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α-Azido Acids for Direct Use in Solid-phase Peptide Synthesis

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Abstract: Several new α-azido acids have been synthesized and their use in solid-phase peptide synthesis has been demonstrated. The azido group allows for high activation of the carboxyl group as an acid chloride without formation of byproducts and with no detectable racemization. An analog of Leu-enkephalin has been prepared and tested in the mouse vas deferens and guinea pig ileum bioassays: it displays moderate activity at the δ-opiod receptor. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

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INTRODUCTION

Protection of the amino group has always been essential in peptide chemistry to avoid side-reactions and several protection schemes have been developed. The Boc/Bzl [1] and Fmoc/*t*Bu [2] combinations have been the most frequently employed. Both are based on the urethane group, which decreases the nucleophilicity of the amino group, reduces racemization at C^{α}, and limits oxazolone formation. However, urethane-protected amino acids have their limits when synthesizing difficult sequences where the resin-bound α-amino group is not easily accessible, either due to formation of secondary structures in the growing peptide chain, or to sterically hindered residues, both resulting in incomplete acylation [3].

Ways to circumvent this problem include decreasing the size of the leaving group and amino protecting group, increasing activation of the carboxyl group and improving the stability of the amino protecting group. All these have been achieved by use of azide as a 'protecting group' and activation of the carboxyl group as the acid chloride. α-Azido acids have been known for decades [4] but were not used in solid-phase peptide synthesis (SPPS) until recently [5] because of the heterogeneous conditions usually employed for reduction. However, it has been demonstrated that Cleland's reagent [6] (dithiothreitol, DTT), used for cleavage of disulfide bonds in proteins, is efficient in azide reductions, and can be used in combination with solid-phase chemistry.

Many peptide mimetics can be prepared by incorporation of different azido acid building blocks. If α,α-disubstituted azido acids are used, the peptide product will be less susceptible to enzymatic degradation because of the modification at C^{α}. The α,α-disubstitution gives a more restricted conformation of the peptide backbone which may be useful in structure–activity relationship studies [7], where a fixed conformation is desirable. The azido acids can be used in the design of secondary structures [8] by inserting α,α-disubstituted residues in specific

Abbreviations: Ahd, (±)-2-aminohexadecanoic acid; Dhbt-OH, 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine; DIPEA, *N*,*N*-diisopropylethylamine; DTT, dithiothreitol; NBS, *N*-bromosuccinimide; NEM, *N*-ethylmorpholine; PEGA, bis-aminopropyl ethylene glycol polyacrylamide copolymer; Pfp, pentafluorophenyl; TBTU, *N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium tetrafluoroborate *N*-oxide. Three letter codes are used for the amino acids according to IUPAC.

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positions of the peptide thereby obtaining, e.g. β-turns [9], 3_{10} helices [10] or extended conformations [11,12].

In the present work, the synthesis of a range of α-mono- and α,α-disubstituted azido acids (Figure 1) is presented. Furthermore, an example of their use in SPPS (a modified enkephalin derivative **15**) is described.

MATERIALS AND METHODS

^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker DRX250 (250 MHz) or a Varian Unity Inova 500 (500 MHz). MALDI-TOF MS was performed on a Finnigan MAT 2000 using a matrix of α-cyano-4-hydroxycinnamic acid [13]. Electrospray mass spectrometry was performed in the positive mode on a Fisons VG Quattro instrument. IR spectra were recorded on a Perkin Elmer 1600 FTIR instrument as neat liquids or as KBr-pellets. Analytical and preparative reverse-phase HPLC separations were performed on a Waters HPLC system using analytical RCM (8 × 100 mm) and Delta PAK (47 × 300 mm) C_{18} columns with a flow rate of 1 cm^3 min^{-1} and 20 cm^3 min^{-1}, respectively. Detection was at 215 and 280 nm on the analytical instrument and with a photodiode array detector (Waters M991) for preparative separations. A solvent system consisting of A: 0.1% TFA in water and B: 0.1% TFA in 90% acetonitrile–10% water was used. A Grass S48 stimulator and a Grass 7D polygraph were used for stimulation and recording of the smooth muscle contractions, respectively. THF was distilled from sodium, CH_2Cl_2 was distilled from CaH_2, DMF was fractionally distilled *in vacuo* and all solvents were stored over molecular sieves (3 or 4 Å). Water was Milli-Q (Millipore, Glostrup, Denmark). Deuterated solvents used for NMR was $CDCl_3$ and d_6-DMSO from Cambridge Isotope Laboratories. All amino acids were L-amino acids and solvents were from Labscan. The following commercially available chemicals were used: (±)-2-bromohexadecanoic acid, (±)-2-bromohexanoic acid, 2-(bromomethyl)-acrylic acid, 2-bromo-2-methylpropionic acid, (±)-2-bromo-2-phenylacetic acid, DTT, 2-ethylbutyric acid, piperidine, (±)-2-methylbutyric acid, 2-propylpentanoic acid and trifluoromethanesulfonic acid anhydride (Aldrich, Gillingham, Dorset, UK), Fmoc-AA-OH/Fmoc-AA-OPfp, Fmoc-Rink-amide linker and *N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethan-aminium tetrafluoroborate *N*-oxide (TBTU) (Bachem or Novabiochem, Läufelfingen, Switzerland), 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine (Dhbt-OH) and *N*-bromosuccinimide (NBS) (Fluka),

