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N^{α}-Fmoc-Protected ω-Azido- and ω-Alkynyl-L-amino Acids as Building Blocks for the Synthesis of "Clickable" Peptides

Alexandra Le Chevalier Isaad,[a,b] Francesca Barbetti,[c] Paolo Rovero,[a,d] Anna Maria D'Ursi,[e] Mario Chelli,[a,b] Michael Chorev,[f,g] and Anna Maria Papini*[a,b]

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The growing interest in the 1,4-disubstituted-1,2,3-triazolyl moiety as an amide bond surrogate and its formation through very mild, chemoselective, and bioorthogonal Cu^{I}-catalyzed Huisgen 1,3-dipolar [3+2] cycloaddition of an alkynyl to an azido function, presented an unmet need for specifically designed α-amino-acid-derived building blocks. Herein we report the synthesis of unnatural homologous series of N^{α}-Fmoc-protected ω-yne- and ω-azido-L-amino acids compatible with the Fmoc/*t*Bu-based solid-phase peptide synthesis. These building blocks can be incorporated into pseudopeptides that can serve as precursors of inter- and intramolecular click reactions. The homologous N^{α}-Fmoc-ω-azido-L-amino acids were prepared from either the ω-amino or the ω-hydroxy precursors by the respective diazo-transfer reactions and sequential nucleophilic substitutions initially by halide followed by azide. The homologous N^{α}-Fmoc-ω-yne-L-amino acids were prepared by alkylation of a chiral auxiliary, which is a Ni^{II} complex of Schiff base derived from glycine and (*S*)-2-(*N*-benzylprolyl)aminobenzophenone, with ω-bromoalkynes. These building blocks will be instrumental for constructing side-chain modified peptides, interside-chain peptide chimera, peptide small molecule conjugates, and cyclopeptides, which were cyclized through 1,4-disubstituted 1,2,3-triazolyl-containing side-chain-to-side-chain bridges.

Introduction

Endogenous as well as exogenous post-translational modifications of proteins and peptides are recognized as important means to alter bioactivity profiles, recognition, metabolism, immunogenicity, physicochemical properties, and to introduce affinity tags and labels. Methods such as expression of proteins containing non-coded amino acids,[1] native and expressed chemical ligations,[2] employment of bioorthogonal chemical reporters and reactions,[3] and introduction of genetically encoded functional tags[4] present the different approaches explored for specific introduction of post-translational modifications. To this end there are several reports in which non-coded α-amino acids modified by ω-azido and ω-alkyne functionalities were used for bioorthogonal introduction of reporters in peptides and proteins.[5–7]

The unique chemoselectivity of the alkyne and azido functionalities results in a very high degree of bioorthogonality.[5–9] This is characterized by their high stability in water and lack of reactivity toward all naturally abundant functional groups that may be present in natural biopolymers.[5–9] The prototypical click reaction, the so called Cu^{I}-catalyzed Huisgen 1,3-dipolar [3+2] cycloaddition between an azido and a terminal alkynyl function generates the 1,4-disubstituted-1,2,3-triazolyl moiety. It is carried out at room temperature, tolerates the presence of water and results in high yields and excellent regioselectivity.[10–12]

The ω-azido- and ω-alkynyl-α-amino acids are used to enable modifications such as intramolecular side-chain-to-side-chain cyclizations,[8–13] macrocyclizations,[6e] conjugation of carbohydrates to amino acids[5] and peptides,[6j] generation of other bioconjugates,[14] and incorporation into recombinant proteins by either the mutated methionyl-tRNA synthetase[15] or the orthogonal amber tRNA/tRNA synthetase pair.[6d,16] To date, the diazo-transfer reaction is frequently used to generate α-azido-acids in solution[17,18] and α-azido-peptides on solid support.[17] However, ω-azido-α-amino acids such as β-azidoalanine were prepared

[a] Laboratory of Peptide & Protein Chemistry & Biology, Polo Scientifico e Tecnologico, University of Firenze, 50019 Sesto Fiorentino, Italy
Fax: +39-055-457-3584
E-mail: annamaria.papini@unifi.it
[b] Dipartimento di Chimica Organica and CNR-ICCOM, Polo Scientifico e Tecnologico, University of Firenze
Via della Lastruccia 13, 50019 Sesto Fiorentino, Italy
[c] Toscana Biomarkers Srl
Via Fiorentina 1, 53100 Siena, Italy
[d] Dipartimento di Scienze Farmaceutiche, Polo Scientifico e Tecnologico, University of Firenze,
Via Ugo Schiff 3, 50019 Sesto Fiorentino, Italy
[e] Dipartimento di Scienze Farmaceutiche, University of Salerno, Via Ponte Don Melillo 11c, 84089 Salerno, Italy
[f] Laboratory for Translational Research, Harvard Medical School,
One Kendal Square, Building 600, Cambridge, MA 02139, USA
[g] Department of Medicine, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, USA

from either the salt of α-amino-β-propiolactone,[19] the protected serinol[5] or diazo-transfer reaction on the N^{α}-Fmoc-protected α,β-diaminopropionic acid.[9] The ω-alkynyl-α-amino acids, in contrast to the commercially available propargylglycine, such as the racemic 2-aminohexynoic acid and the optically active 2-aminopentynoic acid were prepared from diethyl (acetylamino)malonate[20] and protected homoserinal,[5] respectively. Evidently, there is an unmet need for syntheses that will furnish a series of homologous enantiomerically pure N^{α}-protected ω-azido- and ω-alkynyl-α-amino acids as building blocks for the rapidly expanding array of applications mentioned above.

