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Azatides: Solution and Liquid Phase Syntheses of a New Peptidomimetic

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Abstract: An efficient method has been developed for the solution and liquid phase syntheses of a biopolymer mimetic consisting of "α-aza-amino acids" linked in a repetitive manner to form what we term an azatide oligomer. To construct this biopolymer mimetic, three stages of research were pursued as follows: (1) development of general synthetic procedures that allowed the synthesis of a wide variety of Boc-protected aza-amino acid monomers, (2) optimization of solution phase procedures for the coupling of aza-amino acids in a repetitive manner, and (3) design and synthesis of a linker that would support azatide synthesis using a liquid phase synthetic format. The successful completion of these three phases of research demonstrates that oligoazatides can now be rapidly assembled on a homogeneous polymeric support. The long term prospectus of this new biopolymer is the exploration of peptide structure as well as a potential source of new peptidomimetic libraries.

Introduction

Peptidomimetics have become immensely important for both organic and medicinal chemists.[1] Synthetic interest in these surrogate peptide structures has been driven by the pharmaceutical industry's needs for molecules with improved pharmacokinetic properties.[2] Biophysical studies on these pseudopeptides has allowed elucidation of the functional role of the peptide backbone,[3] and with an ever-increasing level of synthetic sophistication, the degree of peptide mimicry within a peptidomimetric can be tailored to a chemist's needs. Indeed, the alteration of peptides to peptidomimetics has included peptide side chain manipulations, amino acid extensions,[4] deletions,[5] substitutions,[1a,b] and, most recently, backbone modifications.[6] It is this latter development that has been exploited for the synthesis of biomimetic polymeric structures. Such progress has been fueled by the suggestion that peptidomimetics may provide novel scaffolds for the generation of macromolecules with new properties of both biological and chemical interest.[6]

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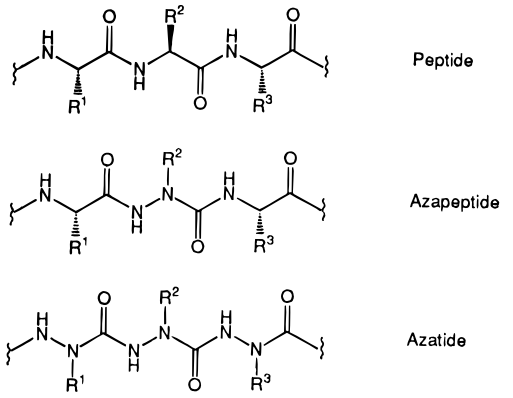


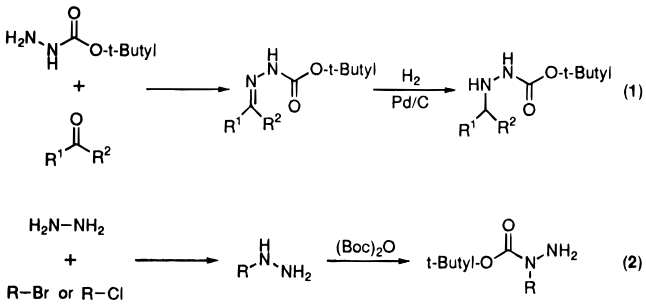
Figure 1. Comparison of a peptide, azapeptide, and azatide.

The most common manipulation involving the α-carbon atom of peptides is the inversion of stereochemistry to yield D-amino acids.[1a] The importance of this substitution in affording compounds with improved biological potencies, altered conformational properties,[7] and increased resistance to enzymatic degradation has been widely recognized and exploited.[8] Replacements of the α-hydrogen of the common amino acids by a methyl group or any other substituent ($NH_2CRR'CO_2H$) are both further examples of α-alkyl modification.[1a] Azapeptides, however, are peptides in which one (or more) of the α-carbon(s) has been replaced by a trivalent nitrogen atom (Figure 1).[9] This transformation results in a loss of asymmetry associated with the α-carbon and yields a structure that can be considered intermediate in configuration between D- and L-amino acids.[1a,10] Interest in this α-carbon replacement unit stems from its ability to provide resistance to enzymatic cleavage and its capacity to act as a selective inhibitor of cysteine[11] and serine proteases.[12] While the synthesis of azapeptides has been reported,[1d,9,13] the synthesis of a "pure azapeptide", or what we will term an "azatide", has yet to be accomplished.[1d,14] Herein, we report how monomeric "α-aza-amino acids" can be coupled in a linear, stepwise, chain-lengthening fashion to construct azatides by either solution phase or liquid phase synthetic methodologies.