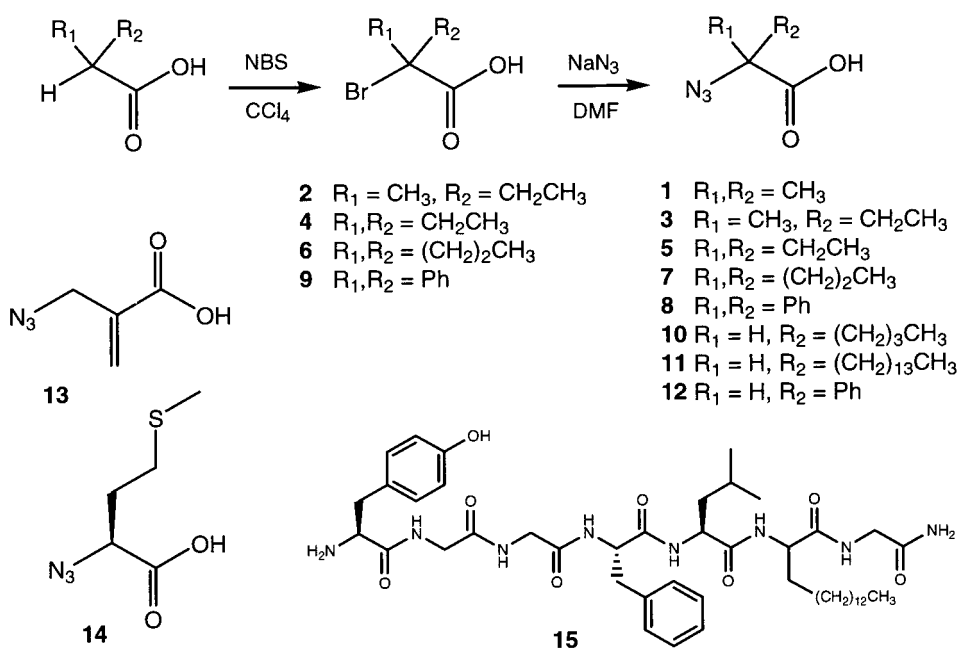

Figure 1 The prepared compounds and a Leu-enkephalin analog synthesized by a combination of an azido acid chloride and Fmoc-amino acid Pfp-esters.

N-ethylmorpholine (NEM), *p*-hydroquinone, trifluoroacetic acid and thionylchloride (Merck, Schuchardt, Germany), bis-aminopropyl ethylene glycol polyacrylamide copolymer (PEGA) resin (Polymer Laboratories), *N*,*N*-diisopropylethylamine (DIPEA), diphenylacetic acid and L-methionine (Sigma, St. Louis, USA). PL017 (a standard μ-agonist) was purchased from Peninsula Laboratories, California, USA and [D-penicillamine(Pen)^2,D-Pen^5]-enkephalin (DPDPE) was a generous gift from Victor J. Hruby.

General Procedures

Substitution reactions with NaN_3 were performed in the dark to avoid photolytic degradation of the products, and the azido acids were stored in the dark at -20°C. Coupling of pentafluorophenyl (Pfp) -esters to amino groups was performed with 3 equivalents of Fmoc-AA-OPfp and 1 equivalent of Dhbt-OH in DMF. Fmoc-deprotection was effected with 20% piperidine in DMF for 2 + 18 min followed by washing of the resin six times with DMF. The resin was washed six times with solvent between each reaction step. TFA (95%, aqueous) and AcOH (95%, aqueous) were prepared at least the day before use to hydrolyse any anhydride present. All reactions took place in DMF unless otherwise stated. Trifluoromethanesulfonyl azide should always be kept in solution because it is explosive in the dry state. When acidifying solutions with NaN_3 present, care was taken to ensure good ventilation due to evolution of toxic HN_3.

2-Azido-2-methylpropionic acid (1). 2-Bromo-2-methylpropionic acid (4.96 g, 29.7 mmol) and NaN_3 (2.88 g, 44.3 mmol, 1.5 equivalents) were mixed in dry DMF (50 mL) and stirred under argon for 2 days. It was concentrated, redissolved in H_2O (30 mL) and acidified to pH 2 in an efficiently ventilated hood with aqueous HCl (3 M). The aqueous phase was extracted with CH_2Cl_2 (3×30 mL) and the organic phases dried, concentrated and purified by VLC (AcOH/EtOAc/heptane 5:20:75) affording **1** (2.68 g, 70%). ^{1}H-NMR (250 MHz) in $CDCl_3$, δ ppm: 1.55 (s, 6H, H-3), 11.87 (s, 1H, COO*H*). ^{13}C-NMR (62.5 MHz) in $CDCl_3$, δ ppm: 26.0 (C-3), 64.6 (C-2), 181.2 (C-1). IR: 1718, 2125 cm^{-1}. The data were identical to those previously reported [5].