Recently, we reported the use of N^{α}-Fmoc-L-Nle(ε-N_3)-OH together with N^{α}-Fmoc-L-propargylglycine (N^{α}-Fmoc-L-Pra-OH) in the synthesis of cyclic peptide via *i*-to-(*i*+4) intramolecular side-chain-to-side-chain azido-to-alkynyl Cu^{I}-catalyzed Huisgen 1,3-dipolar [3+2] cycloaddition generating a topologic mimic of the helical structure stabilized by the corresponding *i*-to-(*i*+4) intramolecular side-chain-to-side-chain lactam-bridged cyclopeptide.[13]

Herein we report the development of efficient and convenient synthetic pathways to non-coded N^{α}-Fmoc-ω-azido- and N^{α}-Fmoc-ω-alkynyl-L-amino acids suitable for Fmoc/*t*Bu solid-phase peptide synthesis (SPPS) (Figure 1).

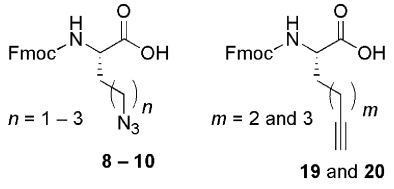


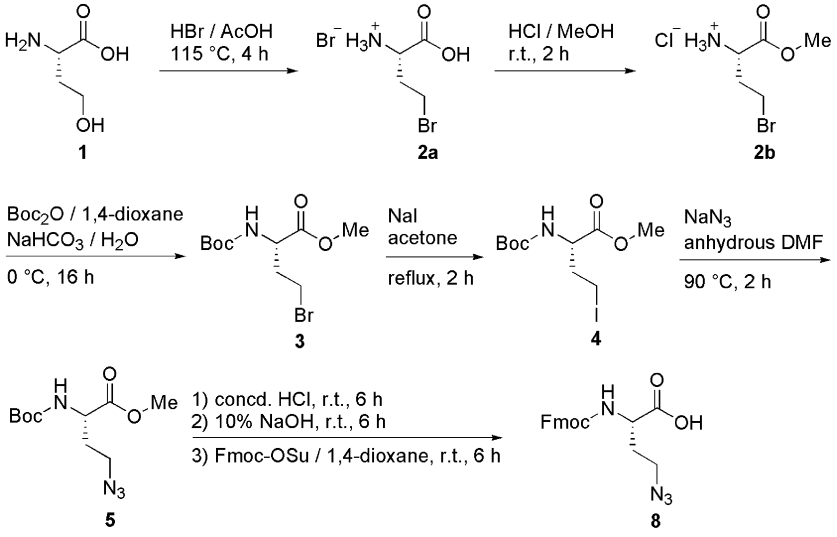
Figure 1. N^{α}-Fmoc-ω-azido- and N^{α}-Fmoc-ω-alkynyl-L-amino acids as building blocks for Fmoc/*t*Bu solid-phase peptide synthesis.

Results and Discussion

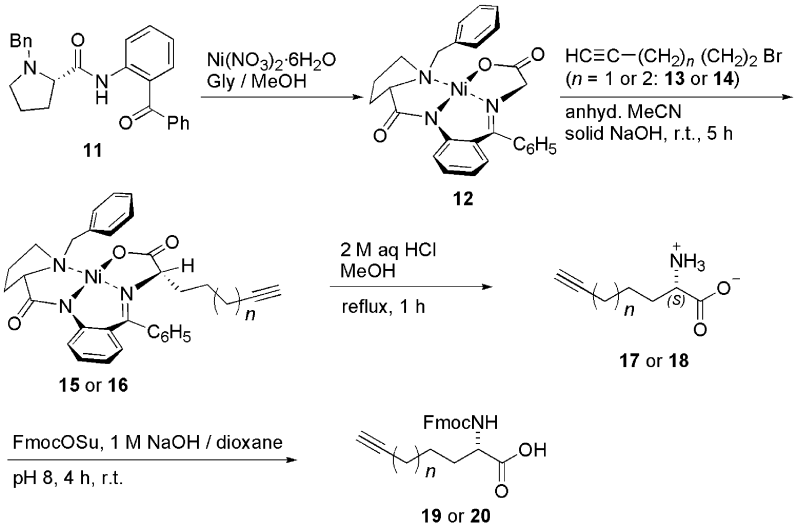
Our preferred synthetic strategy was to take advantage whenever possible of the pre-defined chiralities of commercially available α-amino acid derivatives and use them as the starting materials in the synthesis of the ω-azido- and ω-alkynyl-α-amino acids. An efficient and convenient methodology for generating organic azides is Cu^{II}-catalyzed diazo transfer from amines by trifluoromethanesulfonyl azide.[9,21] In this manner N^{α}-Boc-L-ornithine and N^{α}-Boc-L-lysine were converted in good yield into the respective N^{α}-Fmoc-δ-azido-L-norvaline (**9**) and N^{α}-Fmoc-ε-azido-L-norleucine (**10**) without affecting the original chirality.

The conversion of L-hSer into N^{α}-Fmoc-amino-γ-azido-L-butyric acid (**8**) required a multi-step strategy. The previously reported synthesis of N^{α}-Boc-L-Abu(γ-N_3)-OMe (**5**) started from the L-hSer-derived *N*-protected δ-lactone. It included one-pot ring-opening/substitution reaction to generating the hydrobromide of α-amino-γ-bromo-L-butyric acid that was then transformed into the corresponding γ-iodo derivative and subsequently to the desired azide.[22] Our synthesis of **5** starts with the direct conversion of the unprotected L-hSer to α-amino-γ-bromo-L-butyric acid·HBr (**2a**) (Scheme 1). After saponification of N^{α}-Boc-L-Abu(γ-N_3)-OMe (**5**), we removed the Boc group and replaced it with Fmoc to obtain the desired N^{α}-Fmoc-L-Abu(γ-N_3)-OH (**8**). This multi-step synthesis was carried out without purification of intermediates and the progress of the reactions was monitored by ^{1}H NMR following the characteristic chemical shifts of the γ-H_2 and the presence of the protecting groups.

The strategy to synthesize ω-alkynyl-L-amino acid homologues higher than propargylglycine containing $(CH_2)_n$ (where n = 3 and 4) in the side chain employed asymmetric synthesis to elaborate glycine used as the starting material.



Scheme 1. Synthesis of N^{α}-Fmoc-L-Abu(γ-N_3)-OH.



