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Ligations from Tyrosine Isopeptides via 12- to 19-Membered Cyclic Transition States

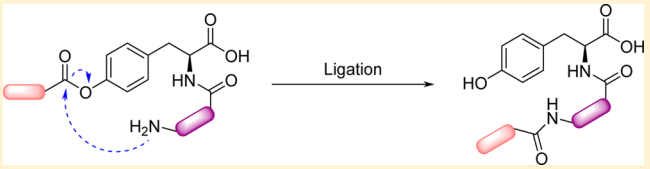
Vadim Popov,[‡] Siva S. Panda,[‡] and Alan R. Katritzky*[,‡,§]

[‡]Center for Heterocyclic Compounds, University of Florida, Department of Chemistry, Gainesville, Florida 32611-7200, United States

[§]Department of Chemistry, King Abdulaziz University, Jeddah 21589, Saudi Arabia

Supporting Information

ABSTRACT: Efficient syntheses of *O*-acyl Tyr-peptides allow chemical long-range ligation (*O*-acyl to *N*-acyl transfer) via each of 12- to 19-membered cyclic transition states. The results represent the first examples of successful isopeptide ligations starting from *O*-acyl Tyr-peptides.



INTRODUCTION

Synthetic methods for peptides are of great interest: native chemical ligation (NCL), first developed by Wieland[1] and developed by Kent,[2,3] is a chemo- and regioselective reaction of a peptide-thioester with an *N*-terminal Cys-peptide that produces a long chain polypeptide with a native amide bond at the ligation site through a rapid *S*- to *N*-acyl transfer within the initial thioester.

Alternative approaches to bypass the requirement in classical NCL of a *N*-terminal Cys-residue have included (i) traceless Staudinger ligation,[4,5] (ii) native chemical ligation with Phe- and Val-analogues bearing a sulfhydryl group at the β-position followed by removal of that group,[6−8] (iii) NCL followed by the conversion of penicillamine to Val,[7] (iv) sugar-assisted ligation,[9−11] (v) Cys free "direct aminolysis" methods,[12] and (vi) desulfurization with the formation of Ala-peptide and protein analogues.[13] However, new ligation strategies are still of significant interest for the synthesis of underivatized and post-translationally modified peptides and proteins.

While of great importance, NCL has limitations including (i) the need of a *N*-terminal Cys-residue at the ligation site to afford a peptide containing an internal Cys and (ii) the low abundance of Cys in globular proteins (1.7% of the residues).[4,14,15] Considerable effort has been devoted to the development of thiol auxiliary groups to overcome the limitation of low abundance of Cys, but subsequent ligations were found (i) difficult to complete because of steric hindrance[15−19] and (ii) problematic since extraneous groups in the ligated product may be difficult to remove.[15−19] Another approach involves the conversion of a Cys-residue into a Ser-residue after NCL,[15] but this requires post-NCL modifications.

Kiso et al.[20] reported that *O*-acyl residues within a backbone significantly altered the secondary structures of native peptides so that "*O*-acyl isopeptides" are more hydrophilic and easier to purify by HPLC than their corresponding native peptides; *N*-terminal Ser-isopeptides can rapidly generate the corresponding native peptide by $O \rightarrow N$ intramolecular acyl migration via a 5-membered transition state.[21]

Our group developed ligations of *S*-acylated Cys-peptides[22−24] and *N*-acylated Trp-peptides[25] to form native peptides through various transition states. Recently we demonstrated that such classic *O*- to *N*-acyl shifts via a 5-membered transition state in *O*-acyl Ser- and *O*-acyl Thr-peptides can be extended to 8-membered and 11-membered transition states. Thus, "traceless" chemical ligation involving *O*- to *N*-acyl shift (at a Ser site) involving neither Cys nor an auxiliary group at the ligation site is possible.[26]

We now report migrations of acyl groups from *O*-acylated Tyr-isopeptides (free of Cys) via 12- to 19-membered cyclic transition states to give natural peptides.

RESULTS AND DISCUSSION

We synthesized the monoisotripeptides **5a−c** as starting materials to study the possibility of diverse *O*- to *N*-acyl migrations via 12- to 19-membered cyclic transition states. Compounds **5a−c** were used for ligation studies via 12-, 13- and 14-membered cyclic transition states and after classical coupling of **5a−c** with α-, β- or γ-amino acids provided the starting monoisotetrapeptides **8a−e** for ligation studies via 15- to 19-membered cyclic transition states. In order to enhance migration rates, we used exclusively Gly-, β-Ala- and GABA-units in these monoisopeptide intermediates.

Preparation of the Monoisotripeptides 5a−c. Benzotriazolides **1a−c** of Boc-protected Gly, β-Ala and GABA were coupled with free Tyr **2** at 20 °C in the presence of base to give the corresponding Boc-protected dipeptides **3a−c** (69−84%) (Scheme 1). Dipeptides **3a−c** were *O*-acylated by Z-L-Ala-Bt **1d** in the presence of TEA to provide *N*-protected monoisotripeptides **4a−c** (71−84%), which after deprotection by HCl solution in 1,4-dioxane yielded the free monoisotripeptides **5a−c** (96−

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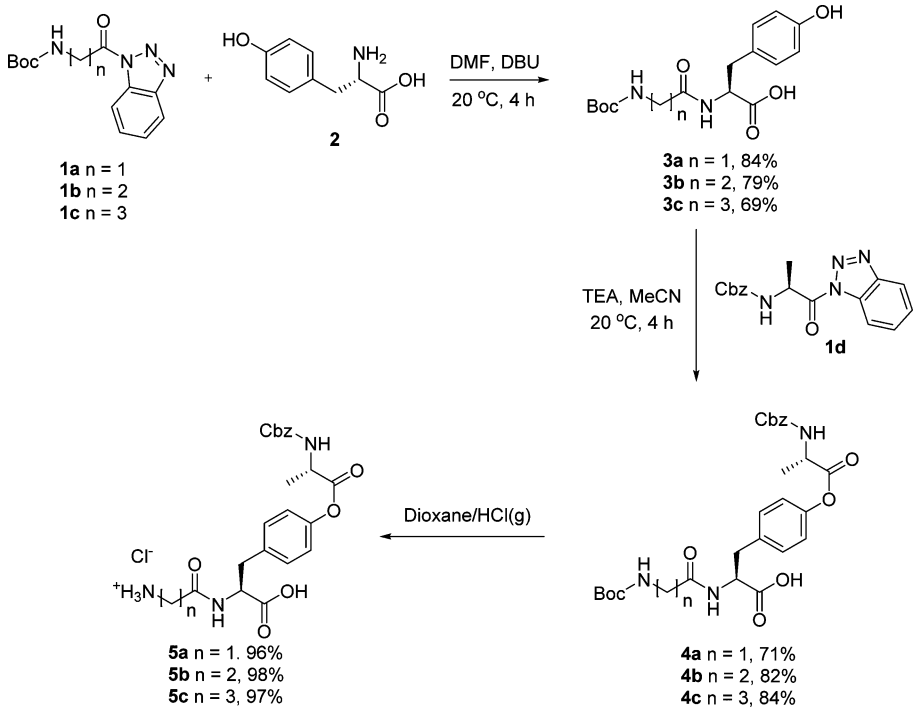
Scheme 1. Synthesis of the Monoisotripeptides 5a−c