(±)-2-Bromo-2-methylbutyric acid (2). (±)-2-Methylbutyric acid (10.39 g, 0.102 mol) and NBS (19.02 g, 0.107 mol, 1.05 equivalents) were refluxed in CCl_4 (100 mL) under argon. The red color from bromine had disappeared overnight and the solid succinimide was filtered off. The filtrate was concentrated affording a slightly yellow oil which was distilled *in vacuo* and **2** was collected at 53°C$^{0.2\ mmHg}$ (14.40 g, 78%). B.p. lit. [14]: 108–110°C$^{6\ mmHg}$. ^{1}H-NMR (250 MHz) in $CDCl_3$, δ ppm: 1.08 (t, 3H, $J = 7$ Hz, H-4), 1.93 (s, 3H, H-3′), 2.19 (dq, 2H, $J = 2$ Hz, $J' = 7$ Hz, H-3), 11.41 (br s, 1H, COO*H*). ^{13}C-NMR (62.5 MHz) in $CDCl_3$, δ ppm: 10.3 (C-4), 27.0 (C-3′), 35.4 (C-3), 61.3 (C-2), 177.8 (C-1).

(±)-2-Azido-2-methylbutyric acid (3). (±)-2-Bromo-2-methylbutyric acid (**2**) (13.69 g, 75.6 mmol) and NaN_3 (7.38 g, 113.5 mmol, 1.5 equivalents) in dry DMF (35 mL) were stirred under argon for 3 days. The solution was concentrated, redissolved in H_2O (40 mL) and acidified to pH 2 in an efficiently ventilated hood with aqueous HCl (3 M). It was extracted with CH_2Cl_2 (3×50 mL) and the combined organic phases were dried, concentrated and purified by VLC (AcOH/EtOAc/heptane 5:10:85) affording **3** (9.86 g, 91%). ^{1}H-NMR (250 MHz) in $CDCl_3$, δ ppm: 1.00 (t, 3H, $J = 7$ Hz, H-4), 1.54 (s, 3H, H-3′), 1.82 (dq, 1H, $J = 7$ Hz, $J' = 7$ Hz, H-3a), 1.90 (dq, 1H, $J = 7$ Hz, $J' = 7$ Hz, H-3b), 8.12 (br s, 1H, COO*H*). ^{13}C-NMR (62.5 MHz) in $CDCl_3$, δ ppm: 8.7 (C-4), 22.3 (C-3′), 31.5 (C-3), 67.2 (C-2), 178.8 (C-1). IR: 1718, 2108 cm^{-1}. Found: C, 41.23%; H, 6.96%; N, 28.78%. Calculated for $C_5H_9N_3O_2$: C, 41.95%; H, 6.34%; N, 29.36%.

2-Bromo-2-ethylbutyric acid (4). 2-Ethylbutyric acid (10.06 g, 86.6 mmol) and *N*-bromosuccinimide (15.60 g, 87.7 mmol, 1 equivalent) were dissolved in CCl_4 (100 mL) and refluxed under argon for 4.5 h, after which the reaction mixture was filtered and concentrated. The remaining liquid was distilled *in vacuo* and **4** was collected at 71°C$^{0.5\ mmHg}$ (10.97 g, 65%). B.p. lit. [14]: 109–112°C$^{5\ mmHg}$. ^{1}H-NMR (250 MHz) in $CDCl_3$, δ ppm: 0.85 (t, 6H, $J = 7$ Hz, H-4), 1.98 (dq, 4H, $J = 2$ Hz, $J' = 7$ Hz, H-3), 12.12 (s, 1H, COO*H*). ^{13}C-NMR (62.5 MHz) in $CDCl_3$, δ ppm: 10.0 (C-4), 32.3 (C-3), 68.9 (C-2), 177.7 (C-1).

2-Azido-2-ethylbutyric acid (5). 2-Bromo-2-ethylbutyric acid (**4**) (3.84 g, 19.7 mmol) and NaN_3 (1.93 g, 29.7 mmol, 1.5 equivalents) were dissolved in dry DMF (50 mL) and allowed to stir for 5 days at 20°C. The DMF was removed *in vacuo* and the residue was dissolved in water and acidified with aqueous HCl (0.1 M) to pH 1–2 in an efficiently ventilated hood. Extraction with EtOAc (4×25 mL) afforded a yellow oil after drying and concentration. This was concentrated three times with pentane (50 mL) and

purified by preparative RP-HPLC affording **5** as a white crystalline compound (0.97 g, 31%) and some 2-hydroxy-2-ethylbutyric acid. M.p. of **5**: 57.1–58.1°C. ^{1}H-NMR (250 MHz) of **5** in d_6-DMSO, δ ppm: 0.85 (t, 6H, $J = 7$ Hz, H-4), 1.70 (m, 4H, H-3). ^{13}C-NMR (62.5 MHz) in d_6-DMSO, δ ppm: 9.3 (C-4), 29.8 (C-3), 71.6 (C-2), 173.9 (C-1). IR: 1693, 2108 cm^{-1}. Found: C, 46.22%; H, 7.06%; N, 26.65%. Calculated for $C_6H_{11}N_3O_2$: C, 45.85%; H, 7.05%; N, 26.74%.