Scheme 2. Asymmetric synthesis of (*S*)-ω-alkynyl-α-amino acids employing the chiral auxiliary [Ni^{II}-(*S*)BPB-Gly] (**12**).

The Ni^{II} complex [Ni^{II}-(*S*)BPB-Gly][23] (**12**) of the Schiff base derived from glycine and (*S*)-2-(*N*-benzylprolyl)aminobenzophenone (BPB) (**11**), was used as a chiral auxiliary during the C^{α}-alkylations.[24,25] Alkylations through the *si*-face of the glycine enolate are largely favored leading to high enantiomeric excess of the (*S*)-ω-alkynyl-α-amino acid residue containing diastereoisomer (Scheme 2).[25]

C^{α}-Alkylation of the glycine moiety, incorporated into the chiral auxiliary [Ni^{II}-(*S*)BPB-Gly] (**12**), by excess of ω-bromoalkynes (1.4 equiv.) **13** and **14** was carried out in anhydrous acetonitrile in the presence of NaOH yielding the corresponding C^{α}-alkylated complexes **15** and **16** in good diastereomeric excess (monitored by Ultra Performance Liquid Chromatography, Table 1). The ω-bromoalkynes **13** and **14** were prepared from the corresponding alcohols by treatment with TsCl followed by LiBr.

Table 1. Diastereomeric ratio of the ω-alkylated complexes.

Products of alkylation of the [Ni^{II}-(*S*)BPB-Gly] complex with	Diastereomeric ratio (*SS*)/(*SR*)
5-Bromopent-1-yne (**13**)	71:29
6-Bromohex-1-yne (**14**)	88:12

The straightforward separation of the diastereoisomeric mixture by FCC was followed by short hydrolysis of the pure diastereomeric alkylated complexes **15** and **16** in 2 M HCl. Treatment of the crude hydrolysis products with H^+ Chelex resin removed the residual Ni^{II}, which was found to interfere in the subsequent N^{α}-protection by Fmoc-OSu.

Finally, the free ω-alkynyl-α-L-amino acids **17** and **18** were N^{α}-protected as Fmoc to yield the building blocks **19** and **20** in adequate quantities for Fmoc/*t*Bu solid-phase peptide synthesis.

Conclusions

Herein we report efficient synthetic pathways for the construction of building blocks comprised of a series of enantiomerically pure N^{α}-Fmoc-protected ω-alkynyl- and ω-azido-L-amino acids in which the alkynyl and azido functions are carried on side-chains of variable length (2–4 methylene groups). These synthetic strategies are suitable for scaling up and are generally applicable for the preparation of higher homologs. In addition, following the reported syntheses we generate not only the N^{α}-Fmoc- but also N^{α}-Boc-protected L-amino acids thus providing building blocks for orthogonal methodologies in solid-phase peptide synthesis. Extensive application of these enantiomerically pure building blocks in syntheses of side-chain-modified peptides, inter-side chain peptide–peptide, and peptide–carbohydrate chimera, peptide–small molecule conjugates and bioconjugates, and linear precursors of side-chain-to-side-chain-bridged cyclopeptides is in progress in our laboratories.

Experimental Section

The chemicals were purchased from Sigma–Aldrich and used without further purification. Protected amino acids were obtained from Novabiochem AG (Laufelfingen, Switzerland). TLC were carried out on silica gel precoated plates (Merck; 60 Å F254) and spots located with: (a) UV light (254 and 366 nm), (b) ninhydrin (solution in acetone), (c) Cl_2/tolidine, (d) fluorescamine, (e) I_2, (f) a basic solution of permanganate [$KMnO_4$ (3 g), K_2CO_3 (20 g), and NaOH (0.25 g) in water (300 mL)]. Flash Column Chromatography (FCC) was performed on Merck silica gel 60 (230–400 mesh) according to Still et al.[26]

^1H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, on a Varian spectrometer in deuterated chloroform solution and are reported in parts per million (ppm), with solvent resonance used as reference. Melting point was determined on a Büchi mod. 510 apparatus and are uncorrected. Elemental analysis was performed on a Perkin–Elmer 240 C Elemental Analyzer. Infrared spectra were recorded on a Perkin–Elmer mod. BX II FT-IR spectrometer. The $[a]_D$ were obtained on Perkin–Elmer mod. 343 Polarimeter in cell of 1 dm. Products were analyzed by ACQUITY UPLC (Waters Corporation, Milford, Massachusetts) coupled

to a single quadrupole ESCI-MS (Micromass ZQ) using a 2.1 × 50 mm 1.7 μm ACQUITY BEH C18 at 30 °C, with a flow rate of 0.45 mL/min. The solvent systems used were A (0.1% TFA in H_2O) and B (0.1% TFA in CH_3CN).

Methyl 4-Azido-2*S*-{[(1,1-dimethylethoxy)carbonyl]amino}butyrate [N^{α}-Boc-L-Abu(γ-N_3)-OMe] (5): N^{α}-Boc-L-Abu(γ-I)-OMe (**4**) (prepared as reported in Scheme 1) (2.58 g, 7.52 mmol) in anhydrous DMF (30 mL) was added to NaN_3 (0.846 g, 13.01 mmol) under nitrogen. The mixture was heated at 90 °C for 2 h and then poured into an ice/water mixture (180 mL). The aqueous layer was extracted with AcOEt (60 mL). The organic phase was washed with brine, dried on anhyd Na_2SO_4, filtered, and evaporated to obtain crude azide as a yellow oil (0.929 g, 48%). ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 5.40 (br. s, 1 H, NH), 4.26 (br. s, 1 H, α-H), 3.64 (s, 3 H, OCH_3), 3.31 (t, *J* = 6.6 Hz, 2 H, γ-H_2), 2.10–1.64 (m, 2 H, β-H_2), 1.33 (s, 9 H, *t*Bu) ppm. IR (KBr): $\tilde{\nu}$ = 2101 (N_3) cm^{-1}.