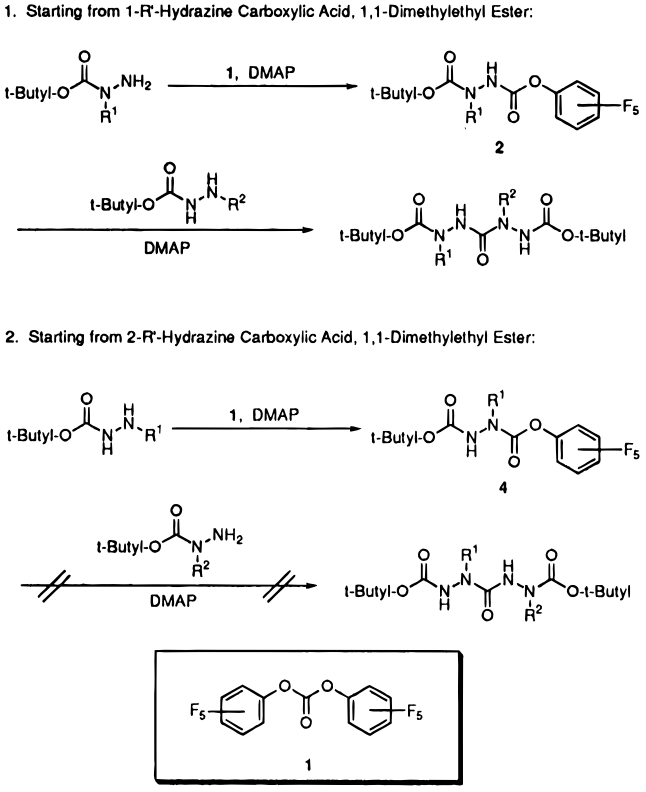
Results and Discussion

For the synthesis of oligoazatides, an alphabet of suitably protected aza-amino acid constituents needed to be prepared. The tactic that we took was to synthesize *de novo* Boc-protected alkylhydrazine monomers substituted with a variety of functional groups. Two principal routes are used in their syntheses (Scheme 1): (1) reduction of Boc-protected hydrazones,[13b] derived from the reaction of Boc-carbazate with either an aldehyde or a ketone (eq 1), and (2) alkylation of hydrazine with an alkyl halide followed by Boc-protection of the resulting alkylhydrazine (eq 2).[15] The outgrowth of these methods is the transient protection of either the "amino- or carboxy-terminal" functionality of the aza-amino acid and an ability to create a unique alphabet of α-aza-amino acid R groups.

Scheme 1. Preparation of Boc-Protected Alkylhydrazine Monomers



Scheme 2. Routes for Solution Phase Diazatide Synthesis



To convert these Boc-protected aza-amino acids into acylating agents that would allow stepwise chain lengthening, the hydrazine portion of the molecule had to be activated (Scheme 2). Activation of this moiety is a challenging problem since the Boc-alkylhydrazines are poorer nucleophiles than simple amines or amino acids. Consequently, we required a highly activated carbonyl synthon that would allow facile coupling of two Boc-protected aza-amino acids to form the azatide linkage. Furthermore, this coupling reaction had to be controllable, such that symmetrical dimer formation could be minimized. Our initial attempts to couple two aza-amino acids together using *p*-nitrophenyl chloroformate, carbonyldiimidazole, bis(2,4-dinitrophenyl) carbonate, or trichloromethyl chloroformate were

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(8) Dooley, C. T.; Chung, N. N.; Wilkes, B. C.; Schiller, P. W.; Bidlack, J. M.; Pasternak, G. W.; Houghten, R. N. *Science* **1994**, *266*, 2019.

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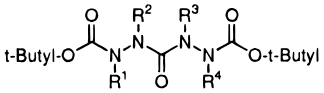
(14) The earliest attempts to make pure azatides can be dated to Gante and co-workers: (a) Gante, J. *Chem. Ber.* **1965**, *98*, 3340. (b) Gante, J.; Krug, M.; Lauterbach, G.; Weitzel, R. *Proc. Am. Pept. Symp. 13th 1993*, **1994**, 299. But the methodology that was reported does not allow azatide stepwise chain lengthening in a repetitive manner of anything but hydrazine units.

(15) Biel, J. H.; Drukker, A. E.; Mitchell, T. F.; Sprengeler, E. P.; Nuhfer, P. A.; Conway, A. C.; Horita, A. *J. Am. Chem. Soc.* **1959**, *81*, 2805.

Table 1. Prepration of Diazatides Starting from 1-R′-Hydrazinecarboxylic acid, 1,1-Dimethylethyl Ester



compd	R^1	R^2	R^3	R^4	yield (%)
5	H	H	H	H	92
6	methyl	H	H	methyl	91
7	H	methyl	H	methyl	90
8	H	methyl	H	benzyl	85
9	H	methyl	H	isobutyl	84
10	H	isobutyl	H	isobutyl	82
11	H	isopropyl	H	isopropyl	84

unsuccessful, as they suffered from complicated side reactions, poor reaction yields, and/or prolonged reaction time. We reasoned that these results were due to either insufficient leaving ability of *p*-nitrophenol and imidazole or steric hindrance of the *o*-nitro group in the case of bis(2,4-dinitrophenyl) carbonate. To overcome these problems, we opted to use bis(pentafluorophenyl) carbonate (**1**) as the carbonyl activation element.[16] Our decision to use this reagent was based on three factors. First, the pentafluorophenol functionality is a powerful electron-withdrawing group, while the fluoro substituents minimize steric problems. Second, the bis(pentafluorophenyl) carbonate can be readily prepared from phosgene and a sodium pentafluorophenolate solution. Third, the compound is a highly crystalline solid which is easy to handle.