2-Bromo-2-propylpentanoic acid (6). 2-Propylpentanoic acid (12.64 g, 87.7 mmol) and *N*-bromosuccinimide (15.84 g, 89.0 mmol, 1.0 equivalent) were mixed in CCl_4 (100 mL) and refluxed under argon. The reaction was stopped after 5 days, filtered and concentrated. The remaining oil was distilled *in vacuo* and **6** was collected at 81°C$^{0.09\ mmHg}$ (6.96 g, 36%). B.p. lit. [14]: 126–130°C$^{5\ mmHg}$. ^{1}H-NMR (250 MHz) in $CDCl_3$, δ ppm: 0.97 (t, 6H, $J = 7$ Hz, H-5), 1.45 (m, 4H, H-4), 2.11 (m, 4H, H-3), 11.15 (br s, 1H, COO*H*). ^{13}C-NMR (62.5 MHz) in $CDCl_3$, δ ppm: 14.7 (C-5), 19.7 (C-4), 42.6 (C-3), 68.0 (C-2), 177.8 (C-1).

2-Azido-2-propylpentanoic acid (7). 2-Bromo-2-propylpentanoic acid **6** (3.90 g, 17.5 mmol) and NaN_3 (1.74 g, 26.8 mmol, 1.5 equivalents) were mixed in dry DMF (50 mL) and stirred at 20°C. The reaction was stopped after 8 days, filtered and concentrated with toluene (50 mL). The residue was acidified to pH 3 in an efficiently ventilated hood with aqueous HCl (1 M) and extracted with EtOAc (3×50 mL). The combined organic phases were dried, concentrated and purified on preparative RP-HPLC affording **7** (1.56 g, 48%). M.p.: 49–49.5°C. ^{1}H-NMR (250 MHz) in $CDCl_3$, δ ppm: 0.77 (t, 6H, $J = 7$ Hz, H-5 and H-5′), 1.15 (m, 2H, H-4a and H-4′a), 1.31 (m, 2H, H-4b and H-4′b), 1.56 (ddd, 2H, $J = 5$ Hz, $J' = 12$ Hz, $J'' = 14$ Hz, H-3a and H-3′a), 1.68 (ddd, 2H, $J = 5$ Hz, $J' = 12$ Hz, $J'' = 14$ Hz, H-3b and H-3′b). ^{13}C-NMR (62.5 MHz) in $CDCl_3$, δ ppm: 12.6 (C-5), 16.0 (C-4), 37.5 (C-3), 76 (C-2), 164 (C-1). IR: 1713, 2119 cm^{-1}. Found: C, 52.04%; H, 8.24%; N, 22.68%. Calculated for $C_8H_{15}N_3O_2$: C, 51.88%; H, 8.16%; N, 22.69%.

2-Azido-2,2-diphenylacetic acid (8). Diphenylacetic acid (6.36 g, 30.0 mmol) and *N*-bromosuccinimide (5.61 g, 31.5 mmol, 1.1 equivalents) were mixed in CCl_4 (50 mL) and refluxed under argon. The reaction was cooled to 0°C after 2 days and filtered to remove succinimide. The filtrate was concentrated with toluene (60 mL). Analytical HPLC of the crude product showed 2-bromo-2,2-diphenylacetic acid (**9**) as the major product. Crude **9** and NaN_3 (3.04 g, 46.8 mmol, 1.6 equivalents) were dissolved in dry DMF (50 mL), stirred for 5 days at 20°C and then the DMF was removed *in vacuo*. The residue was repeatedly concentrated with toluene, dissolved in toluene and filtered twice. The oil was concentrated with toluene. It was purified by flash chromatography (AcOH/EtOAc/hexane 1:4:95) and preparative RP-HPLC yielding diphenylketene (2%) and **8** (0.84 g, 11% from diphenylacetic acid). M.p. 149.7–150.5°C. ^{1}H-NMR (250 MHz) of **5** in $CDCl_3$, δ ppm: 7.32 (H-4, H-5, H-6). ^{13}C-NMR (62.5 MHz) in d_6-DMSO, δ ppm: 78.5 (C-2), 130.0, 130.60, 130.63 (C-4, C-5, C-6), 141.1 (C-3), 173.6 (C-1). IR: 711, 772, 1490, 1748, 2115, 3185 cm^{-1}. Data were identical to those previously reported [5].