4-Azido-2*S*-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}butanoic Acid [N^{α}-Fmoc-L-Abu(γ-N_3)-OH] (8): Cleavage of the Boc protecting group of N^{α}-Boc-L-Abu(γ-N_3)-OMe (**5**) (0.748 g, 2.9 mmol) was achieved by treatment with an excess of TFA (10 mL) at room temp. for 10 min. The reaction was checked by TLC: $R_{f(a)}$ = 0.10 (AcOEt/*n*-hexane, 1:1). TFA was removed by flushing with N_2 and the residue dissolved in water and lyophilized. The methyl ester was hydrolyzed by stirring with 1 M NaOH (5 mL) at room temp. for 6 h. The solution was then treated with concd HCl until pH 7 and lyophilized to afford the free amino acid H-L-Abu(γ-N_3)-OH. A solution of Fmoc-OSu (0.843 g, 2.5 mmol) in dioxane (6 mL) was added dropwise to a solution of the deprotected amino acid in dioxane (10 mL). A solution of 1 M NaOH was subsequently slowly added until pH 8 and the reaction mixture was stirred at room temp. for 3 h. The reaction was monitored by TLC: $R_{f(a)}$ = 0.58 (CH_2Cl_2/MeOH, 9:2). Water (7.5 mL) was added and the solution was acidified with concd HCl until pH 3. The product was extracted with CH_2Cl_2 (3 × 20 mL), dried with anhyd Na_2SO_4, filtered and the solvent evaporated under vacuum. The crude was purified by FCC on silica gel employing a step-gradient of MeOH in CH_2Cl_2, 0%-10% to obtain the N^{α}-Fmoc-L-Abu(γ-N_3)-OH (124 mg, 27%) as yellow oil. RP-UPLC: R_t = 1.31 min (50 to 100% of B in 3 min). IR ($CHCl_3$): $\tilde{\nu}$ = 2100 (N_3) cm^{-1}. ESI-MS: calcd. for $C_{19}H_{18}N_4O_4$ [M + Na]$^+$ 389.12; found 389.4. $[\alpha]_D$ = –11.5 (*c* = 1.0, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.75 (*pseudo* d, *J* = 7.6 Hz, 2 H, fluorenyl 4-H and 5-H), 7.54 (*pseudo* d, *J* = 7.4 Hz, 2 H, fluorenyl 1-H and 8-H), 7.39 (*pseudo* t, 2 H, fluorenyl 3-H and 6-H), 7.31 (*pseudo* t, 2 H, fluorenyl 2-H and 7-H), 6.14 (br. s, COOH), 5.63 (m, 1 H, NH), 4.53–4.41 (m, 3 H, CH_2-O and α-H), 4.21 (t, *J* = 6.8 Hz, 1 H, fluorenyl 9-H), 3.42–3.39 (m, 2 H, γ-H_2), 2.19–1.96 (m, 2 H, β-H_2) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 172.71 (COOH), 156.26 (CONH), 143.53 and 141.29 (fluorenyl C-4a, C-4b, C-8a, and C-9a), 127.76, 127.08, 125.04, 124.99 (fluorenyl C-2 to C-7), 120.00 (fluorenyl C-1 and C-8), 67.17 (CH_2-O), 51.70 (C-α), 47.68 (C-γ), 47.09 (fluorenyl C-9), 31.21 (C-β) ppm. $C_{19}H_{18}N_4O_4$ (366.37): calcd. C 62.29, H 4.95, N 15.29; found C 62.36, H 4.99, N 15.24.

5-Azido-2*S*-{[(1,1-dimethylethoxy)carbonyl]amino}pentanoic Acid [N^{α}-Boc-L-Nva(δ-N_3)-OH] (6): N^{α}-Boc-L-Orn(N^{δ}-Cbz)-OH (1.0 g, 2.73 mmol) in MeOH (25 mL) was treated with H_2 and 10% Pd/C (0.1 g, 0.094 mmol Pd) at atmospheric pressure for 16 h. The mixture was filtered through Celite and washed with MeOH. After solvent evaporation, the deprotected N^{α}-Boc-L-ornithine was obtained as a white powder (669 mg) that was used without further purification. A solution of TfN_3 in CH_2Cl_2, was obtained as follows: Tf_2O (0.92 mL, 5.54 mmol) was added dropwise to a vigorously stirred mixture of NaN_3 (1.775 g, 27.3 mmol) in H_2O (4.5 mL) and CH_2Cl_2 (7.5 mL) at 0 °C. The resulting mixture was warmed to room temp. and stirring was continued for 2 h. The water layer was extracted with CH_2Cl_2 (2 × 3 mL) and the combined organic layers were washed with saturated aqueous Na_2CO_3 (8.5 mL). The resulting solution of TfN_3 in CH_2Cl_2 was then slowly added to a solution of N^{α}-Boc-L-norvaline (669 mg, 2.88 mmol), K_2CO_3 (600 mg, 4.32 mmol), and $CuSO_4 \cdot 5H_2O$ (7 mg, 0.028 mmol) in H_2O (8 mL) and MeOH (17 mL). The mixture was stirred overnight and the reaction was monitored by TLC: $R_{f(a\ \&\ b)}$ = 0.76 (*i*PrOH/AcOEt/H_2O, 6:1:3). The organic phase was evaporated under vacuum. The water layer was acidified to pH 6 with concd. HCl, diluted with 0.25 M of phosphate buffer at pH 6.2 (25 mL), and extracted with CH_2Cl_2 (4 × 50 mL). The organic layers were washed with brine (25 mL), dried with anhydrous Na_2SO_4, filtered and the solvent was evaporated under vacuum. The crude colorless oil was purified on a RP-18 LiChroprep column employing a step-gradient of CH_3CN in H_2O to afford the N^{α}-Boc-L-Abu(δ-N_3)-OH (324 mg, 44%). IR ($CHCl_3$): $\tilde{\nu}$ = 2101 (N_3) cm^{-1}. ESI-MS: calcd. for $C_{10}H_{18}N_4O_4$ [M + Na]$^+$ 281.12; found 281.02. $[\alpha]_D$ = –1.8 (*c* = 1.0, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.10 (br. s, 1 H, COOH), 6.58 (br. s, 1 H, NH), 4.34–4.17 (m, 1 H, α-H), 3.33 (t, *J* = 6.8 Hz, 2 H, δ-H_2), 1.99–1.65 (m, 4 H, 2 × CH_2), 1.45 (s, 9 H, *t*Bu) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 176.71 (COOH), 155.58 (CONH), 80.56 (Me_3C), 52.85 (C-α), 50.82 (C-δ), 29.70 (CH_2), 28.27 (3 × CH_3), 24.90 (CH_2) ppm. $C_{10}H_{18}N_4O_4$ (258.27): calcd. C 46.50, H 7.02, N 21.69; found C 46.48, H 7.05, N 21.71.