Scheme 2. Acyl Migration of *O*-Acyl Isotripeptides 5a−c

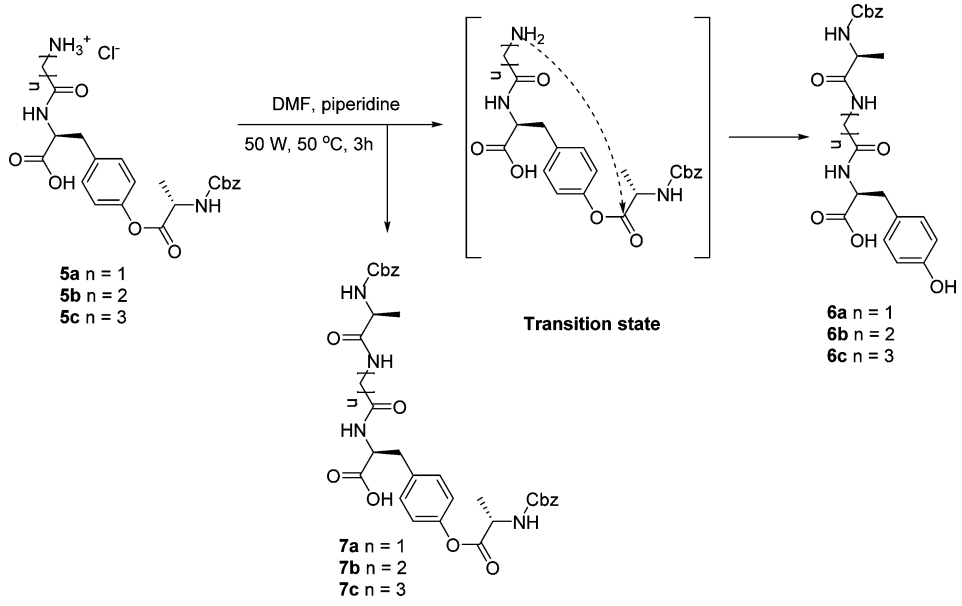


Table 1. Chemical Ligation of *O*-Acyl Isotripeptides 5a−c

			relative area (%)[a,b]			product characterization by HPLC−MS			
						ligated peptide (LP)		bis-acylated product (BA)	
react	cyclic TS size	total yield (%) of crude products isolated	react	LP	BA	LP	$[M + H]^+$ found	BA	$[M + H]^+$ found
5a	12	87	4.72	95.29	0.00	**6a**	444.2	**7a**	–
5b	13	93	5.00	85.21	9.79	**6b**	458.1	**7b**	663.2
5c	14	92	3.03	96.97	0.00	**6c**	472.2	**7c**	–

[a]Semiquantitative determination HPLC−MS. The area of ion-peak resulting from the sum of the intensities of the $[M + H]^+$ and $[M + Na]^+$ ions for each compound was integrated (corrected for starting material). [b]React = Reactant; LP = ligated peptide; BA = bis-acylated product.

98%), which were used both directly for ligation studies and also as intermediates to prepare the monoisotetrapeptides **8a−e**.

Study of the Feasibility of *O* → *N* Acyl Migrations via a 12-, 13- and 14-Membered Cyclic Transition States.