(±)-2-Azidohexanoic acid (10). (±)-2-Bromohexanoic acid (10.46 g, 53.6 mmol) and NaN_3 (5.23 g, 80.5 mmol, 1.5 equivalents) were mixed in dry DMF (100 mL) and stirred at 20°C under argon. The DMF was removed *in vacuo* after 3 days. The residue was acidified with aqueous HCl (1 M) in an efficiently ventilated hood, extracted with CH_2Cl_2 (3×30 mL) and the organic phase was dried and concentrated. The crude material was purified by VLC (AcOH/CH_2Cl_2/heptane 5:20:75) affording **10** (8.20 g, 97%). ^{1}H-NMR (250 MHz) in $CDCl_3$, δ ppm: 0.96 (t, 3H, $J = 7$ Hz, H-6), 1.44 (m, 4H, H-4, H-5), 1.88 (m, 2H, H-3), 3.91 (dd, 1H, $J = 5$ Hz, $J' = 8$ Hz, H-2), 9.51 (s, 1H, COO*H*). ^{13}C-NMR (62.5 MHz) in $CDCl_3$, δ ppm: 12.8 (C-6), 21.2 (C-5), 26.8 (C-4), 30.0 (C-3), 60.8 (C-2), 176.0 (C-1). IR: 1721, 2097 cm^{-1}. Found: C, 45.53%; H, 6.88%; N, 26.61%. Calculated for $C_6H_{11}N_3O_2$: C, 45.85%; H, 7.05%; N, 26.74%.

(±)-2-Azidohexadecanoic acid (11). (±)-2-Bromohexadecanoic acid (4.78 g, 14.3 mmol) and NaN_3 (1.44 g, 22.2 mmol, 1.5 equivalents) were mixed in dry DMF (50 mL) and stirred at 20°C for 2 days. The DMF was removed *in vacuo* and the white solid was triturated in cyclohexane (75 mL) to precipitate excess sodium azide. It was filtered off and the filtrate was concentrated. The crude material was recrystallized twice by dissolving it in DMF and slowly adding H_2O. The solution was cooled, filtered and the crystals were washed with cold water (50 mL) and lyophilized affording **11** (1.73 g, 41%). M.p.: 73.7–74.2°C. ^{1}H-NMR (250 MHz) in d_6-DMSO, δ ppm: 1.08 (t, 3H, $J = 6$ Hz, H-16), 1.47 (m, 24H, H-4, . . . , H-15), 1.76 (m, 1H, H-3a), 1.91 (m, 1H, H-3b), 3.89 (dd, 1H, $J = 5$ Hz, $J' = 8$ Hz, H-2). ^{13}C-NMR (62.5 MHz) in d_6-DMSO, δ ppm: 14.8 (C-16),

23.0 (C-15), 26.6 (C-14), 29.5 (C-13), 29.6 (C-12), 29.8 (C-10, C-11), 29.9 (C-3, . . . , C-9), 63.6 (C-2), 173.4 (C-1). IR: 1718, 2110 cm^{-1}. ES-MS: $(M + H^+ - N_2)$ m/z 269.9 (calculated 270). Found: C, 62.49%; H, 9.72%; N, 13.77%. Calculated for $C_{16}H_{31}N_3O_2 \cdot 0.5H_2O$: C, 62.71%; H, 10.53%; N, 13.71%.

(±)-2-Azido-2-phenylacetic acid (12). (±)-2-Bromo-2-phenylacetic acid (11.83 g, 55.0 mmol) and NaN_3 (5.40 g, 83.1 mmol, 1.5 equivalents) were mixed in dry DMF (100 mL) and stirred at 20°C under argon. The reaction was stopped after 2.5 h by removing the DMF *in vacuo*. The residue was acidified with aqueous HCl (1 M) in an efficiently ventilated hood, extracted with EtOAc (3 × 30 mL) and the organic phase was dried and concentrated. The crude was purified by VLC (AcOH/EtOAc/heptane 5:25:70) affording **12** (8.76 g, 90%). M.p.: 98.7–100.3°C. ^{1}H-NMR (250 MHz) in d_6-DMSO, δ ppm: 5.31 (s, 1H, H-2), 7.41 (5H, H-3, H-4, H-5), 13.68 (br s, 1H, COO*H*). ^{13}C-NMR (62.5 MHz) in d_6-DMSO, δ ppm: 66.6 (C-2), 129.7), 130.7, 130.9 (C-4, C-5, C-6), 137.4 (C-3), 172.5 (C-1). IR: 1712, 2109 cm^{-1}. Found: C, 54.13%; H, 4.17%; N, 23.76%. Calculated for $C_8H_7N_3O_2$: C, 54.24%; H, 3.98%; N, 23.72%.