Shown in Scheme 2 are two solution phase routes to diazatides. In the first case, carbamate **2** is utilized for the coupling reaction. Thus, a Boc-protected aza-amino acid is added dropwise to **1** granting activation of the 1-R′-hydrazinecarboxylic acid, 1,1-dimethylethyl ester. The activated complex formed, **2**, is not isolated but instead immediately reacted *via* the addition of a second Boc-protected alkylhydrazine to complete the diazatide coupling. This coupling procedure provides diazatides in good yield with few side reactions in an acceptable reaction time. Results using this coupling method are summarized in Table 1. From this table, it is evident that the coupling process seems to be quite general, as both simple Gly^a-Gly^a (superscript a refers to an aza-amino acid linkage) and sterically demanding (Val^a-Val^a) azatides can be synthesized in <1 h. The latter result is extremely important as it dictates whether the stepwise coupling of aza-amino acids is feasible. Whereas coupling through activated 1-R′-hydrazinecarboxylic acid, 1,1-dimethylethyl ester was successful, the coupling of activated 2-R′-hydrazinecarboxylic acid, 1,1-dimethylethyl ester was not (Scheme 2). From these findings, we surmise that the activated complex is not carbamate **2** but rather the isocyanate **3**.[17]

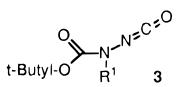
The techniques described above allow α-azatide chain building to be performed in an iterative manner. To prepare a small well-defined α-azatide, we chose to use polymer-supported liquid phase synthesis.[18] Liquid phase synthesis uses a soluble linear homopolymer [poly(ethylene glycol) monomethyl ether (MeO-PEG)] which serves as a terminal protecting group for the compound to be synthesized. The essence of this technology is that it avoids a number of difficulties found in solid phase synthesis and preserves the positive aspects of solution phase synthesis. We have demonstrated the advantages of using liquid phase synthesis through the construction of both peptide and small molecule combinatorial libraries.[19]

A leucine−enkephalin peptide sequence (YGGFL) was chosen as the first azatide mimetic to be synthesized. This pentamer was selected as the N-terminal sequence within this unit (YGGF) is common to most natural opioid peptides.[20] The successful diazatide-coupling procedure described in Scheme 2 implies N-to-C-terminal construction of the azatide. A *p*-substituted benzyl ester spacer unit that would accommodate directional synthesis on MeO-PEG and withstand the rigors of Boc-chemistry was designed (**12**; Scheme 3). It was reasoned that **12** attached to MeO-PEG would be stable against acidolysis due to the presence of the *p*-benzoate substituent, and the oligoazatide could be liberated by catalytic hydrogenation generating a free amino group. Thus methyl *p*-(hydroxymethyl)-benzoate was *O*-protected as the *tert*-butyl ether by treatment with isobutylene and acid. Subsequent hydrolysis of the methyl ester with lithium hydroxide provided **12**. Linker **12** was coupled to MeO-PEG with the aid of DCC/DMAP, and upon deprotection with trifluoroacetic acid (TFA) gave the MeO-PEG-benzyl-OH (**13**) support ready for azatide synthesis. Synthesis of the azatide pentamer $Y^aG^aG^aF^aL^a$ was accomplished in a repetitive stepwise fashion as shown in Scheme 3. Because of the unique physical properties of the MeO-PEG homopolymer, each coupling/deprotection reaction could be purified by precipitation of the modified homopolymer. Furthermore MeO-PEG allows reaction progress to be conveniently monitored by either proton-NMR spectroscopy or the Kaiser ninhydrin test.[21] On the basis of our linker strategy, the pentamer and the benzyl protecting group of aza-tyrosine could be liberated in a single step using catalytic hydrogenation to give the Boc-protected pentamer (overall yield: 56.7% from **13**). This compound was converted to the desired Leu-enkephalin azatide by treatment with trifluoroacetic acid (Scheme 3).

Tandem mass spectrometry,[22] when coupled with any soft ionization method, has emerged as an important tool for the elucidation of sequences of peptides and nucleotides. We used this technique for the sequence determination of our azatide. Thus, the Leu-enkephalin azatide was subjected to ESI-tandem mass spectrum analysis. In the acidic matrix employed for ESI experiments, Leu-enkephalin azatide would exist as a $(M + H)^+$ ion with a proton located on the α-nitrogen atom of Leu^a (i.e., the most basic residue). In the gas phase, $(M + H)^+$ ions undergo proton transfer to other basic sites to allow charge delocalization. For an azatide, proton trnasfer would preferentially occur on the more basic tertiary amide nitrogens over the secondary amide nitrogens. The protonation of a tertiary amide nitrogen causes bond cleavage between the α-nitrogen and carbonyl carbon to generate X- and A-type fragments (Figure 2). When there is no preferential protonation between two secondary amide nitrogens such as in the urea linkage involving the Gly^a residues, cleavage is possible on either side

(16) Efimov, V. A.; Kalinkina, A. L.; Chakhmakhcheva, O. G. *Nucleic Acids Res.* **1993**, *21*, 5337.

(17) We hypothesized this to be the case based on findings reported by Abeles (see ref 11). For an activated 2-R′-hydrazinecarboxylic acid, 1,1-dimethylethyl ester, the intermediate is untenable because of the carbamate's substitution pattern.



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(19) Han, H.; Wolfe, M. M.; Brenner, S.; Janda, K. D. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 6419.