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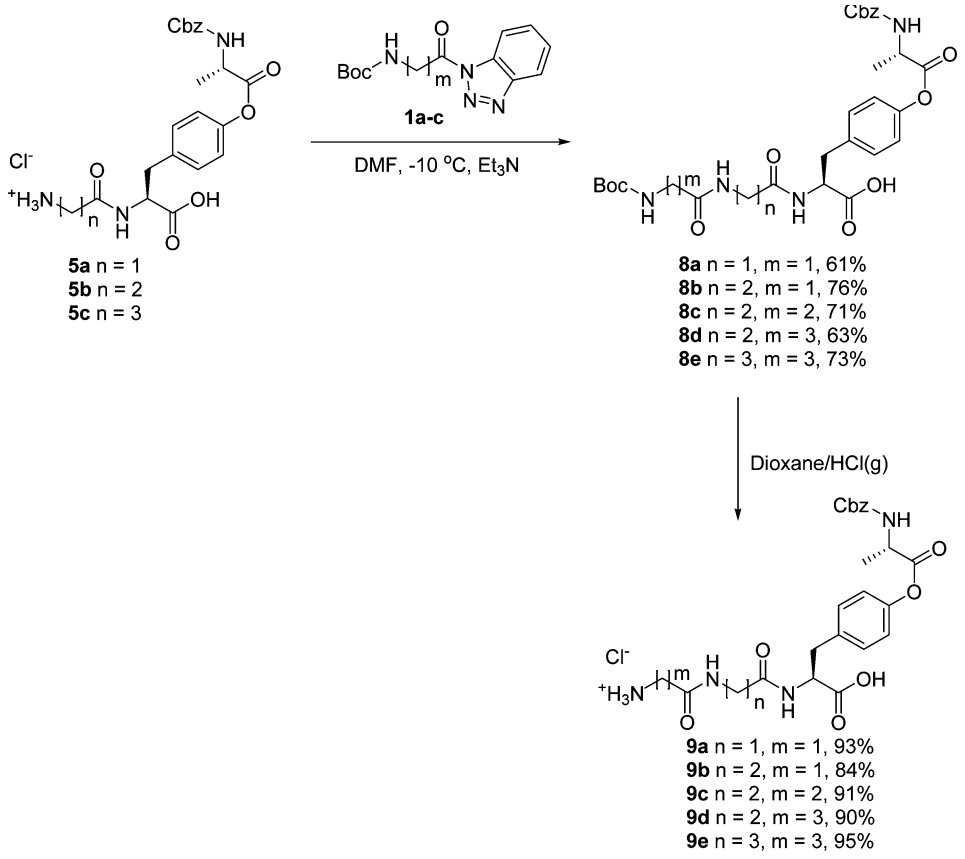
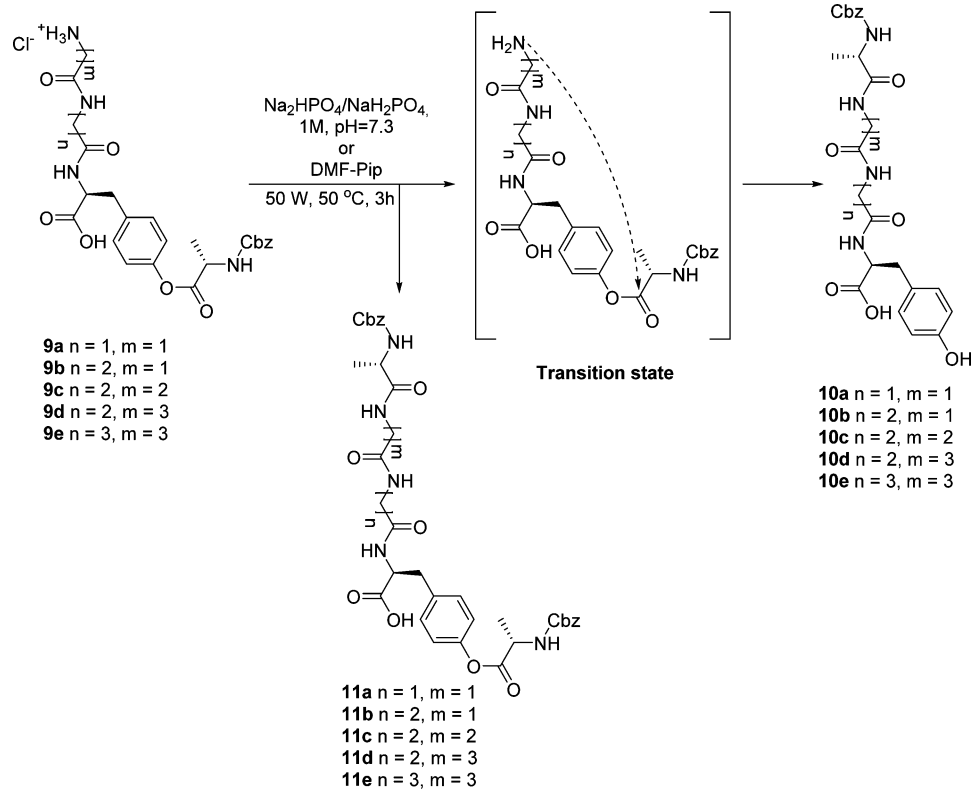
Scheme 3. Synthesis of *O*-Acyl Isotetrapeptides 9a–e



Scheme 4. Acyl Migration of *O*-Acyl Isotetrapeptides 9a–e



Attempts to ligate **5b** (Scheme 2) under aqueous conditions, (pH 7.3, 1 M buffer strength, μw 50 °C, 50 W, 3 h), failed to yield the ligated product via a 13-membered transition state. However when the reaction was carried out under microwave irradiation in

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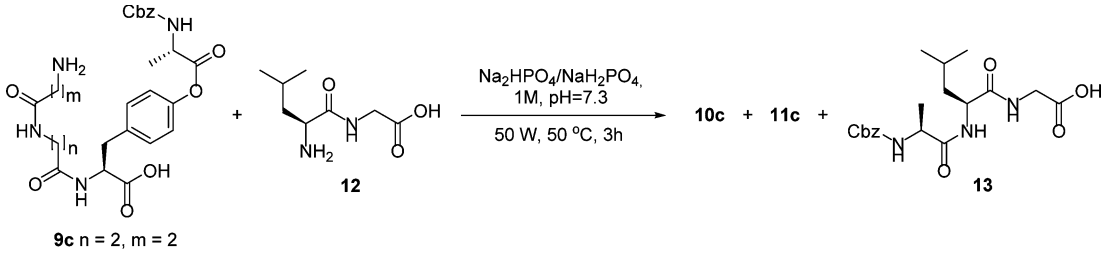
Table 2. Chemical Ligation of *O*-Acyl Isotripeptides 9a−e

			relative area (%)[a,b]			product characterization by HPLC−MS			
						ligated peptide (LP)		bis-acylated product (BA)	
react	cyclic TS size	total crude yield (%) of products isolated	react	LP	BA	LP	$[M + H]^+$ found	BA	$[M + H]^+$ found
9a	15	90	0.54	94.6	4.88	**10a**	501.0	**11a**	706.0
9b	16	86	3.62	87.0	9.43	**10b**	515.1	**11b**	720.1
9c	17	91	0.1	96.8	3.10	**10c**	529.1	**11c**	734.2
9d	18	88	0.1	99.9	0.00	**10d**	543.1	**11d**	–
9e	19	94	1.91	98.1	0.00	**10e**	557.2	**11e**	–

[a]Semiquantitative determination HPLC−MS. The area of ion-peak resulting from the sum of the intensities of the $[M + H]^+$ and $[M + Na]^+$ ions for each compound was integrated (corrected for starting material). [b]React = Reactant, LP = ligated peptide, BA = bis-acylated product

Scheme 5. Competitive Ligation Experiment



piperidine−DMF at 50 °C, 50 W for 1 h (Scheme 2), HPLC−MS indicated the formation of 85% of the desired intramolecular ligated products **6b** (retention time 28.09 min), via the 13-membered transition state together with bis-acylated product **7b** (10%, retention time 60.19 min) and 5% of the starting material **5b** (retention time 37.10 min). The retention times and fragmentation patterns of **5b** and **6b** were also studied in control experiments (HPLC−MS of pure **5b**). Thus HPLC−MS, via (−)ESI-MS/MS, confirmed that compounds **5b** and **6b**, each with MW 457, have very different fragmentation patterns, proving the formation of the intramolecular ligated product **6b**.

Similar *O* → *N* acyl migration via 12- and 14-membered cyclic transition states were studied by irradiating compound **5a** and **5c** in piperidine−DMF at 50 °C, 50 W for 3 h. The HPLC−MS results are summarized in Table 1. The results indicate the formation of compounds **6a−c** and demonstrate that *O*- to *N*-acyl group migrations via 12- and 14-membered transition states are even more highly preferred compared to intermolecular acylation then for the 13-membered cyclic transition state.

