC chromatographic analysis to be ≤0.5%, so that the Staudinger ligation proceeds with ≥99.5% retention of chirality. This latter value is greater than values for analogous intermolecular acyl transfer reactions to an iminophosphorane (ref 7g).

TABLE 1. Staudinger Ligation of $AcGlySCH_2PPh_2$ (21) and Non-Glycyl α-Azido Acids

α-azido acid	peptide	yield (%)[b]
 **18-L**	AcGly(L)PheNHBn **22-L**	90
 **18-D**	AcGly(D)PheNHBn **22-D**	93
 **19-L**	AcGly(L)Asp(OMe)NHBn **23-L**	91
 **19-D**	AcGly(D)Asp(OMe)NHBn **23-D**	95
 **20-L**	AcGly(L)Ser(Bn)NHBn **24-L**	92
 **20-D**	AcGly(D)Ser(Bn)NHBn **24-D**	99

[a] Reaction conditions: THF/H_2O (3:1) at room temperature for 12 h. [b] Isolated yield of product after purification by flash chromatography.

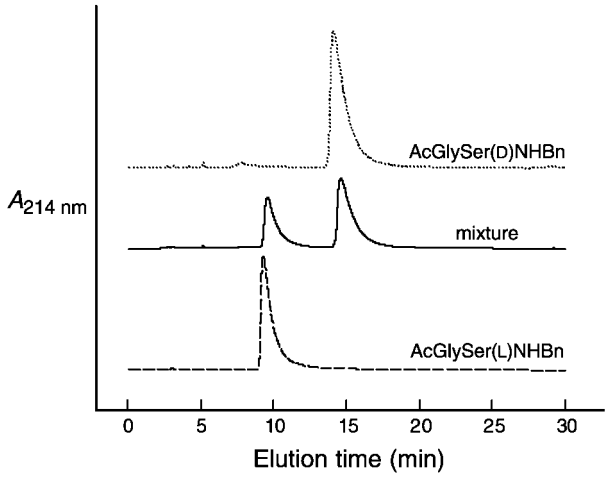


FIGURE 1. HPLC elution profile of AcGly(D)SerNHBn, AcGly(L)SerNHBn, and a mixture of the two enantiomers.[18]

pore size. Chiral HPLC was performed with a D-phenylglycine analytical chiral column. NMR spectra were obtained with a 500 or 300 MHz spectrometer at the University of Wisconsin nuclear magnetic resonance facility. Carbon-13 and phosphorus-31 NMR spectra were both proton-decoupled, and phosphorus-31 spectra were referenced against an external standard of deuterated phosphoric acid (0 ppm). Mass spectra were obtained with electrospray ionization (ESI) techniques.

Borane−Thioacetic Acid *S*-[(Diphenylphosphanyl)methyl] Ester Complex (10). Borane−diphenylphosphine complex **9** (10.33 g, 51.6 mmol) was dissolved in dry DMF under Ar(g) and cooled to 0 °C. NaH (1.24 g, 51.6 mmol) was added slowly, and the mixture was stirred at 0 °C until bubbling ceased. Alkylating agent **8**[13] (8.73 g, 51.6 mmol) was then added, and the mixture was allowed to warm to room temperature and stirred for 12 h. Solvent was removed under reduced pressure, and the residue was purified by flash chromatography (silica gel, 10% v/v EtOAc in hexanes). Compound **10** was isolated as a colorless oil in 86% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.74−7.67 (m, 4 H), 7.54−7.41 (m, 6 H), 3.72 (d, J = 6 Hz, 2 H), 2.23 (s, 3 H), 1.51−0.53 (broad m, 3 H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.94, 132.26 (d, J = 9.2 Hz), 131.61 (d, J = 2.3 Hz), 128.71 (d, J = 10.2 Hz), 127.43 (d, J = 55.4 Hz), 29.87, 23.59 (d, J = 35.5 Hz) ppm; ^{31}P NMR (121 MHz, $CDCl_3$) 19.40 (d, J = 59.3 Hz) ppm; MS (ESI) m/z 311.0806 (MNa^+ $[C_{15}H_{18}BOPSNa]$ = 311.0807).