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(21) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1979**, *34*, 595.

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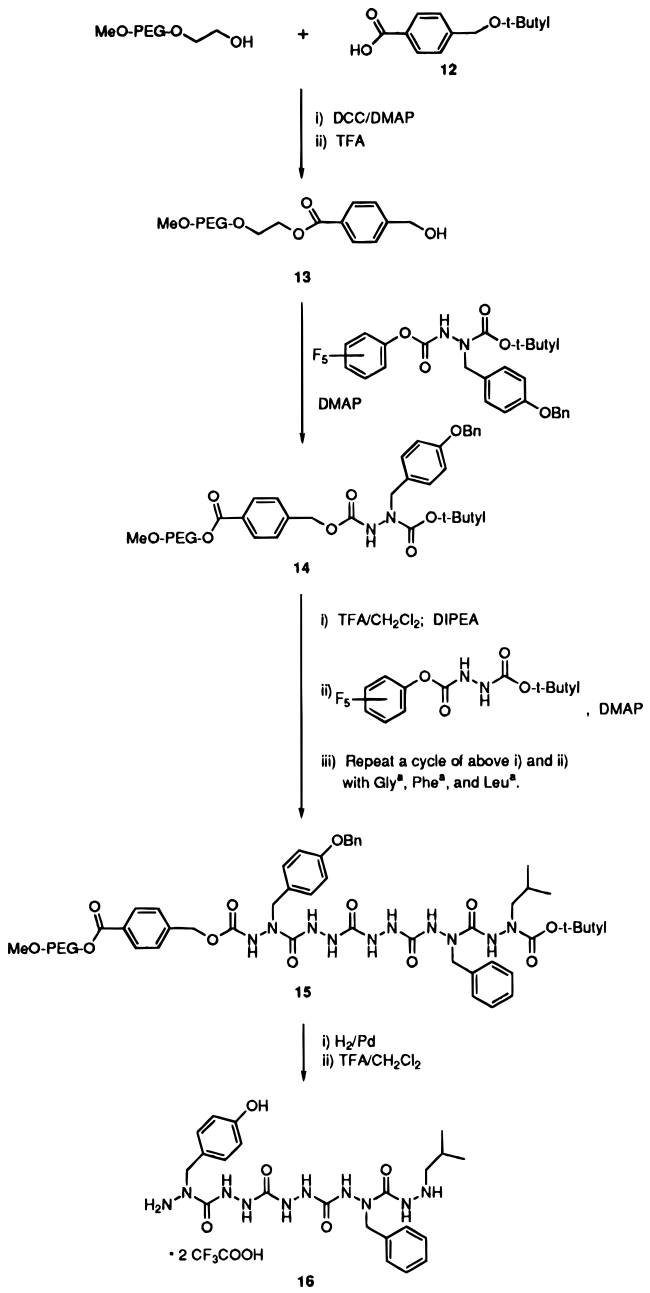
Scheme 3. MeO-PEG-Supported Leu-Enkephalin Azatide Synthesis



of the carbonyl group. Conversely, Y- and B-type fragmentations of peptides result from protonation of amide nitrogens and hence cleavage of amide bonds (Figure 2). This prediction was manifested in the collision-induced dissociation (CAD) pattern of Leu-enkephalin azatide **16** shown in Figure 3. The MS-MS of the $(M + H^+)$ ion at *m/z* 517 produced daughter peaks at *m/z* 403, 255, 197 (A-type), 321, and 263 (X-type), and MS-MS-MS on *m/z* 403 $(M - Leu^a + H^+)$ gave granddaughter peaks at *m/z* 255, 197, 139 (A-type), 207, 149 (X-type), 239, 197, and 123 (Y-type). Peaks at *m/z* 297, 149, 107, and 91 represent A-type fragments involving cleavage of side chain of Tyr^a. Mass difference between homologous A-type ions corresponds to elements -CONHNR-. Predicted *m/z* values for A_2-A_5 fragments were obtained by sequentially adding the incremental masses of Gly^a, Gly^a, Phe^a, and Leu^a to that for A_1 at *m/z* 139. A similar arguement can be made for X- and Y-type fragments, confirming the Tyr^a-Gly^a-Gly^a-Phe^a-Leu^a sequence of Leu-enkephalin azatide.

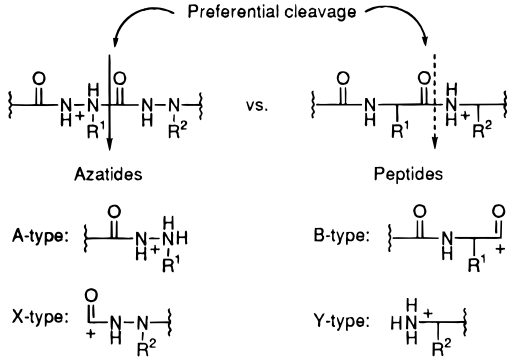


Figure 2. Fragmentation patterns of $(M + 1)^+$ ion of peptides and azatides.