Preparation of the *O*-Acyl Isotetrapeptides 9a−e. Boc-protected monoisotetrapeptides **8a−e** were synthesized in solution phase, by coupling the benzotriazolides of Boc-protected Gly, *β*-Ala and GABA **1a−c** with the unprotected monoisotripeptides **5a−c** at −10 °C in 96, 98 and 97% yields, respectively. The Boc-group of **8a−e** were removed by stirring each with concentrated HCl in 1,4-dioxane for 2 h to afford the HCl salts of the unprotected monoisotetrapeptides **9a−e**. Compounds **8a−e** and **9a−e** were fully characterized by ^{1}H, ^{13}C NMR analysis (Scheme 3).

Study of the Feasibility of *O* → *N* Acyl Migrations via a 15- to 19-Membered Cyclic Transition States. Compounds **9a−e** reacted under aqueous conditions (pH 7.3, 1 M buffer strength, MW 50 °C, 50 W, 3 h) to produce the expected ligation products **10a−e**, in yields of 87−100% (Scheme 4). In the case of **9a**, **9b** and **9c** the intermolecular bis-acylated products **11a**, **11b** and **11c** were also formed as shown by HPLC−MS (ESI) analysis of the mixtures after ligation. Small amounts of unreacted **9a−e** were sometimes also present in the ligation mixtures. HPLC−MS, via (−)ESI-MS/MS, confirmed that the ligated products **10a−e** each produced different MS fragmentation patterns from those of the starting monoisohexapeptides **9a−e**. The relative abundances of the crude ligated mixtures as analyzed by analytical HPLC are shown in Table 2.

Competitive Ligation Experiment. To further support the intramolecular nature of the ligation of *N*-terminus unprotected *O*-isopeptides **5a−c** and **9a−e** to form native peptide **6a−c** and **10a−e**, respectively, by chemical ligation via a 12- to 19-membered transition state, we carried out the chemical ligation of *N*-terminus unprotected *O*-isotetrapeptide **9c** in the presence of 5 equiv of dipeptide **12** (H-Leu-Gly-OH) under aqueous conditions (Scheme 5). HPLC−MS analysis of the isolated crude product (S49, Supporting Information) confirmed the formation of 96% of the desired ligation product **10c** having a retention time at 42.73 along with 3% of intermolecular bis-acylated product **11c** having a retention time at 56.75. The starting material **9c** (1%, retention time 40.20) was revealed as well. *Z*-Protected tripeptide **13**, which is the *N*-acylated product of dipeptide **12**, was not observed in the HPLC−MS analysis.

■ CONCLUSION

Efficient and convenient syntheses of novel *O*-acyl isopeptides containing Tyr-residues and subsequent chemical ligation studies have demonstrated that successful intramolecular *O*- to *N*-acyl transfer via 12- to 14-membered transition states occurs in DMF-Pip solution as well as 15- to 19-membered transition states in aqueous solution. Microwave assisted isopeptide ligation offers the following advantages: (i) short reaction times (3 h) at moderate temperature (50 °C), (ii) traceless chemical ligation from Tyr-containing isopeptides, and (iii) avoidance of ligation auxiliaries. Compared with the classical native chemical ligation approach, our methodology allows the isolation of the *O*-acyl isopeptide intermediates, which may be useful in synthetic and biological applications.

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EXPERIMENTAL SECTION

All commercial materials were used without further purification. All solvents were reagent grade or HPLC grade. Melting points were determined on a capillary point apparatus equipped with a digital thermometer and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$, DMSO-d_6 or CD_3OD using a 300 MHz spectrometer (with TMS as an internal standard). Chemical shifts are reported in parts per million relative to residual solvent $CDCl_3$ (^{1}H, 7.26 ppm; ^{13}C, 77.16 ppm), DMSO-d_6 (^{1}H, 2.50 ppm; ^{13}C, 39.52 ppm), CD_3OD (^{1}H, 3.31 ppm; ^{13}C, 49.00 ppm). All ^{13}C NMR spectra were recorded with complete proton decoupling. All microwave assisted reactions were carried out with a single mode cavity Discover Microwave Synthesizer (CEM Corporation, NC). The reaction mixtures were transferred into a 10 mL glass pressure microwave tube equipped with a magnetic stirrer bar. The tube was closed with a silicon septum and the reaction mixture was subjected to microwave irradiation (Discover mode; run time 60 s.; PowerMax-cooling mode). HPLC−MS analyses were performed on reverse phase gradient Phenomenex Synergi Hydro-RP (2.1 × 150 mm; 5 um) + guard column (2 × 4 mm) or Thermoscientific Hypurity C8 (5um; 2.1 × 100 mm + guard column) using 0.2% acetic acid in H_2O/methanol as mobile phases; wavelength = 254 nm; and mass spectrometry was done with electrospray ionization (ESI).

Procedure for Preparation of Dipeptides 3a−c. L-Tyr-OH (**2**) (0.91 g, 5 mmol) and Boc-AA-Bt (**1a−c**) (5 mmol) were dissolved in mixture of DMF (5−8 mL) and DBU (1.52 g, 1.49 mL, 10 mmol) and left to stir at room temperature for 4 h. The reaction mixture was diluted with 2 N hydrochloric acid, and product was extracted with EtOAc (3 × 30 mL). The combined organic layers were washed with 2 N HCl (3 × 10 mL) and dried over sodium sulfate. Evaporation and recrystallization from diethyl ether gave desired dipeptides (**3a−c**).

Boc-Gly-L-Tyr-OH (3a). 1.42 g, 84%: mp 135−137 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 7.02 (d, J = 8.5 Hz, 2H), 6.70 (d, J = 8.5 Hz, 2H), 4.63 (dd, J = 7.3, 5.3 Hz, 1H), 3.73 (d, J = 17.1 Hz, 1H), 3.66 (d, J = 16.7 Hz, 1H), 3.08 (dd, J = 14.0, 5.2 Hz, 1H), 2.94 (dd, J = 14.0, 7.4 Hz, 1H), 1.43 (s, 9H); ^{13}C NMR (75 MHz, CD_3OD) δ 174.4, 172.1, 158.2, 157.3, 131.3, 128.5, 116.2, 80.8, 55.0, 44.4, 37.5, 28.7. Anal. Calcd for $C_{16}H_{22}N_2O_6$: C, 56.80; H, 6.55; N, 8.28. Found: C, 56.77; H, 6.57; N, 8.30.