6-Azido-2*S*-{[(1,1-dimethylethoxy)carbonyl]amino}hexanoic Acid [N^{α}-Boc-L-Nle(ε-N_3)-OH] (7): Tf_2O (1.35 mL, 8.13 mmol) was added dropwise to a vigorously stirred mixture of NaN_3 (2.635 g, 40.5 mmol) in H_2O (6.5 mL) and CH_2Cl_2 (11 mL) at 0 °C. The resulting mixture was warmed to room temp. and stirring was continued for 2 h. The water layer was extracted with CH_2Cl_2 (2 × 4 mL) and the combined organic layers were washed with saturated aqueous Na_2CO_3 (12.5 mL). The resulting solution of TfN_3 in CH_2Cl_2 was then slowly added to a solution of N^{α}-Boc-lysine (1.0 g, 4.06 mmol), K_2CO_3 (0.84 g, 6.08 mmol), and $CuSO_4 \cdot 5H_2O$ (10 mg, 0.04 mmol) in H_2O (13 mL) and MeOH (27 mL). The mixture was stirred overnight and the reaction was monitored by TLC: $R_{f(a\ \&\ b)}$ = 0.81 (*i*PrOH/AcOEt/H_2O, 6:1:3). The organic phase was evaporated under vacuum. The water layer was acidified to pH 6 with concd. HCl, diluted with 0.25 M phosphate buffer at pH 6.2 (25 mL), and extracted with CH_2Cl_2 (4 × 50 mL). The organic layers were washed with brine (25 mL), dried with anhydrous Na_2SO_4, filtered and the solvent was evaporated under vacuum. The colorless oil was purified on a RP-18 LiChroprep column using a step-gradient of CH_3CN in H_2O to afford N^{α}-Boc-L-Nle(ε-N_3)-OH (451 mg, 41%). IR ($CHCl_3$): $\tilde{\nu}$ = 2100 (N_3) cm^{-1}. ESI-MS: calcd. for $C_{11}H_{20}N_4O_4$ [M – H]$^-$ 271.14; found 271.06. $[\alpha]_D$ = –1.1 (*c* = 1.0, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.93 (br. s, 1 H, COOH), 6.50 (br. s, 1 H, NH), 4.31–4.14 (m, 1 H, α-H), 3.28 (t, *J* = 6.8 Hz, 2 H, ε-H_2), 1.90–1.48 (m, 6 H, 3 × CH_2), 1.45 (s, 9 H, *t*Bu) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 177.14 (COOH), 155.53 (CONH), 80.32 (Me_3C), 54.29 (C-α), 51.17 (C-ε), 31.95 (CH_2), 28.42 (CH_2), 28.30 (3 × CH_3), 22.55 (CH_2) ppm. $C_{11}H_{20}N_4O_4$ (272.30): calcd. C 48.52, H 7.40, N 20.58; found C 48.56, H 7.44, N 20.61.

General Procedure for the Synthesis of N^{α}-Fmoc-L-Nva(δ-N_3)-OH (9) and N^{α}-Fmoc-L-Nle(ε-N_3)-OH (10): Cleavage of the Boc protecting group of N^{α}-Boc-ω-azido-L-α-amino acids **6** and **7** (3.96 mmol) was achieved by treatment with an excess of concd. HCl (2.5 mL) at room temp. for 6 h. The residue was dissolved in water (5 mL) and lyophilized. A solution of (2,5-dioxo-1-pyrrolidinyl) (9*H*-flu-

oren-9-ylmethyl) carbonate (Fmoc-OSu, 4.36 mmol) in dioxane (20 mL) was then added dropwise to a stirred solution of the deprotected amino acid in dioxane (30 mL). A solution of 1 M NaOH was subsequently slowly added until pH 8–9 and the reaction mixture was stirred at room temp. for 2.5 h. The reaction was monitored by TLC (CH_2Cl_2/MeOH, 9:2). Water (12 mL) was added and the solution was acidified with 2 M HCl until pH 3. The product was extracted with CH_2Cl_2 (3 × 30 mL), dried with anhydrous Na_2SO_4, filtered and the solvent removed under vacuum. The crude material was purified by FCC on silica gel (employing a step-gradient of MeOH in CH_2Cl_2, 0–10%) to obtain the corresponding pure N^{α}-Fmoc-L-ω-azido-α-amino acids **9** and **10** as yellow oils.