2-(Azidomethyl)-acrylic acid (13). 2-(Bromomethyl)-acrylic acid (4.49 g, 27.2 mmol), NaN_3 (2.06 g, 31.7 mmol, 1.2 equivalents) and *p*-hydroquinone (33 mg, 0.30 mmol, 0.01 equivalents) were mixed in dry THF (50 mL) and stirred under argon in the dark at 20°C. The reaction was stopped after 2 days, filtered and the THF was removed *in vacuo* at 20°C in the dark and all the following operations were carried out in the dark. The residue was extracted with CH_2Cl_2 (3 × 50 mL) and the organic phase was concentrated. The residue was purified by preparative RP-HPLC in the dark without TFA in the buffers. Each pure fraction was shaken with CH_2Cl_2 (20 mL) and a concentrated citric acid solution (0.5 mL) to protonate the azido acid for extraction into the organic phase. This was concentrated affording **13** (1.12 g, 32%) to which a crystal of *p*-hydroquinone was added. It was possible to record ^{1}H-NMR, ^{13}C-NMR and IR spectra but with time the product decomposed and further reactions were difficult to perform. ^{1}H-NMR (250 MHz) in $CDCl_3$, δ ppm: 4.00 (s, 2H, H-3′), 5.93 (br s, 1H, H-3a), 6.46 (br s, 1H, H-3b), 9.47 (br s, 1H, COO*H*). ^{13}C-NMR (62.5 MHz) in $CDCl_3$, δ ppm: 50.8 (C-3′), 130.3 (C-3), 134.4 (C-2), 170.6 (C-1). IR: 1636, 1701, 2105 cm^{-1}.

(S)-2-Azido-4-methylsulfonyl-butyric acid (14). Trifluoromethanesulfonyl azide was prepared by a literature procedure [15] and used directly without purification. L-Methionine (0.886 g, 5.94 mmol) and $CuSO_4 \cdot 5H_2O$ (32.4 mg, 0.130 mmol, 0.02 equivalents) were dissolved in H_2O (18 mL) and the pH was adjusted to 9–10 with K_2CO_3. Methanol (36 mL) and the crude trifluoromethanesulfonyl azide (11.9 mmol) in CH_2Cl_2 (30 mL) were added, the pH was again adjusted to 9–10 with K_2CO_3 and the reaction was stirred vigorously overnight (20 h). The two phases were separated by addition of CH_2Cl_2, the organic phase was washed with H_2O (2 × 40 mL) and the combined aqueous phases were acidified with aqueous HCl (3 M) to pH 2. The aqueous phase was extracted with CH_2Cl_2 (4 × 50 mL). The combined organic phases were dried (Na_2SO_4), filtered, concentrated to approximately 7 mL and purified directly by VLC (AcOH/EtOAc/heptane 5:20:75) affording **14** (0.960 g, 92%). $[\alpha]_D^{25} = -99°$ ($c = 1.0$, $CHCl_3$). ^{1}H-NMR (250 MHz) in $CDCl_3$, δ ppm: 2.10 (m, 2H, H-3), 2.12 (s, 3H, H-6), 2.64 (m, 2H, H-4), 4.24 (dd, 1H, $J = 5$ Hz, $J' = 9$ Hz, H-2), 11.11 (br s, 1H, COO*H*). ^{13}C-NMR (62.5 MHz) in $CDCl_3$, δ ppm: 15.3 (C-6), 30.0 and 30.4 (C-3, C-4), 60.2 (C-2), 176.0 (C-1). IR: 1719, 2110 cm^{-1}. Found: C, 34.43%; H, 5.18%; N, 22.47%. Calculated for $C_5H_9N_3O_2S \cdot 0.25$AcOH: C, 34.73%; H, 5.30%; N, 22.09%.

H-Tyr-Gly-Gly-Phe-Leu-Ahd-Gly-NH$_2$ (15). The peptide **15** was synthesized manually by the syringe method [16] on a PEGA resin [17] derivatized with the Fmoc-Rink-amide linker [18] using TBTU [19] activation for linker attachment. The Fmoc-group was removed with 20% piperidine in DMF and Fmoc-Gly-OPfp was coupled with Dhbt-OH [20,21] catalysis. After removal of the Fmoc-group, (±)-2-azidohexadecanoic acid (5 equivalents) was converted into the acid chloride with a binary mixture of CH_2Cl_2 (0.4 mL) and freshly distilled $SOCl_2$ (0.4 mL, 10 equivalents) by refluxing for 2 h. The acid chloride was coupled to the resin in CH_2Cl_2 with *N*-ethylmorpholine (10 equivalents) present. The azido-group was reduced quantitatively with DTT (1 M) and DIPEA (0.5 M) in DMF at 50°C for 2 h. The rest of the amino acids were coupled as their Fmoc-AA-OPfp esters with Dhbt-OH catalysis. The resin was lyophilized after the last coupling. The peptide was cleaved off the resin with TFA (95%, aqueous) for 2 h, concentrated with acetic acid (95%, aqueous) and water and purified on RP-HPLC affording the two diastereomeric peptides **15a**