Boc-β-Ala-L-Tyr-OH (3b). 1.39 g, 79%: mp 160−162 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 7.04 (d, J = 8.3 Hz, 2H), 6.71 (d, J = 8.4 Hz, 2H), 4.61 (dd, J = 8.7, 5.1 Hz, 1H), 3.23 (t, J = 6.7 Hz, 1H), 3.10 (dd, J = 14.0, 5.0 Hz, 1H), 2.86 (dd, J = 13.9, 8.8 Hz, 1H), 2.35 (t, J = 6.8 Hz, 2H), 1.42 (s, 9H); ^{13}C NMR (75 MHz, CD_3OD) δ 174.8, 173.6, 158.1, 157.2, 131.2, 128.9, 116.1, 80.2, 55.2, 37.9, 37.6, 36.9, 28.7. Anal. Calcd for $C_{17}H_{24}N_2O_6$: C, 57.94; H, 6.86; N, 7.95. Found: C, 58.14; H, 7.05; N, 7.78.

Boc-GABA-L-Tyr-OH (3c). 1.26 g, 69%: mp 65−67 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 7.04 (d, J = 8.5 Hz, 2H), 6.70 (d, J = 8.5 Hz, 2H), 4.60 (dd, J = 9.2, 5.0 Hz, 1H), 3.11 (dd, J = 13.9, 5.1 Hz, 1H), 2.98 (t, J = 6.8 Hz, 2H), 2.84 (dd, J = 14.0, 9.2 Hz, 1H), 2.23−2.14 (m, 2H), 1.66 (p, J = 7.0 Hz, 2H), 1.43 (s, 9H); ^{13}C NMR (75 MHz, CD_3OD) δ 175.4, 174.9, 158.5, 157.2, 131.2, 129.1, 116.1, 79.9, 55.2, 40.6, 37.6, 34.0, 28.8, 27.3. Anal. Calcd for $C_{18}H_{26}N_2O_6$: C, 59.00; H, 7.15; N, 7.65. Found: C, 58.77; H, 7.59; N, 7.55.

Procedure for Preparation of Isotripeptides 4a−c. Boc-AA-L-Tyr-OH (**3a−c**) (2 mmol) and Z-L-Ala-Bt (**1d**) (0.65 g, 2 mmol) were dissolved in mixture of acetonitrile (30 mL) and TEA (0.30 g, 0.42 mL, 3 mmol) and left to stir at room temperature for 6 h. After evaporation the reaction mixture was diluted with EtOAc (30 mL) and washed with 1 N citric acid (3 × 30 mL). Organic layer was dried over sodium sulfate and evaporated to give isotripeptides (**4a−c**).

Boc-Gly-L-Tyr(Z-L-Ala)-OH (4a). 0.77 g, 71%: mp 62−64 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.33 (s, 5H), 7.15 (d, J = 8.1 Hz, 2H), 6.96 (d, J = 8.1 Hz, 2H), 5.62 (d, J = 7.8 Hz, 1H), 5.49 (s, 1H), 5.25−4.99 (m, 2H), 4.80 (s, 1H), 4.58 (p, J = 7.2 Hz, 1H), 3.92−3.56 (m, 2H), 3.27−2.89 (m, 2H), 1.53 (d, J = 6.9 Hz, 3H), 1.41 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.6, 171.9, 170.1, 156.5, 156.0, 149.5, 136.2, 134.2, 130.6, 128.7, 128.2, 121.4, 80.6, 67.2, 53.8, 50.0, 36.9, 29.3, 28.4, 18.5. Anal. Calcd for $C_{27}H_{33}N_3O_9$: C, 59.66; H, 6.12; N, 7.73. Found: C, 59.45; H, 6.22; N, 7.72.

Boc-β-Ala-L-Tyr(Z-L-Ala)-OH (4b). 0.91 g, 82%: mp 124−125 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.33 (s, 5H), 7.15 (d, J = 7.7 Hz, 2H), 6.97 (d, J = 7.4 Hz, 1H), 6.76 (br s, 1H), 5.59 (d, J = 7.1 Hz, 1H), 5.29 (s, 1H), 5.14 (d, J = 12.0 Hz, 1H), 5.09 (d, J = 15.2 Hz, 1H), 4.80 (q, J = 6.1 Hz, 1H), 4.58 (t, J = 6.9 Hz, 1H), 3.39−3.24 (m, 2H), 3.19 (dd, J = 13.8, 5.1 Hz, 1H), 3.01 (dd, J = 13.4, 6.2 Hz, 1H), 2.36 (s, 2H), 1.53 (d, J = 6.9 Hz, 3H), 1.42 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.7, 171.9, 156.6, 155.9, 149.4, 136.2, 134.4, 130.6, 128.7, 128.3, 128.2, 121.3, 80.0, 67.2, 53.4, 50.0, 37.0, 36.4, 28.5, 18.5. Anal. Calcd for $C_{28}H_{35}N_3O_9$: C, 60.31; H, 6.33; N, 7.54. Found: C, 60.27; H, 6.59; N, 7.47.

Boc-GABA-L-Tyr(Z-L-Ala)-OH (4c). 0.96 g, 84%: mp 134−136 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.41−7.22 (m, 7H), 7.00 (d, J = 8.3 Hz, 2H), 5.11 (s, 2H), 4.68 (dd, J = 9.2, 4.9 Hz, 1H), 4.41 (q, J = 7.3 Hz, 1H), 3.22 (dd, J = 14.0, 4.9 Hz, 1H), 3.09−2.88 (m, 3H), 2.21−2.13 (m, 2H), 1.65 (p, J = 7.1 Hz, 2H), 1.52 (d, J = 7.3 Hz, 3H), 1.43 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.4, 174.5, 173.4, 158.4, 150.9, 138.1, 136.4, 131.3, 129.4, 128.9, 128.7, 122.4, 79.9, 67.6, 54.8, 51.2, 40.6, 37.7, 34.0, 28.8, 27.3, 17.4. Anal. Calcd for $C_{29}H_{37}N_3O_9$: C, 60.93; H, 6.52; N, 7.35. Found: C, 60.57; H, 6.57; N, 7.15.