5-Azido-2*S*-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}pentanoic Acid [N^{α}-Fmoc-L-Nva(δ-N_3)-OH] (9): Yield 69%. TLC: $R_{f(a)}$ = 0.58 (CH_2Cl_2/MeOH; 9:2). RP-UPLC: R_t = 1.37 min (50 to 100% of B in 3 min). IR ($CHCl_3$): $\tilde{\nu}$ = 2100 (N_3) cm^{-1}. ESI-MS: calcd. for $C_{20}H_{20}N_4O_4$ [M + Na]$^+$ 403.14; found 403.3. $[\alpha]_D$ = –2.3 (*c* = 1.0, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.76 (d, $J_{3,4} = J_{5,6}$ = 7.6 Hz, 2 H, fluorenyl 4-H and 5-H), 7.61 (*pseudo* d, $J_{1,2} = J_{7,8}$ = 7.6 Hz, 2 H, fluorenyl 1-H and 8-H), 7.40 (*pseudo* t, 2 H, fluorenyl 3-H and 6-H), 7.31 (*pseudo* t, 2 H, fluorenyl 2-H and 7-H), 6.16 (br. s, COOH), 5.34 (m, 1 H, NH), 4.45–4.40 (m, 3 H, CH_2-O and α-H), 4.22 (t, *J* = 6.6 Hz, 1 H, fluorenyl 9-H), 3.37–3.30 (m, 2 H, δ-H_2), 2.01–1.46 (m, 4 H, 2× CH_2) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 175.72 (COOH), 156.72 (CONH), 143.75, 143.57, and 141.33 (fluorenyl C-4a, C-4b, C-8a, and C-9a), 127.76, 127.08, and 125.00 (fluorenyl C-2 to C-7), 120.02 (fluorenyl C-1 and C-8), 67.12 (CH_2-O), 53.16 (C-α), 50.76 (C-δ), 47.15 (fluorenyl C-9), 29.62 (CH_2), 24.81 (CH_2) ppm. $C_{20}H_{20}N_4O_4$ (380.40): calcd. C 63.15, H 5.30, N 14.73; found C 63.09, H 5.25, N 14.80.

6-Azido-2*S*-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}hexanoic Acid [N^{α}-Fmoc-L-Nle(ε-N_3)-OH] (10): Yield 64%. TLC: $R_{f(a)}$ = 0.58 (CH_2Cl_2/MeOH, 9:2). RP-UPLC: R_t = 1.51 min (50 to 100% of B in 3 min). IR ($CHCl_3$): $\tilde{\nu}$ = 2100 (N_3) cm^{-1}. ESI-MS: calcd. for $C_{21}H_{22}N_4O_4$ [M + Na]$^+$ 417.15; found 417.2. $[\alpha]_D$ = –2.5 (*c* = 1.0, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.74 (d, $J_{3,4} = J_{5,6}$ = 7.4 Hz, 2 H, fluorenyl 4-H and 5-H), 7.54 (d, $J_{1,2} = J_{7,8}$ = 7.4 Hz, 2 H, fluorenyl 1-H and 8-H), 7.37 (*pseudo* t, 2 H, fluorenyl 3-H and 6-H), 7.28 (*pseudo* t, 2 H, fluorenyl 2-H and 7-H), 6.19 (br. s, COOH), 5.46 (m, 1 H, NH), 4.49–4.33 (m, 3 H, CH_2-O and α-H), 4.18 (t, *J* = 6.4 Hz, 1 H, fluorenyl 9-H), 3.24–3.21 (m, 2 H, ε-H_2), 1.70–1.42 (m, 6 H, 3× CH_2) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 176.97 (COOH), 156.35 (CONH), 143.75, 143.60, and 141.28 (fluorenyl C-4a, C-4b, C-8a, and C-9a), 127.74, 127.06, and 125.01 (fluorenyl C-2 to C-7), 120.00 (fluorenyl C-1 and C-8), 67.14 (CH_2-O), 53.90 (C-α), 51.02 (C-ε), 47.07 (fluorenyl C-9), 31.68 (CH_2), 28.31 (CH_2), 22.55 (CH_2) ppm. $C_{21}H_{22}N_4O_4$ (394.42): calcd. C 63.95, H 5.62, N 14.20; found C 64.01, H 5.58, N 14.23.

Synthesis of (*S*)-2-(*N*-Benzylprolyl)aminobenzophenone (BPB) (11): *N*-Benzyl-L-proline (prepared from proline as described previously)[24] (3.467 g, 16.9 mmol) was added at room temp. under N_2 to a stirred freshly prepared transparent solution of PCl_5 (7.035 g, 33.8 mmol) in anhydrous CH_2Cl_2 (55 mL). After 30 min, cold petroleum ether was added and the acyl chloride precipitated as an oil. The oil was dissolved in anhydrous CH_2Cl_2 (60 mL) under N_2 and 2-aminobenzophenone (3.33 g, 16.9 mmol) was added in one portion, followed by Et_3N until pH 8. The mixture was stirred for 4 h at room temp., then washed with a saturated solution of Na_2CO_3 and twice with H_2O. The organic layer was dried with anhydrous Na_2SO_4, filtered and the solvents evaporated under vacuum. The BPB was recrystallized from dried crude using EtOH. Some product was also recovered from the EtOH washings. The material was dried under vacuum to afford BPB (1.873 g, 29%). NMR spectroscopic data were in accordance with the literature.[27] ESI-MS: calcd. for $C_{25}H_{24}N_2O_2$ [M + H]$^+$: 385.19; found 385.2. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 11.52 (s, 1 H, NH), 8.56 (d, *J* = 8.4 Hz, 1 H, ArH), 7.79–7.36 (m, 9 H, ArH), 7.15 (m, 4 H, ArH), δ_A = 3.92, δ_B = 3.59 (syst AB, J_{AB} = 12.8 Hz, 2 H, $PhCH_2$), 3.32 (dd, $J_{\alpha,\beta}$ = 4.4, $J_{\alpha,\beta'}$ = 10.0 Hz, 1 H, α-H), 3.22 (dd, $J_{\delta,\delta'} = J_{\delta,\gamma}$ = 6.4 Hz, 1 H, δ-H), 2.41 (dd, $J_{\beta,\beta'}$ = 8.8, $J_{\beta,\gamma}$ = 16 Hz, 1 H, β′-H), 2.26 (ddd, $J_{\delta\delta'}$ = 6.4, $J_{\delta\gamma}$ = 12.8, $J_{\delta\gamma'}$ = 22 Hz, 1 H, δ′-H), 1.96 (ddd, $J_{\beta,\beta'}$ = 8.8, $J_{\beta,\gamma}$ = 4.4, $J_{\beta\gamma'}$ = 16.4 Hz, 1 H, β-H), 1.85–1.76 (m, 2 H, γ-H and γ′-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 198.03 (Ph-CO-Ph), 174.64 (N-C=O), 139.16, 138.54, 138.12, 133.37, 132.55, 132.48, 130.11, 129.12, 128.30, 128.15, 127.05, 125.32, 122.19, 121.52 (18 Ar), 68.25 (C-α), 59.82 (Ph*C*H_2), 53.85 (C-δ), 30.98 (C-β), 24.14 (C-γ) ppm.