The azatide oligomer sequence synthesized (Tyr^a-Gly^a-Gly^a-Phe^a-Leu^a) provided a chance to assess any biological activity that this azatide biopolymer sequence may possess (*vide supra*). Monoclonal antibody 3-E7 was raised by Meo et al. against the antigen β-endorphin and, like the δ-opioid receptor, recognized the N-terminal portion of the protein.[20] The antibody also binds tightly to [Leu^5]enkephalin [Tyr-Gly-Gly-Phe-Leu] (K_d = 7.1 nM) and a variety of related opioid peptides.[24] A competition ELISA method was used to investigate if the Tyr^a-Gly^a-Gly^a-Phe^a-Leu^a sequence could bind to IgG 3-E7.[19] At 1 mM the azatide pentamer showed no propensity to compete with the natural peptide for 3-E7. While this result at first glance appears to be disappointing, it was not completely unexpected. The bound conformation(s) of enkephalin has been studied extensively for over the past 15 years.[25] While the exact bioactive conformation of this peptide remains shrouded, it is thought that the active form of this peptide resides in some sort of a β-turn,[26] this being based on X-ray crystallographic data which showed that the glycine residues at the second and third position of enkephalin force a type I′ 4 → 1 β-turn.[27] Although we have yet to obtain an X-ray structure on **16**, physiochemical data do exist on diacylhydrazines.[28] Simple unsubstituted diacyl hydrazines (i.e., glycine azatides) contain a dihedral (ϕ) angle of ca. −175°, while *N*-substituted ones (i.e., all other azatides) possess a dihedral (ϕ) angle of ca. −110°. Taken as a whole, these data suggest that **16** should adopt a more extended conformation within the critical glycine region. In essence then this azatide oligomer would have difficulty in achieving the orientation displayed by the antigenic determinant (Tyr-Gly-Gly-Phe-Leu) that elicited IgG 3-E7, the outcome being that **16** is nonligand for 3-E7.

Summary

In conclusion, we have developed solution and liquid phase methodologies for the stepwise synthesis of azatides. In spite of our inability to show biological activity for azatide **16**, we believe that azatides will be of considerable interest as an accessible new material with potential for novel biological properties. Furthermore, the structural and pharmacological

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(28) (a) Olivato, P. R.; Guerrero, S. A. *J. Chem. Soc., Perkin Trans. II* **1983**, 1053. (b) Graybill, T. L.; Ross, J. T.; Gauvin, B. R.; Gregory, J. S.; Harris, A. L.; Ator, M. A.; Rinker, J. M.; Dolle, R. E. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1375.

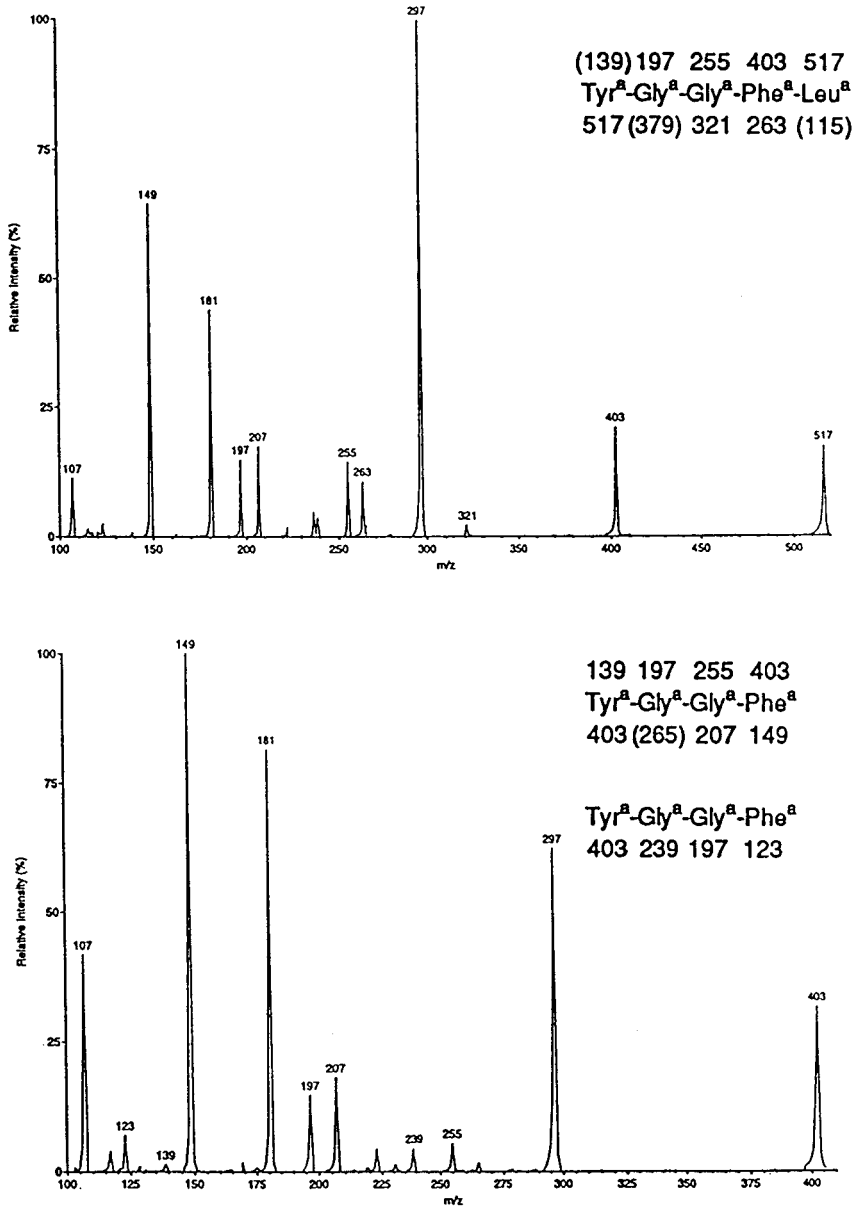

Figure 3. CAD spectra of *m/z* 517 $(M + 1)^+$ and 403 peaks for compound **16**.