Procedure for the Preparation of Hydrogen Chlorides of Unprotected Isotripeptides 5a−c. Boc-protected isotripeptides (**4a−c**) (1.00 mmol) were dissolved in 4 N HCl in 1,4-dioxane (15 mL) at 20 °C and stirred for 2 h. The reaction mixture was evaporated, and the residue was recrystallized from diethyl ether to give the corresponding hydrogen chloride salts of unprotected isodipeptides (**5a−c**).

H-Gly-L-Tyr(Z-L-Ala)-OH Hydrochloride (5a). 0.46 g, 96%: mp 73−75 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.92 (d, J = 8.0 Hz, 1H), 8.23 (br s, 3H), 7.99 (d, J = 6.6 Hz, 1H), 7.38−7.24 (m, 7H), 6.99 (d, J = 7.9 Hz, 2H), 5.06 (s, 2H), 4.56−4.45 (m, 1H), 4.31 (p, J = 6.8 Hz, 1H), 3.62−3.41 (m, 2H), 3.11 (dd, J = 13.7, 3.9 Hz, 1H), 2.91 (dd, J = 13.3, 9.0 Hz, 1H), 1.41 (d, J = 7.1 Hz, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.3, 171.4, 166.0, 156.1, 149.2, 137.0, 135.0, 130.4, 128.5, 128.0, 127.9, 121.3, 65.7, 53.9, 49.7, 42.8, 36.1, 16.8; HRMS (+ESI-TOF) m/z for $C_{22}H_{26}N_3O_7$ $[M - HCl + H]^+$ calcd 444.1765, found 444.1764.

H-β-Ala-L-Tyr(Z-L-Ala)-OH Hydrochloride (5b). 0.48 g, 98%: mp 182−184 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.55 (d, J = 8.0 Hz, 1H), 8.05−7.80 (m, 4H), 7.35−7.25 (m, 7H), 6.96 (d, J = 8.1 Hz, 2H), 5.05 (s, 2H), 4.47−4.38 (m, 1H), 4.30 (p, J = 6.5 Hz, 1H), 3.06 (dd, J = 13.8, 4.6 Hz, 1H), 2.90−2.76 (m, 3H), 2.65−2.35 (m, 3H), 1.40 (d, J = 7.2 Hz, 3H); ^{13}C NMR (300 MHz, DMSO-d_6) δ 172.8, 171.8, 169.5, 156.0, 149.0, 136.9, 135.4, 130.2, 128.4, 127.9, 127.8, 121.2, 65.6, 53.6, 49.7, 36.1, 35.2, 31.9, 16.8. Anal. Calcd for $C_{23}H_{28}ClN_3O_7$: C, 55.93; H, 5.71; N, 8.51. Found: C, 55.62; H, 5.82; N, 8.42.

H-GABA-L-Tyr(Z-L-Ala)-OH Hydrochloride (5c). 0.49 g, 97%: mp 164−166 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 7.47−7.14 (m, 7H), 7.00 (d, J = 8.2 Hz, 2H), 5.12 (s, 2H), 4.72 (dd, J = 9.9, 4.4 Hz, 1H), 4.41 (q, J = 7.2 Hz, 1H), 3.33−3.21 (m, 1H), 2.94 (dd, J = 13.9, 10.1 Hz, 1H), 2.88−2.65 (m, 2H), 2.42−2.20 (m, 2H), 1.82 (p, J = 7.0 Hz, 2H), 1.53 (d, J = 7.4 Hz, 3H); ^{13}C NMR (300 MHz, CD_3OD) δ 174.4, 174.3, 173.7, 158.5, 150.9, 138.1, 136.5, 131.4, 129.5, 129.0, 128.7, 122.5, 67.7, 54.8, 51.3, 40.2, 37.7, 33.4, 24.3, 17.3; HRMS (+ESI-TOF) m/z for $C_{24}H_{30}ClN_3O_7$ $[M - HCl + H]^+$ calcd 472.2078, found 472.2093.

Procedure for the Preparation of Isotetrapeptides 8a−c. Unprotected isodipeptides (**5a−c**) (1 mmol) and Boc-AA-Bt (1 mmol) were dissolved in DMF (5 mL) at −10 °C; TEA (0.15 g, 0.21 mL, 1.50 mmol) was added, and the reaction mixture was stirred at room temperature for 8 h. The mixture was diluted with 2 N HCl and extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with 2 N HCl (3 × 10 mL) and then dried over sodium sulfate. Evaporation and recrystallization from diethyl ether gave isotetrapeptides (**8a−e**).

Boc-Gly-Gly-L-Tyr(Z-L-Ala)-OH (8a). 0.37 g, 61%: mp 71−73 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 7.49−7.12 (m, 7H), 7.00 (d, J = 7.4 Hz, 2H), 5.12 (s, 2H), 4.72−4.52 (m, 1H), 4.41 (q, J = 6.9 Hz, 1H), 3.98−3.63 (m, 4H), 3.17 (dd, J = 13.8, 4.7 Hz, 1H), 3.03 (dd, J = 13.8, 9.1 Hz, 1H), 1.52 (d, J = 7.1 Hz, 3H), 1.45 (s, 9H); ^{13}C NMR (75 MHz, CD_3OD) δ 174.5, 173.4, 172.9, 171.2, 158.5, 158.4, 150.9, 138.1, 136.3,

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131.4, 129.4, 129.0, 128.7, 122.4, 80.8, 67.6, 55.3, 51.3, 44.8, 43.2, 37.7, 28.7, 17.4. Anal. Calcd for $C_{29}H_{36}N_4O_{10}$: C, 57.99; H, 6.04; N, 9.33. Found: C, 57.72; H, 6.28; N, 9.32.

Boc-Gly-β-Ala-L-Tyr(*Z*-L-Ala)-OH (8b). 0.47 g, 76%: mp 108−110 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 7.51−7.17 (m, 7H), 7.01 (d, *J* = 8.0 Hz, 2H), 5.11 (s, 2H), 4.69 (dd, *J* = 8.7, 5.0 Hz, 1H), 4.42 (q, *J* = 7.1 Hz, 1H), 3.65 (s, 2H), 3.38 (q, *J* = 6.2 Hz, 2H), 3.21 (dd, *J* = 13.8, 4.7 Hz, 1H), 2.97 (dd, *J* = 13.8, 9.1 Hz, 1H), 2.38 (q, *J* = 6.0 Hz, 2H), 1.51 (d, *J* = 7.2 Hz, 4H), 1.44 (s, 9H); ^{13}C NMR (75 MHz, CD_3OD) δ 174.4, 173.6, 173.4, 172.4, 158.3, 158.2, 150.9, 138.0, 136.3, 131.2, 129.4, 128.9, 128.7, 122.4, 80.7, 67.6, 54.8, 51.2, 44.6, 37.6, 36.8, 36.2, 28.7, 17.4. Anal. Calcd for $C_{30}H_{38}N_4O_{10}$: C, 58.62; H, 6.23; N, 9.12. Found: C, 58.69; H, 5.77; N, 9.36.