General Procedure for the Alkylation of [Ni^{II}-(*S*)BPB-Gly] Complex with ω-Alkynyl Bromides: To a stirred mixture of [Ni^{II}-(*S*)BPB-Gly] (**12**) (prepared from BPB following the procedure previously described)[23] (1.99 g, 4 mmol) in anhydrous CH_3CN (17.5 mL) were added, under N_2, finely powdered NaOH (0.4 g, 10 mmol) and ω-alkynyl bromide (6.01 mmol). After 5 h, the reaction mixture was treated with 0.1 M HCl (59 mL) and the red product extracted with CH_2Cl_2 (4 × 40 mL), dried with $MgSO_4$, the mixture was filtered and the solvent removed under vacuum. The crude was purified by FCC on silica gel (CH_2Cl_2/Me_2CO, 2:1) affording the product as a red amorphous solid.

Alkylation of [Ni^{II}-(*S*)BPB-Gly] Complex with 5-Bromopent-1-yne (13): Yield of **15**: 59%. M.p. 83.5–85 °C; TLC: $R_{f(a\,\&\,e)}$ = 0.57 [CH_2Cl_2/Me_2CO, 2:1]. $[\alpha]_D$ = –1522 (*c* = 0.5, MeOH). ESI-MS: calcd. for $C_{32}H_{31}N_3NiO_3$ [M + H]$^+$: 564.18; found 564.5. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.12 (d, *J* = 8.4 Hz, 1 H, ArH), 8.04 (d, *J* = 7.6 Hz, 2 H, ArH), 7.50–7.41 (m, 3 H, ArH), 7.33 (t, *J* = 7.6 Hz, 2 H, ArH), 7.25–7.10 (m, 3 H, ArH), 6.95 (d, *J* = 7.6 Hz, 1 H, ArH), 6.64–6.60 (m, 2 H, ArH), 4.43 (d, *J* = 12.6 Hz, 1 H, $PhCH_2$), 3.86 (dd, J_1 = 3.4; J_2 = 8.8 Hz, 1 H, Pro α-H), 3.56 (d, *J* = 12.6 Hz, 1 H, $PhCH_2$), 3.52–3.43 (m, 3 H), 2.79–2.72 (m, 1 H), 2.54–2.48 (m, 1 H), 2.35–2.20 (m, 1 H), 2.20–2.05 (m, 3 H), 2.05–1.95 (m, 2 H), 1.93 (t, *J* = 2.6 Hz, 1 H, C≡CH), 1.78–1.70 (m, 2 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 180.37 (O-C=O), 179.15 (N-C=O), 170.54 (C=N), 142.27, 133.67, 133.24, 132.16, 131.54, 129.71, 129.01, 128.89, 128.86, 127.63, 127.07, 126.38, 123.71, 120.71 (18× Ar C), 83.47 (HC≡*C*), 70.27 (H*C*≡C), 69.99 (Pro C-α), 69.06 (Ph*C*H_2), 63.11 (*C*-COO), 57.05 (Pro C-δ), 34.53 (Pro C-β), 30.75 (C≡C-CH_2-*C*), 24.36 [HC≡C-$(CH_2)_2$-*C*], 23.73 (Pro C-γ), 18.15 (C≡C-*C*) ppm. $C_{32}H_{31}N_3NiO_3$ (564.30): calcd. C 68.11, H 5.54, N 7.45; found C 68.08, H 5.52, N 7.49.

Alkylation of [Ni^{II}-(*S*)BPB-Gly] Complex with 6-Bromohex-1-yne (14): Yield of **16**: 68%. M.p. 87.5–88.5 °C; TLC: $R_{f(a\,\&\,e)}$ = 0.53 [CH_2Cl_2/Me_2CO, 2:1]. $[\alpha]_D$ = –1429 (*c* = 0.5, MeOH). ESI-MS: calcd. for $C_{33}H_{33}N_3NiO_3$ [M + H]$^+$: 578.19; found 578.4. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.12 (d, *J* = 8.4 Hz, 1 H, ArH), 8.04 (d, *J* = 7.6 Hz, 2 H, ArH), 7.50–7.44 (m, 3 H, ArH), 7.33 (t, *J* = 7.6 Hz, 2 H, ArH), 7.26–7.10 (m, 3 H, ArH), 6.92 (d, *J* = 7.6 Hz, 1 H, ArH), 6.66–6.60 (m, 2 H, ArH), 4.42 (d, *J* = 12.8 Hz, 1 H, $PhCH_2$), 3.91 (dd, J_1 = 3.4; J_2 = 7.8 Hz, 1 H, Pro α-H), 3.57 (d, *J* = 12.8 Hz, 1 H, $PhCH_2$), 3.52–3.43 (m, 3 H), 2.79–2.73 (m, 1 H), 2.56–2.46 (m, 1 H), 2.36–2.30 (m, 1 H), 2.16–2.02 (m, 4 H), 1.97–1.88 (m, 1 H), 1.91 (t, *J* = 2.4 Hz, 1 H, C≡CH), 1.76–1.61 (m, 2 H), 1.44–1.32 (m, 2 H) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 180.32 (O-C=O), 179.30 (N-C=O), 170.46 (C=N), 142.27, 133.81, 133.21, 132.12, 131.54, 129.73, 128.94, 128.89, 128.85, 127.55, 127.14, 126.47, 123.66, 120.69 (18× Ar), 83.86 (HC≡*C*), 70.28 (H*C*≡C),

70.10 (Pro C-α), 68.83 (Bn CH_2), 63.13 (*C*-COO), 56.99 (Pro C-δ), 34.53 (Pro C-β), 30.76 (HC≡C-CH_2-*C*), 27.93 [HC≡C-$(CH_2)_3$-*C*], 24.30 (Pro C-γ), 23.67 [C≡C-$(CH_2)_2$-*C*], 18.14 (HC≡C-*C*) ppm. $C_{33}H_{33}N_3NiO_4$ (578.33): calcd. C 68.53, H 5.75, N 7.27; found C 68.55, H 5.71, N 7.32.