properties of these azatides may provide important leads for the drug industry, and biophysical studies of these polymers could enhance our understanding of receptor−ligand interactions. Combinatorial library construction of this new biomimetic polymer may provide a means to fabricate global peptidomimetic libraries. Future studies will address these possibilities.

Experimental Section

General. Methylene chloride and methanol were dried over CaH_2 and powdered magnesium, respectively. Poly(ethylene glycol) monomethyl ether (MeO-PEG, MW = 5000) was purchased from Aldrich and dried over P_2O_5 under vacuum before use. All other solvents and chemicals were obtained from commercial sources and used without further purification, unless otherwise stated. NMR spectra were obtained on a Bruker AM-300 spectrometer. Boc-protected methylhydrazines, isopropylhydrazines, isobutylhydrazines, benzylhydrazines, (*p*-*O*-benzylhydroxybenzyl)hydrazines,[13b,15] and pentafluorophenyl carbonate[16] were prepared according to literature procedures.

General Solution Phase Diazatide-Coupling Procedure. To a stirred solution of pentafluorophenyl carbonate (50.0 mg, 13.0 mmol) in methylene chloride (5 mL) over a period of 20 min was added a solution of 1-*N*-Boc-alkylhydrazine (1 equiv) and DMAP (1 equiv) in methylene chloride (2 mL) dropwise. Upon completion of the addition, a solution of 2-*N*-Boc-alkylhydrazine (1 equiv) and DMAP (1 equiv) in methylene chloride (2 mL) was added. The resulting mixture was stirred for 30 min at room temperature. Removal of solvent and flash chromatography[23] gave the desired unsymmetrical diazatide.

Boc-Gly[a]-Gly[a]-Boc: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.35 (s, 18H), 7.33 (br s, 2H), 7.77 (s, 2H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 27.9, 81.3, 156.2, 157.1; HRMS (FAB) calcd for $C_{11}H_{22}N_4O_5{\cdot}Cs^+$ 423.0645, found 423.0655.

Boc-Ala[a]-Ala[a]-Boc (symmetrical): ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.42 (s, 18H), 3.09 (s, 6H), 7.43 (s, 2H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 28.1, 38.3, 81.5, 156.1, 157.0; HRMS (FAB) calcd for $C_{13}H_{26}N_4O_5{\cdot}Cs^+$ 451.0958, found 451.0976.

Boc-Ala[a]-Ala[a]-Boc: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.43 (s, 9H), 1.45 (s, 9H), 3.08 (s, 3H), 3.09 (s, 3H), 6.45 (br s, 1H), 7.05 and 7.62 (br s, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 28.2, 28.3, 37.9, 81.2, 81.4, 155.4, 156.6, 156.8; HRMS (FAB) calcd for $C_{11}H_{22}N_4O_5{\cdot}Cs^+$ 451.0958, found 451.0965.

Boc-Ala[a]-Phe[a]-Boc: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.37 (s, 9H), 1.41 (s, 9H), 3.1 (s, 3H), 4.50 (br s, 2H), 6.10 and 6.59 (br s, 1H), 7.22 (m, 5H), 7.37 and 7.55 (br s, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 27.8, 28.2, 38.5, 54.3, 81.5, 81.6, 127.3, 128.3, 128.6, 137.3, 155.5, 156.4, 156.5; HRMS (FAB) calcd for $C_{19}H_{30}N_4O_5{\cdot}Cs^+$ 527.1271, found 527.1289.

Boc-Ala[a]-Leu[a]-Boc: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 0.90 (d, J = 7

Hz, 6H), 1.40 (s, 9H), 1.45 (s, 9H), 1.84 (m, 1H), 3.07 (s, 3H), 3.35 (br s, 2H), 6.30 and 6.56 (br s, 1H), 7.20 and 7.36 (br s, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 19.9, 26.3, 28.1, 28.3, 38.0, 55.7, 81.1, 82.0, 154.5, 156.1, 157.6; HRMS (FAB) calcd for $C_{16}H_{32}N_4O_5{\cdot}Cs^+$ 493.1427, found 493.1447.

Boc-Leu^a-Leu^a-Boc: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 0.88 (d, J = 7 Hz, 6H), 0.90 (d, J = 7 Hz, 6H), 1.42 (s, 9H), 1.46 (s, 9H), 1.86 (m, 2H), 3.27 (br s, 4H), 6.33 and 6.57 (br s, 1H), 7.11 and 7.23 (br s, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 20.4, 20.4, 27.6, 27.8, 28.5, 28.6, 56.7, 59.5, 81.8, 82.3, 156.3, 156.5, 158.1; HRMS (FAB) calcd for $C_{19}H_{38}N_4O_5{\cdot}Cs^+$ 535.1897, found 535.1881.