(23.8 mg, 40%) and **15b** (26.2 mg, 44%). MALDI-TOF on **15a** and **15b**: m/z 865.7 $(M+H)^+$, 886.9 $(M+Na)^+$, 903.6 $(M+K)^+$, calculated 865.1. Proton, COSY and NOESY NMR spectra were recorded at 310 K in d_6-DMSO and the COSY spectrum was used for assigning the resonances. The spectra of **15a** and **15b** were practically identical and therefore only the spectrum of **15a** is reported (the amide proton of the (±)-2-aminohexadecanoic acid (Ahd)-residue is moved 0.1 ppm downfield in **15b**). $^1H,^1H$-COSY-NMR (500 MHz) in d_6-DMSO of **15a**, δ ppm: 0.855 (3H, Leu $C^{\delta}H_3$), 0.859 (3H, Ahd CH_3), 0.903 (3H, Leu $C^{\delta'}H_3$), 1.228 (24H, Ahd CH_2s), 1.466 (1H, Leu $C^{\beta}H_2$), 1.499 (1H, Leu $C^{\beta'}H_2$), 1.563 (1H, Ahd $C^{\beta}H_2$), 1.584 (1H, Leu $C^{\gamma}H$), 1.689 (1H, Ahd $C^{\beta'}H_2$), 2.753 (1H, Phe $C^{\beta}H_2$), 2.819 (1H, Tyr $C^{\beta}H_2$), 3.009 (1H, Phe $C^{\beta'}H_2$), 3.012 (1H, Tyr $C^{\beta'}H_2$), 3.637 (2H, Gly^7 $C^{\alpha}H_2$), 3.699 (2H, Gly^3 $C^{\alpha}H_2$), 3.808 (2H, Gly^2 $C^{\alpha}H_2$), 3.988 (1H, Tyr $C^{\alpha}H$), 4.201 (1H, Ahd $C^{\alpha}H$), 4.307 (1H, Leu $C^{\alpha}H$), 4.572 (1H, Phe $C^{\alpha}H$), 7.003 and 7.093 (2H, $CONH_2$), 7.174 and 7.240 (9H, Phe and Tyr aromatic protons), 7.995 (1H, Tyr NH), 8.014 (1H, Phe NH), 8.027 (1H, Gly^7 NH), 8.051 (1H, Gly^3 NH), 8.092 (1H, Ahd NH), 8.152 (1H, Leu NH), 8.636 (1H, Gly^2 NH).

In vitro bioassays. Tissues came from male ICR mice weighing 30–40 g and from male Hartley guinea pigs weighing 500–700 g. The mouse vas deferens (MVD) and guinea pig ileum (GPI) assays were electrically induced smooth muscle contractions from MVD and GPI longitudinal muscle-myenteric plexus [22]. The tissues were tied to gold chains with suture silk and suspended in 37°C 20-mL baths containing oxygenated (95% O_2, 5% CO_2) Krebs bicarbonate solution (no magnesium for MVD assay) and allowed to equilibrate for 15 min before being stretched with 1 g tension (0.5 g for MVD) and allowed to equilibrate again. The tissues were stimulated transmurally between platinum plate electrodes at 0.1 Hz for 0.4 ms pulses (2.0 ms pulses for MVD) and 2 V. Drugs were added to the baths in 14–40-μL volumes dissolved in H_2O/DMSO.

Percent inhibition was calculated by dividing the height for 1 min preceding the addition of the drug by the contraction height 3 min after exposure to the drug. The IC_{50} values represent the mean of not less than four tissues. The results can be seen in Figure 2.

RESULTS

Ten azido acids have been synthesized, and one example of their use in SPPS has been given. A large variety of such compounds can be produced either by the diazo transfer [15] reaction, by direct azide-substitution on commercially available α-bromo carboxylic acids or by free radical bromination of carboxylic acids followed by azide substitution. In general, azido acids should be stored in the dark to avoid the slow photolytic degradation by loss of nitrogen. To avoid decomposition of azido acids, temperatures above 50°C should not be applied.

Preparation of the more hindered α,α-disubstituted azido acids (**5**, **7** and **8**) gave rise to some of the hydroxy-compounds when prolonged reaction times (2–3 days) were required but dry conditions and an inert atmosphere greatly improved the yields. When **13** was prepared in the dark, under argon, and in the presence of *p*-hydroquinone then it was possible to analyse the azido acid, however, it