Boc-β-Ala-β-Ala-L-Tyr(*Z*-L-Ala)-OH (8c). 0.45 g, 71%: mp 125−126 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 7.50−7.15 (m, 7H), 7.00 (d, *J* = 8.2 Hz, 2H), 5.11 (s, 2H), 4.70 (dd, *J* = 9.1, 4.9 Hz, 1H), 4.42 (q, *J* = 7.2 Hz, 1H), 3.43−3.15 (m, 5H), 2.97 (dd, *J* = 14.0, 9.3 Hz, 1H), 2.47−2.25 (m, 4H), 1.52 (d, *J* = 7.3 Hz, 3H), 1.42 (s, 9H); ^{13}C NMR (75 MHz, CD_3OD) δ 174.5, 173.8, 173.6, 173.4, 158.3, 158.1, 150.9, 138.0, 136.3, 131.2, 129.4, 128.9, 128.7, 122.4, 80.1, 67.6, 54.8, 51.2, 38.0, 37.6, 37.3, 36.9, 36.3, 28.8, 17.4; HRMS (ESI) calcd for $C_{31}H_{40}N_4O_{10}$ $[M + H]^+$ 629.2817, found 629.2818.

Boc-GABA-β-Ala-L-Tyr(*Z*-L-Ala)-OH (8d). 0.40 g, 63%: mp 169−171 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 7.41−7.25 (m, 7H), 7.01 (d, *J* = 8.4 Hz, 2H), 5.12 (s, 2H), 4.69 (dd, *J* = 9.2, 5.0 Hz, 1H), 4.42 (q, *J* = 7.3 Hz, 1H), 3.40−3.31 (m, 2H), 3.22 (dd, *J* = 13.9, 4.9 Hz, 1H), 3.12−2.90 (m, 3H), 2.48−2.31 (m, 2H), 2.14 (t, *J* = 7.5 Hz, 2H), 1.71 (p, *J* = 7.1 Hz, 2H), 1.52 (d, *J* = 7.3 Hz, 3H), 1.42 (s, 9H); ^{13}C NMR (75 MHz, CD_3OD) δ 175.6, 174.5, 173.6, 173.4, 158.4, 151.0, 138.1, 136.4, 131.2, 129.4, 129.0, 128.7, 122.4, 79.9, 67.6, 54.9, 51.3, 40.8, 37.6, 36.9, 36.3, 34.3, 28.8, 27.2, 17.4. Anal. Calcd for $C_{32}H_{42}N_4O_{10}$: C, 59.80; H, 6.59; N, 8.72. Found: C, 59.83; H, 6.64; N, 8.69.

Boc-GABA−GABA-L-Tyr(*Z*-L-Ala)-OH (8e). 0.48 g, 73%: mp 98−100 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 7.45−7.15 (m, 7H), 7.00 (d, *J* = 8.2 Hz, 2H), 5.11 (s, 2H), 4.70 (dd, *J* = 9.5, 4.9 Hz, 1H), 4.42 (q, *J* = 7.3 Hz, 1H), 3.23 (dd, *J* = 14.2, 4.9 Hz, 1H), 3.13−2.90 (m, 5H), 2.18 (t, *J* = 6.7 Hz, 4H), 1.70 (dp, *J* = 14.1, 6.8 Hz, 4H), 1.52 (d, *J* = 7.3 Hz, 3H), 1.43 (s, 9H); ^{13}C NMR (75 MHz, CD_3OD) δ 175.5, 175.3, 174.5, 173.4, 158.4, 150.9, 138.1, 136.3, 131.2, 129.4, 129.0, 128.7, 122.4, 79.9, 67.6, 54.7, 51.3, 40.8, 39.7, 37.7, 34.3, 34.1, 28.8, 27.3, 26.6, 17.4. Anal. Calcd for $C_{33}H_{44}N_4O_{10}$: C, 60.35; H, 6.75; N, 8.53. Found: C, 60.08; H, 6.88; N, 8.81.

Procedure for the Preparation of Hydrogen Chlorides of Unprotected Isotetrapeptides 9a−e. Boc-protected isotetrapeptides (**8a−c**) (1 mmol) were dissolved in 4 N HCl in 1,4-dioxane (15 mL) and stirred at 0 °C for 2 h. After evaporation the residue was recrystallized from diethyl ether to give the corresponding hydrogen chloride salts of unprotected isodipeptides (**9a−e**).

H-Gly-Gly-L-Tyr(*Z*-L-Ala)-OH Hydrochloride (9a). 0.50 g, 93%: mp 78−80 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 7.41−7.19 (m, 7H), 7.00 (d, *J* = 7.8 Hz, 2H), 5.12 (s, 2H), 4.67 (dd, *J* = 7.5, 5.4 Hz, 1H), 4.41 (q, *J* = 7.3 Hz, 1H), 3.92 (d, *J* = 3.3 Hz, 2H), 3.72 (s, 3H), 3.20 (dd, *J* = 13.6, 4.2 Hz, 1H), 3.02 (dd, *J* = 13.6, 8.8 Hz, 1H), 1.52 (d, *J* = 7.2 Hz, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ 174.3, 173.5, 170.8, 167.8, 158.5, 151.0, 138.1, 136.2, 131.4, 129.4, 129.0, 128.7, 122.4, 67.7, 55.2, 51.3, 43.0, 41.5, 37.7, 17.4; HRMS (+ESI-TOF) *m*/*z* for $C_{24}H_{29}ClN_4O_8$ $[M - HCl + H]^+$ calcd 501.1980, found 501.1984.