Hydrolysis of the Alkylated Complexes 15 and 16 and Fmoc Protection of the Free Amino Acids. General Procedure: A solution of the alkylated complex (1.33 mmol) in MeOH (22.5 mL) was added to warm 2 M HCl (16 mL), the mixture was refluxed for 1 h. After cooling to room temp., 1 M NaOH was added until pH 6 and the solvent was removed under vacuum. The solid residue was washed with acetone, the dried solid product was dissolved in MeOH/H_2O (15:20 v/v) (70 mL) and then gently swirled ON with Chelex 100 resin in the H^+ form. The mixture was filtered and the resin washed with water, the combined filtrates were evaporated under vacuum, and the residue lyophilized.

A solution of FmocOSu (1.51 mmol) in dioxane (15 mL) was added dropwise to the lyophilized product (1.37 mmol) dissolved in dioxane (15 mL) and then treated with 1 M NaOH to obtain pH 8. The reaction mixture was stirred at room temp. for 4 h, followed by the addition of water (7.5 mL) and acidification to pH 3 with 2 M HCl. The product was then extracted with CH_2Cl_2 (3 × 20 mL), dried with anhydrous Na_2SO_4 and the solvent removed under vacuum. The crude was purified by FCC employing a step-gradient of MeOH in CH_2Cl_2 (0%-10%) to obtain the pure amino acid as a yellow oil.

2*S*-{[(9*H*-Fluoren-9-ylmethoxy)carbonyl]amino}-6-heptynoic Acid (19): Yield 24%. RP-UPLC: R_t = 1.49 min (50–100% of B in 3 min). $[\alpha]_D$ = –3.0 (*c* = 1.0, MeOH). ESI-MS: calcd. for $C_{22}H_{21}NO_4$ [M + Na]$^+$ 386.14; found 386.2. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.73 (d, *J* = 7.2 Hz, 2 H, fluorenyl 4-H and 5-H), 7.57 (d, *J* = 7.4 Hz, 2 H, fluorenyl 1-H and 8-H), 7.39 (*pseudo* t, 2 H, fluorenyl 3-H and 6-H), 7.30 (*pseudo* t, 2 H, fluorenyl 2-H and 7-H), 6.60 (br. s, COOH), 5.51 (m, 1 H, NH), 4.43–4.35 (m, 3 H, CH_2-O and α-H), 4.18 (t, *J* = 6.6 Hz, 1 H, fluorenyl 9-H), 2.08–1.99 (m, 3 H), 1.94 (t, *J* = 2.4 Hz, 1 H, HC≡C), 1.80–1.75 (m, 1 H), 1.58–1.42 (m, 2 H, CH_2) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 177.06 (COOH), 156.26 (CONH), 143.81, 143.62 and 141.27 (fluorenyl C-4a, C-4b, C-8a, and C-9a), 127.70, 127.06 and 125.05 (fluorenyl C-2 to C-7), 119.96 (fluorenyl C-1 and C-8), 83.49 (HC≡*C*), 69.11 (CH_2-O), 67.06 (H*C*≡C), 53.77 (C-α), 47.11 (fluorenyl C-9), 31.33 (C-β), 24.28 (C-γ), 18.01 (C-δ) ppm. $C_{22}H_{21}NO_4$ (363.41): calcd. C 72.71, H 5.82, N 3.85; found C 72.80, H 5.87, N 3.80.

2*S*-{[(9*H*-Fluoren-9-ylmethoxy)carbonyl]amino}-7-octynoic Acid (20): Yield 35%. RP-UPLC: R_t = 1.49 min (50–100% of B in 3 min). $[\alpha]_D$ = –3.1 (*c* = 1.0, MeOH). ESI-MS: calcd. for $C_{23}H_{23}NO_4$ [M + Na]$^+$ 400.15; found 400.3. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.75 (*pseudo* d, *J* = 7.6 Hz, 2 H, fluorenyl 4-H and 5-H), 7.59 (*pseudo* d, *J* = 7.6 Hz, 2 H, fluorenyl 1-H and 8-H), 7.37 (*pseudo* t, 2 H, fluorenyl 3-H and 6-H), 7.28 (*pseudo* t, 2 H, fluorenyl 3-H and 6-H), 5.79 (br. s, COOH), 5.48 (m, 1 H, NH), 4.44–4.38 (m, 3 H, CH_2-O and α-H), 4.21 (t, *J* = 6.8 Hz, 1 H, fluorenyl 9-H), 2.08–1.99 (m, 3 H), 1.94 (t, *J* = 2.4 Hz, 1 H, HC≡C), 1.80–1.75 (m, 1 H), 1.58–1.42 (m, 4 H, 2× CH_2) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 176.63 (COOH), 156.17 (CONH), 143.83, 143.67 and 141.29 (fluorenyl C-4a, C-4b, C-8a, and C-9a), 127.71, 127.06, 125.04 (fluorenyl C-2 to C-7), 119.98 (fluorenyl C-1 and C-8), 83.97 (HC≡*C*), 68.69 (CH_2-O), 67.06 (H*C*≡C), 53.83 (C-α), 47.15 (fluorenyl C-9), 31.73 and 27.81 (C-β and δ), 24.31 (C-γ), 18.15 (C-ε) ppm. $C_{23}H_{23}NO_4$ (377.43): calcd. C 73.19, H 6.14, N 3.71; found C 73.09, H 6.19, N 3.81.

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