Thioacetic Acid *S*-[(Diphenylphosphanyl)methyl] Ester (11). Compound **10** (4.00 g, 13.9 mmol) was dissolved in toluene (0.14 L) under Ar(g). DABCO (1.56 g, 13.9 mmol) was added, and the mixture was heated at 40 °C for 4 h. Solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2 and washed with both 1 N HCl and saturated brine. The organic layer was dried over $MgSO_4$(s), and the solvent was removed under reduced pressure. Compound **11** was isolated in 95% yield and was used without further purification. Spectral data was the same as that reported previously.[4b]

2(*S*)-Azido-*N*-benzyl-3-phenylpropionamide (18-L). N_3(L)PheOH (**15-L**) was synthesized from L-phenylalanine essentially by the procedure of Lundquist and Pelletier.[20] N_3(L)PheOH (1.08 g, 5.7 mmol) was dissolved in anhydrous DMF (40 mL). HOBt (0.87 g, 5.7 mmol) was then added, followed by DCC (1.17 g, 5.7 mmol). Once precipitate was observed in the reaction, benzylamine (0.62 mL, 5.7 mmol) was added. The reaction was allowed to stir under Ar(g) for 3 h. The resulting precipitate (DCU) was removed by filtration, and the filtrate was concentrated under reduced pressure to give a yellow oil. This oil was purified by flash chromatography (silica gel, 35% v/v ethyl acetate in hexanes). N_3(L)PheNHBn (**18-L**) was isolated as an off-white solid in 90% yield. The procedure was repeated with D-phenylalanine to give N_3(D)PheNHBn (**18-D**) as a white solid in 92% yield. The spectral data for both N_3PheNHBn products (D and L enantiomers) are identical. **N_3(L)PheNHBn (18-L)** ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.31−7.12 (m, 8 H), 7.11 (m, 2 H), 6.55 (bs, 1 H), 4.38 (m, 2 H), 4.22 (dd, J = 7.8, 4.6 Hz, 1 H), 3.34 (dd, J = 14.0, 4.5 Hz, 1 H), 3.07 (dd, J = 14.1, 7.5 Hz, 1 H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.30, 137.35, 135.96, 129.51, 128.61, 128.66, 128.61, 128.55, 127.70, 127.68, 127.66, 127.57, 127.16, 65.40, 43.41, 38.41 ppm; MS (ESI) m/z 303.1235 (MNa^+ $[C_{16}H_{16}N_4ONa]$ = 303.1222).

3(*S*)-Azido-*N*-benzylsuccinamic Acid Methyl Ester (19-L). Benzyl-protected L-aspartate was used in the procedure of Lundquist and Pelletier[20] to give N_3(L)Asp(OMe)OH (**16-L**). Under these conditions, we observed transesterification to give the methyl ester product as opposed to the benzyl ester. N_3(L)Asp(OMe)OH (**16-L**) was produced as a yellowish oil in 78% yield. N_3(L)Asp(OMe)OH (**16-L**) was then coupled with benzylamine as above to give N_3(L)Asp(OMe)NHBn (**19-L**) as a yellowish oil in 90% yield (70% overall, two steps). The procedure above was repeated with benzyl-protected D-aspartate to give N_3(L)Asp(OMe)NHBn (**19-D**) as a yellowish oil in 67% overall yield. The spectral data for both N_3Asp(OMe)OH (D and L enantiomers) and both N_3Asp(OMe)NHBn (D and L enantiomers) products are identical. **N_3(L)Asp(OMe)OH (16-L)** ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.24 (bs, 1H), 4.47 (dd, J = 7.4, 5.3 Hz, 1 H), 3.76 (s, 3 H), 2.91 (dd, J = 16.9, 5.1 Hz, 1 H), 2.79 (dd, J = 16.8, 7.6 Hz) ppm; ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.68, 170.12, 50.09,

(20) Lundquist, J. T., IV.; Pelletier, J. C. *Org. Lett.* **2001**, *3*, 781−783.