H-Gly-β-Ala-L-Tyr(*Z*-L-Ala)-OH Hydrochloride (9b). 0.46 g, 84%: mp 104−106 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 7.45−7.19 (m, 7H), 7.01 (d, *J* = 7.8 Hz, 2H), 5.11 (s, 2H), 4.69 (dd, *J* = 8.5, 4.6 Hz, 1H), 4.42 (q, *J* = 7.1 Hz, 1H), 3.65 (s, 2H), 3.44 (t, *J* = 6.3 Hz, 2H), 3.22 (dd, *J* = 13.6, 4.1 Hz, 1H), 2.99 (dd, *J* = 13.7, 9.3 Hz, 1H), 2.56−2.31 (m, 2H), 1.52 (d, *J* = 7.2 Hz, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ 174.6, 173.5, 173.4, 167.1, 158.4, 150.8, 138.0, 136.3, 131.3, 129.4, 128.9, 128.7, 122.4, 67.6, 54.9, 51.2, 41.6, 37.5, 36.9, 36.0, 17.4; HRMS (ESI) (+ESI-TOF) *m*/*z* for $C_{25}H_{31}ClN_4O_8$ $[M - HCl + H]^+$ calcd 513.1991, found 513.2004.

H-β-Ala-β-Ala-L-Tyr(*Z*-L-Ala)-OH Hydrochloride (9c). 0.51 g, 91%: mp 114−116 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 7.46−7.19 (m, 7H), 7.00 (d, *J* = 7.9 Hz, 2H), 5.11 (s, 2H), 4.68 (dd, *J* = 9.2, 5.0 Hz, 1H), 4.41 (q, *J* = 7.2 Hz, 1H), 3.39 (t, *J* = 6.7 Hz, 2H), 3.27−3.10 (m, 3H), 2.99 (dd, *J* = 14.2, 9.3 Hz, 1H), 2.56 (t, *J* = 6.4 Hz, 2H), 2.42 (hept, *J* = 7.8, 7.2 Hz, 3H), 1.52 (d, *J* = 7.3 Hz, 3H); ^{13}C NMR (300 MHz, CD_3OD) δ 174.5, 173.6, 173.5, 172.1, 158.4, 150.9, 138.1, 136.3, 131.3, 129.4, 129.0, 128.7, 122.4, 67.6, 54.9, 51.3, 37.6, 37.2, 36.9, 36.2, 32.8, 17.4; HRMS (+ESI-TOF) *m*/*z* for $C_{26}H_{33}ClN_4O_8$ $[M - HCl + H]^+$ calcd 529.2293, found 529.2301.

H-GABA-β-Ala-L-Tyr(*Z*-L-Ala)-OH Hydrochloride (9d). 0.52 g, 90%: mp 116−118 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 7.38−7.23 (m, 7H), 7.01 (d, *J* = 8.2 Hz, 2H), 5.12 (s, 2H), 4.68 (dd, *J* = 9.1, 4.9 Hz, 1H), 4.41 (q, *J* = 7.2 Hz, 1H), 3.37 (t, *J* = 6.4 Hz, 2H), 3.22 (dd, *J* = 14.0, 4.7 Hz, 1H), 3.04−2.88 (m, 3H), 2.40 (q, *J* = 6.6 Hz, 2H), 2.30 (t, *J* = 6.9 Hz, 3H), 1.90 (p, *J* = 6.9 Hz, 3H), 1.52 (d, *J* = 7.3 Hz, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ 174.4, 173.7, 173.5, 173.5, 157.3, 150.9, 138.1, 136.1, 131.1, 129.4, 128.9, 128.7, 122.5, 116.2, 67.5, 55.5, 50.9, 40.4, 37.6, 36.9, 36.1, 33.7, 24.3, 17.6; HRMS (+ESI-TOF) *m*/*z* for $C_{27}H_{35}ClN_4O_8$ $[M - HCl + H]^+$ calcd 541.2304, found 541.2315.

H-GABA−GABA-β-Ala-L-Tyr(*Z*-L-Ala)-OH (9e). 0.56 g, 95%: mp 81−83 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 7.40−7.22 (m, 7H), 7.00 (d, *J* = 7.9 Hz, 2H), 5.12 (s, 2H), 4.68 (dd, *J* = 9.2, 4.8 Hz, 1H), 4.41 (q, *J* = 7.2 Hz, 1H), 3.23 (dd, *J* = 14.0, 4.6 Hz, 1H), 3.11 (t, *J* = 6.6 Hz, 2H), 3.01−2.91 (m, 3H), 2.36 (t, *J* = 6.5 Hz, 2H), 2.21 (t, *J* = 7.2 Hz, 2H), 2.00−1.86 (m, 2H), 1.70 (p, *J* = 6.8 Hz, 2H), 1.52 (d, *J* = 7.2 Hz, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ 175.3, 174.4, 173.6, 173.3, 158.3, 157.3, 150.9, 138.1, 136.1, 131.2, 131.1, 129.4, 128.9, 128.7, 122.4, 116.2, 67.6, 55.0, 51.3, 40.4, 39.7, 37.5, 33.9, 33.7, 26.5, 24.4, 17.4 HRMS (+ESI-TOF) *m*/*z* for $C_{28}H_{37}ClN_4O_8$ $[M - HCl + H]^+$ calcd 557.2606, found 557.2610.

General Procedure for Chemical Ligation of *O*-Acyl-isotripeptides 5a−e in DMF−Piperidine. Isotripeptides (**5a−c**) (0.20 mmol) were each dissolved in a mixture of DMF−piperidine (5 mL/1.5 mL), and the mixture was irradiated with microwave (50 °C, 50 W, 3 h) in a microwave tube. After cooling to room temperature the reaction mixtures were acidified with 2 N HCl to pH = 1. Each mixture was extracted with ethyl acetate (3 × 10 mL), the combined organic extracts were dried over sodium sulfate, and the solvent was removed under reduced pressure. Each ligation mixture was weighed, and then a solution in methanol (1 mg mL^{-1}) was analyzed by HPLC−MS.

General Procedure for Chemical Ligation of *O*-Acyl-isotetrapeptides 9a−e in Buffer. Isotetrapeptides (**9a−e**) (0.20 mmol) were each suspended in deoxygenated phosphate buffer (NaH_2PO_4/Na_2HPO_4) (1 M, pH = 7.4, 8 mL) and irradiated with microwave (50 °C, 50 W, 3 h). Each reaction mixture was allowed to cool to room temperature, acidified with 2 N HCl to pH = 1, and extracted with ethyl acetate (3 × 10 mL). The combined organic extracts were dried over Na_2SO_4, and the solvent was removed under reduced pressure. Each ligation mixture was weighed, and a solution in methanol (1 mg mL^{-1}) was analyzed by HPLC−MS.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H, ^{13}C NMR and CHN/HRMS for all the novel compounds and the chromatograms from the HPLC experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: katritzky@chem.ufl.edu.

Notes

The authors declare no competing financial interest.

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