Boc-Val^a-Val^a-Boc: mp 101−102 °C; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 0.09 (br s, 12H), 1.40 (s, 9H), 1.45 (s, 9H), 4.32 (br s, 1H), 4.61 (m, 2H), 6.27 (br s, 1H), 6.79 (br s, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 19.3, 19.8, 28.0, 28.3, 48.4, 48.6, 81.0, 81.6, 157.1, 157.4, 157.9; HRMS (FAB) calcd for $C_{17}H_{34}N_4O_5{\cdot}Cs^+$ 507.1584, found 507.1599.

Linker Preparation: Methyl *p*-*O*-*tert*-Butyl(hydroxymethyl)benzoate. Isobutylene was liquidified in a sealed bottle at −78 °C. A solution of sulfuric acid (0.5 mL) and methyl *p*-(hydroxymethyl)-benzoate (2.00 g, 12.0 mmol) in dry ethyl ether (20 mL) was added to the isobutylene solution (8 mL) at −78 °C and stirred overnight at room temperature. The resulting mixture was cooled to 4 °C; then ice-cooled water was added. The ether layer was dried over magnesium sulfate and evaporated to give the desired product as a white solid (2.59 g, 96.8%): mp 34−36 °C; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.29 (s, 9H), 3.89 (s, 3H), 4.49 (s, 2H), 7.40 (d, J = 6.7 Hz, 2H), 7.98 (d, J = 6.7 Hz, 2H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 27.5, 51.8, 63.4, 73.6, 126.8, 128.7, 129.5; HRMS (FAB) calcd for $C_{13}H_{18}O_5{\cdot}Cs^+$ 355.0310, found 355.0323.

***p*-*O*-*tert*-Butyl(hydroxymethyl)benzoic Acid (12).** Methyl *p*-*O*-*tert*-butyl(hydroxymethyl)benzoate (2.02 g, 9.10 mmol) was dissolved in a 0.8 M LiOH solution in methanol and H_2O (34 mL; methanol: H_2O, 3:1). The reaction mixture was stirred until the starting material disappeared as judged by TLC (methylene chloride:ethyl ether, 9:1). The reaction mixture was acidified by the addition of 1 N HCl and extracted with methylene chloride. The methylene chloride layer was dried over magnesium sulfate and then evaporated to give the desired product as a white solid (1.72 g, 90.9%): mp 147−149 °C; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.39 (s, 9H), 4.50 (s, 2H), 7.42 (d, J = 6.8 Hz, 2H), 8.06 (d, J = 6.8 Hz, 2H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 27.6, 63.6, 73.9, 127.0, 128.0, 130.3, 146.4, 171.8; HRMS (FAB) calcd for $C_{12}H_{16}O_3{\cdot}Na^+$ 231.0997, found 231.0986.

Synthesis of MeO-PEG-Linker-Y^{a}G^{a}G^{a}F^{a}L^a: Attachment of *p*-*O*-*tert*-Butyl(hydroxymethyl)benzoic Acid (12) to MeO-PEG: MeO-PEG-benzyl-OH (13). Compound **12** (125 mg, 601 μmol), MeO-PEG (1.00 g, 200 μmol), and DMAP (611 μg, 50.0 μmol) were dissolved in methylene chloride (10 mL), and DCC (124 mg, 601 μmol) was added. The resulting mixture was stirred for 12 h. The precipitated urea was filtered through Celite. Diethyl ether was slowly added to the filtrate in order to precipitate the polymer. The polymer precipitate was washed with cold absolute ethanol and ether and dried over P_2O_5 under vacuum. This solid was dissolved in trifluoroacetic acid, and the resulting solution was stirred for 9 min at room temperature. The whole reaction mixture was poured onto an ice-cold diethyl ether solution with vigorous stirring. The precipitate was collected, washed with cold absolute ethanol and diethyl ether, and dried over P_2O_5 under vacuum (935 mg, 91.1%): ^{1}H-NMR (300 MHz, $CDCl_3$) δ 4.45 (t, J = 7 Hz, 2H), 4.71 (s, 2H), 7.41 (d, J = 7 Hz, 2H), 7.98 (d, J = 7 Hz, 2H).

Construction of (*O*-Benzyl)Tyr^a-Gly^a-Gly^a-Phe^a-Leu^a-Boc on 13. A mixture of **13** (195 mg, 38.0 μmol), pentafluorophenyl carbamate of Boc-(*p*-*O*-benzylhydroxybenzyl)hydrazine (102 mg, 5 equiv), and DMAP (23.2 mg, 5 equiv) in methylene chloride (5 mL) was stirred for 24 h at room temperature. Diethyl ether was slowly added to this mixture to precipitate the polymer product **14**. The polymer product was washed with absolute ethanol and diethyl ether and dried over P_2O_5 under vacuum: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.40 (s, 9H), 4.45 (2H), 4.55 (2H), 5.00 (2H), 5.15 (2H), 6.55 (1H), 6.88 (2H), 7.15 (2H), 7.38 (7H), 8.00 (2H).