52.44, 35.84 ppm; MS (ESI) *m*/*z* 196.0340 (MNa^+ [$C_5H_7N_3O_4Na$] = 196.0334). **N_3(L)Asp(OMe)NHBn (19-L)** ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38−7.27 (m, 5 H), 6.83 (bs, 1 H), 4.54 (m, 3 H), 3.75 (s, 3 H), 3.18 (dd, J = 17.1, 3.7 Hz, 1 H), 2.75 (dd, J = 17.3, 8.7 Hz, 1 H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.77, 167.90, 137.35, 128.81, 127.80, 127.77, 60.32, 52.24, 43.71, 37.00 ppm; MS (ESI) *m*/*z* 285.0953 (MNa^+ [$C_{12}H_{12}N_4O_3Na$] = 285.0964).

2(*S*)-Azido-*N*-benzyl-3-benzyloxypropionamide (20-L). Benzyl-protected L-serine was used in the procedure above to give N_3(L)Ser(Bzl)NHBn (**20-L**) as a yellowish oil in 93% yield. The procedure was repeated with benzyl-protected D-serine to give N_3(D)Ser(Bzl)NHBn (**20-D**) as a yellowish oil in 90% yield. The spectral data for both N_3Ser(Bzl)NHBn products (D and L enantiomers) are identical. **N_3(L)Ser(Bzl)NHBn (20-L)** ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36−7.23 (m, 10 H), 6.86 (bs, 1 H), 4.57 (s, 2 H), 4.43 (m, 2 H), 4.25 (dd, J = 6.9, 3.5 Hz, 1 H), 4.01 (dd, J = 10.3, 3.5 Hz, 1 H), 3.83 (10.1, 6.7 Hz, 1 H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.81, 137.40, 137.22, 128.65, 128.40, 127.81, 127.59, 127.54, 73.45, 70.54, 63.28, 43.38 ppm; MS (ESI) *m*/*z* 333.1337 (MNa^+ [$C_{17}H_{18}N_4O_2Na$] = 333.1327).

(Acetylamino)thioacetic Acid *S*-[(Diphenylphosphanyl)-methyl] Ester (21). *N*-Acetylglycine (1.90 g, 16.2 mmol) was dissolved in anhydrous DMF (75 mL). HOBt (2.48 g, 16.2 mmol) was added to the resulting solution followed by DCC (3.34 g, 16.2 mmol). Once precipitate (DCU) was observed, phosphinothiol **1** was added (3.77 g, 16.2 mmol). The reaction mixture was allowed to stir under Ar(g) for 3 h. The precipitate was removed by filtration, and the filtrate was concentrated under reduced pressure to give a white solid. This solid was dissolved in ethyl acetate and purified by flash chromatography (silica gel, ethyl acetate). Compound **21** was isolated in 96% yield. Spectral data was the same as that reported previously.[4b]

2(*S*)-(2-(Acetylamino)acetylamino)-*N*-benzyl-3-phenylpropionamide (22-L). *N*-Acetylglycylphosphinothioester **9** (0.166 g, 0.5 mmol) and N_3(L)PheNHBn (**18-L**) (0.140 g, 0.5 mmol) was dissolved in THF/H_2O (3:1, 4 mL), and the mixture was stirred at room temperature for 12 h. Solvent was removed under reduced pressure, and the residue was purified by flash chromatography (silica gel, 5% v/v methanol in dichloromethane). AcGly(L)PheNHBn (**22-L**) was obtained in as a white solid in 90% yield. The procedure was repeated with N_3(D)PheNHBn (**18-D**) to give AcGly(D)PheNHBn (**22-D**) in 93% yield. The spectral data for both dipeptide products (D and L enantiomers) are identical. **AcGly(L)PheNHBn** (**22-L**) ^{1}H NMR (300 MHz, $CDCl_3$:CD_3OD 1:1) δ 7.30−7.22 (m, 6 H), 7.19−7.16 (m, 2 H), 7.16−7.11 (m, 2H), 4.63 (t, J = 7.3 Hz, 1 H), 4.33 (dd, J = 31.1, 14.6 Hz, 2 H), 3.79 (dd, J = 33.1, 16.7 Hz, 2 H), 3.12 (dd, J = 13.8, 7.2 Hz, 1 H), 2.98 (dd J = 13.7, 7.2 Hz, 1 H), 1.98 (s, 3 H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$:CD_3OD 1:1) δ 171.98, 170.93, 169.33, 137.29, 136.00, 128.76, 128.05, 127.97, 127.03, 126.75, 126.41, 54.16, 42.79, 42.37, 37.52, 21.56 ppm; MS (ESI) *m*/*z* 376.1624 (MNa^+ [$C_{20}H_{23}N_3O_3Na$] = 376.1637).