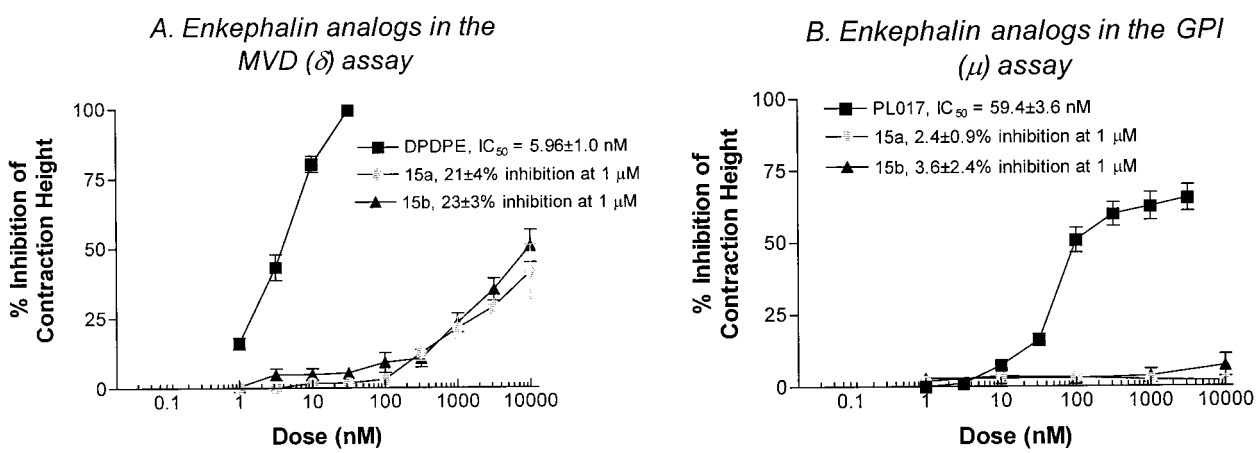

Figure 2 Mean concentration–response data for **15a** and **15b** in the MVD assay (A) and in the GPI assay (B).

polymerized after some hours at −20°C and was too unstable to use for synthesis. Some of the azido acids, **1** [4,5,23], **8** [5,23,24], **10** [25], **11** [26], **12** [25,27,28], and **13**[29] have previously been prepared but data have not been reported for compound **11** or **13** and was included.

APPLICATION OF AZIDO ACIDS IN FMOC-BASED PEPTIDE SYNTHESIS

Enkephalin, which is isolated as a mixture of Leu-enkephalin and Met-enkephalin with the structure H-Tyr-Gly-Gly-Phe-Met/Leu-OH, is an endogenous peptide in the brain targeted at the μ, δ and κ receptors with morphine-like activity [30], i.e. it relieves pain. A variety of enkephalin analogs with high *in vitro* agonist activity have previously been synthesized, but many of these reports did not deal with the bioavailability of the drug, a general problem in drug development. Many drugs acting on receptors in the brain show high activity for their receptor, but only a few of them possess the crucial ability to be taken up in the blood and transported across the blood–brain barrier (BBB). The BBB is lipophilic, so charged or polar compounds such as peptides cannot easily cross the barrier, and it is therefore necessary to investigate possible ways to circumvent this problem [31]. One way is to label the drug with a specific carrier which transports the drug across the BBB by an active transport, as described by Polt *et al.* [32] where β-D-glucose is coupled to an enkephalin analog and the conjugate is actually transported into the brain, probably via a glucose transport system. Another approach is to increase the lipophilicity of the drug by adding alkyl substituents [33].

[Leu^5, Ahd^6, Gly^7]-Enkephalinamide (**15**) was synthesized by standard SPPS methods combined with coupling of racemic 2-azidohexadecanoic acid, incorporated as its acid chloride and later reduced with DTT. The two diastereomeric peptides **15a** and **15b** were analysed by ^{1}H-NMR, COSY, NOESY, MALDI-TOF MS and analytical HPLC. The peptides were tested in the classical MVD and GPI bioassays and both diastereomeric peptides had approximately the same activity which is not surprising since the first five amino acids correspond to enkephalin in both peptides. Moderate activity was observed at the δ-receptor (MVD assay) at high concentrations (21–23% inhibition at 1 μM) and no activity at the μ-receptor (GPI assay). Leu-enkephalin is a δ-selective agonist, so it was expected that **15** would not be active at the μ-receptor. An explanation of the low activity could be that the long C_{14}-alkyl chain inserts into the membrane and reduces the activity of the peptide because it cannot bind the receptor in the correct orientation. Increased bioavailability was expected by making the peptides more lipophilic, however, they turned out to be much less active than the very potent δ-agonist, DPDPE.

CONCLUSION

The α-azido acid building blocks are accessible via nucleophilic substitution on α-bromo carboxylic acids or diazo transfer between triflyl azide and amino acids. Incorporation of α-azido acids into peptides synthesized by the Fmoc-protocol is straightforward, and solves the problems of synthesizing difficult sequences by high activation of the carboxyl group as the acid chloride, thus allowing sterically hindered peptides to be easily synthesized. Many new amino acid derivatives are conveniently derived through the azido acid intermediates and the facile direct use of azido acids in SPPS greatly improves the incorporation of these often valuable compounds into peptides. The azido group is small, and reduction to an amino group is orthogonal to most other protection schemes. Compounds **1**, **5**, **7** and **8** have been incorporated in chemotactic peptides [34], and compound **11** was used in the synthesis of an enkephalin analog (**15**). Biological tests of **15a** and **15b** showed only moderate activity at the δ-opiod receptor and none at the μ-receptor.

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