The polymer **14** was dissolved in TFA/methylene chloride and stirred for 30 min to remove the Boc-group. Precipitation with ether and a separate wash with absolute ethanol and diethyl ether followed by drying over P_2O_5 under vacuum gave the trifluoroacetate salt of (*p*-*O*-benzylhydroxybenzyl)Tyr^a-*O*-benzyl-PEG-OMe. This salt was dissolved in methylene chloride and neutralized with diisopropylethylamine (DIPEA; 1 equiv). To the resulting mixture were added the pentafluorophenyl carbamate of Boc-carbazate (5 equiv) and DMAP (5 equiv). The reaction mixture was stirred for 4 h. Precipitation with diethyl ether followed by washing with absolute ethanol and diethyl ether and then drying over P_2O_5 under vacuum gave the product Boc-Gly^a-(*O*-benzyl)-Tyr^a-*O*-benzyl-PEG-OMe. Repetition of this cycle of deprotection, neutralization, and coupling with Gly^a, Phe^a, and Leu^a produced the Leu-enkephalin azatide **15** (137 mg, 62.4% from **13**): ^{1}H-NMR (300 MHz, CD_3OD) δ 0.87 (6H), 1.42 (9H), 1.91 (1H), 4.43 (2H), 4.96 (2H), 5.12 (2H), 6.83 (2H), 7.13 (2H), 7.37 (7H), 7.95 (2H). The multiplicity of peaks is not described due to the peak broadening.

Tyr^a-Gly^a-Gly^a-Phe^a-Leu^a-Boc. Compound **15** (137 mg, 23.7 μmol) was hydrogenated with 10% Pd/C (100 mg) in methanol (5 mL) under a balloon containing 1 atm of hydrogen for 4 h. All volatiles were removed *in vacuo*, and the residue was extracted with absolute ethanol. This ethanol solution was concentrated and purified by preparative thin layer chromatography. The desired material was observed as a single band at R_f = 0.4 (13.25 mg, 90.7%; TLC solvent, methylene chloride: methanol, 9:1): ^{1}H-NMR (300 MHz, CD_3OD) δ 0.93 (d, J = 7 Hz, 6H), 1.43 (s, 9H), 1.47 (s, 9H), 1.95 (m, 1H), 3.27 (br s, 2H), 4.17 and 5.19 (br s, 2H), 4.50 (br s, 2H), 6.76 (d, J = 6.7 Hz, 2H), 7.12 (d, J = 6.7 Hz, 2H), 7.33 (m, 5H); MS *m/z* (ESI, positive) 639 $(M + Na)^+$, 617 $(M + 1)^+$.

Tyr^a-Gly^a-Gly^a-Phe^a-Leu^a·2CF$_3$COOH (16). Tyr^a-Gly^a-Gly^a-Phe^a-Leu^a-Boc (13.25 mg, 21.5 μmol) was dissolved in TFA/methylene chloride (5 mL) and stirred for 30 min. All volatiles were removed *in vacuo* to give the desired product as a white hygroscopic solid (16.0 mg, 100%): ^{1}H-NMR (300 MHz, CD_3OD) δ 1.05 (d, J = 6.7 Hz, 6H), 2.09 (m, 1H), 3.07 (br s, 2H), 4.22 and 5.26 (br s, 2H), 4.65 (br s, 2H), 6.77 (d, J = 6.8 Hz, 2H), 7.17 (d, J = 6.8 Hz, 2H), 7.35 (m, 5H); MS *m/z* (ESI, positive) 539 $(M + Na)^+$, 517 $(M + 1)^+$.

Azatide 16 Competition ELISA for Anti-β-Endorphin Monoclonal Antibody. Each well of a Costar 96-well plate that was used in the competition was initially coated with 25 μL of Tyr-Gly-Gly-Phe-Leu-CO-NH-$(CH_2)_2$-NH-CO-$(CH_2)_2$-SS-BSA (5−20 mg/mL) in 60 mM sodium bicarbonate/30 mM sodium carbonate, pH 9.3, overnight. The wells were washed 10 times with deionized water and blocked with 100 μL of 3% BSA (all in PBS with 0.5% Tween) to prevent nonspecific adsorption. After incubating for 30 min at 37 °C in a moist chamber, the 3% BSA was then shaken out and 25 μL of 3% BSA and 25 μL of **16** (competing antigen) were added to the first well and serially diluted across the plate; the same process was then continued in the first well of the second row. Well 12 was used as the positive control. The anti-β-endorphin antibody (diluted in 1% BSA/PBS with 0.5% Tween) was added to each well (25 μL), and the plate was incubated at 37 °C for 2 h. The plate was washed 20 times with deionized water, 25 μL of a 1:1000 dilution of goat anti-mouse IgG glucose oxidase conjugate (Cappel) in 1% BSA was added to each well, and the plate was incubated at 37 °C for 1 h. The plates were washed 20 times with deionized water, and bound antibody was detected by the addition of 50 μL of developing agent [0.6 mL of 20% glucose, 40 μL of 92 mM 2,2′-azinobis(3-ethylbenzothiazolinesulfonate), and 40 μL of 25 μM horseradish peroxidase in 5 mL of phosphate buffer, pH 6.0] to each well. Thirty minutes later, the plates were read at 405 nm.

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