3(*S*)-(2-(Acetylamino)acetylamino)-*N*-benzylsuccinamic Acid Methyl Ester (23-L). N_3(L)Asp(OMe)NHBn (**19-L**) was used in the procedure above to give AcGly(L)Asp(OMe)-NHBn (**23-L**) as a white solid in 91% yield. The procedure was repeated with N_3(D)Asp(OMe)NHBn (**19-D**) to give AcGly(D)Asp-(OMe)NHBn (**23-D**) as a white solid in 95% yield. The spectral data for both AcGlyAsp(OMe)NHBn products (D and L enantiomers) are identical. **AcGly(L)Asp(OMe)NHBn** (**23-L**) ^{1}H NMR (500 MHz, $CDCl_3$:CD_3OD 1:1) δ 7.34−7.23 (m, 5 H), 4.84 (t, J = 5.7 Hz, 1 H), 4.34 (s, 2 H), 3.84 (q, J = 16.6 Hz, 2H), 3.69 (s, 3 H), 2.87 (m, 2 H), 2.01 (s, 3 H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$:CD_3OD 1:1) δ 177.24, 176.43, 175.18, 174.58, 142.56, 133.17, 133.03, 131.93, 131.78, 56.46, 54.13, 47.91, 47.81, 47.67, 40.11, 26.59 ppm; MS (ESI) *m*/*z* 358.1388 (MNa^+ [$C_{16}H_{21}N_3O_5Na$] = 358.1379).

2(*S*)-(2-(Acetylamino)acetylamino)-*N*-benzyl-3-benzyloxypropionamide (24-L). N_3(L)Ser(Bzl)NHBn (**20-L**) was used in the procedure above to give AcGly(L)Ser(Bzl)NHBn (**24-L**) as a white solid in 92% yield. The procedure was repeated with N_3(D)PheNHBn (**20-D**) to give AcGly(D)Ser(Bzl)NHBn (**24-D**) as a white solid in 99% yield. The spectral data for both AcGlySer(Bzl)NHBn products (D and L enantiomers) are identical. **AcGly(L)Ser(Bzl)NHBn (24-L)** ^{1}H NMR (500 MHz, $CDCl_3$:CD_3OD 1:1) δ 7.34−7.21 (m, 10 H), 4.60 (t, J = 4.4 Hz, 1 H), 4.43 (dd, J = 23.9, 14.9 Hz, 2 H), 3.85 (m, 3 H), 3.69 (dd, J = 9.6, 4.6 Hz, 1 H), 1.98 (s, 3 H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$:CD_3OD 1:1) δ 172.19, 169.86, 169.61, 137.49, 127.87, 127.31, 127.23, 127.76, 126.59, 72.88, 69.07, 52.93, 42.71, 42.49, 21.38 ppm; MS (ESI) *m*/*z* 406.1750 (MNa^+ [$C_{21}H_{25}N_3O_4Na$] = 406.1743).

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Supporting Information Available: ^{1}H, ^{13}C, and ^{31}P (where applicable) spectra for all novel compounds; chiral HPLC traces for each peptide in Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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