2-(4-Acetyl-2-nitrophenyl)ethyl: A New Base-Labile Carboxyl Protecting Group

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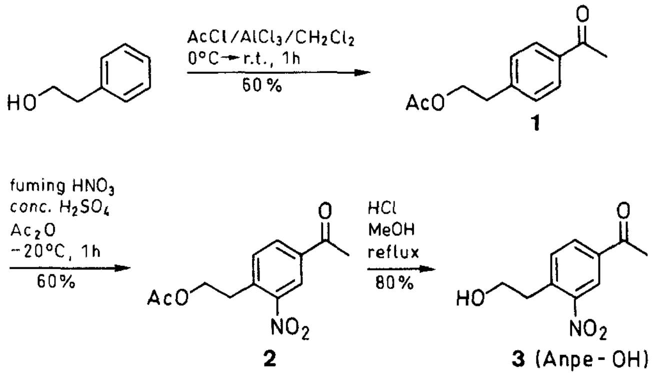
A new base-labile carboxyl protecting group, the 2-(4-acetyl-2-nitrophenyl)ethyl (Anpe) group is reported and compared with other base-labile protecting groups. The synthesis of the alcohol precursor, 1-[4-(2-hydroxyethyl)-3-nitrophenyl]-1-ethanone, the amino acid derivatives Boc-Asp(Anpe)-OH and Boc-Glu(Anpe)-OH and the conditions under which the carboxyl protecting group can be eliminated are also described.

One of the key steps in the synthetic design of multifunctional compounds, as in the case of peptides, oligonucleotides and many other natural and non-natural products, is the correct choice of temporary and permanent protecting groups. In connection with a project directed towards the synthesis of nucleopeptides with a phosphodiester bond between the hydroxyl group of a serine residue and the 5′ end of an oligonucleotide,[1] we have prepared protected peptides with a free side chain hydroxyl group in order to carry out their subsequent phosphitylation and coupling with an oligonucleotide. In particular, for the synthesis of a fragment of adenovirus-2 nucleoprotein,[2] we have obtained a protected peptide corresponding to the sequence -Ser-Gly-Asp- of the protein. The synthesis of the protected peptide was carried out by the subsequent coupling of Boc-amino acids on a *p*-MeBHA (*p*-methylbenzhydrylamine) resin, acetylation of the *N*-terminus and acidolysis with hydrogen fluoride. It is then obvious, from this outline of the synthetic procedure, that in order to obtain the target peptide, Ac-Ser-Gly-Asp(X)-NH_2, the aspartic acid β-carboxyl protecting group has to be stable to acids. Moreover, in an attempt to simplify the overall strategy and to reduce to a minimum the number of deprotection steps at the end of the synthesis of the nucleopeptide, we decided to use a carboxyl protecting group removable under basic conditions, which are also used to eliminate the permanent protecting groups of the phosphate groups, of the nucleobases and of the 3′-OH of the oligodeoxyribonucleotide.[1]

Many base-labile protecting groups, such as the methyl, phenyl,[3] 2-methylthioethyl,[4,5] 2-(*p*-toluenesulfonyl)-ethyl,[6] 1,3-dithianyl-2-methyl,[7] 2-(2′pyridyl)ethyl[8,9] or 9-fluorenylmethyl[10,11] groups, among others, have been described for the protection of the side chain carboxyl groups of aspartic and glutamic acids. Of these, the most labile, and cleavable with a single base treatment without a prior oxidation or alkylation step, is the fluorenylmethyl (Fm) group, which offers the additional advantage that its N^{α}-Boc-aspartic acid derivative is commercially available. We therefore prepared the protected tripeptide Ac-Ser-Gly-Asp(Fm)-NH_2 as described above, and found that the target peptide was soluble only in solvents such as dimethylformamide. As it has been reported that the presence of a fluorenylmethyl or fluorenylmethyl-derived protecting group can have a negative effect on the solubility of amino acids and peptides,[12-14] we decided to search for an alternative base-labile protection for the β-carboxyl of aspartic acid with a stability similar to that of the 9-fluorenylmethyl group but which could improve the solubility of the peptide.

On the basis of the experience of the lability and use of *p*-nitrophenylethyl-type protecting groups,[15-19] we prepared three aspartic acid β-esters: the 2-(4-nitrophenyl)ethyl (Npe) ester, the 2-(4-acetyl-2-nitrophenyl)ethyl (Anpe) ester and the 2-(2,4-dinitrophenyl)ethyl (Dnpe) ester, in order to compare these protecting groups with the β-fluorenylmethyl ester. We also synthesised the 2-cyanoethyl (Cne) and 2-nitroethyl (Ne) β-esters of aspartic acid for the same comparison purpose. All those base-labile side chain protecting groups were chosen as potential candidates for use in peptide synthesis in combination with the N^{α}-Boc protecting group.

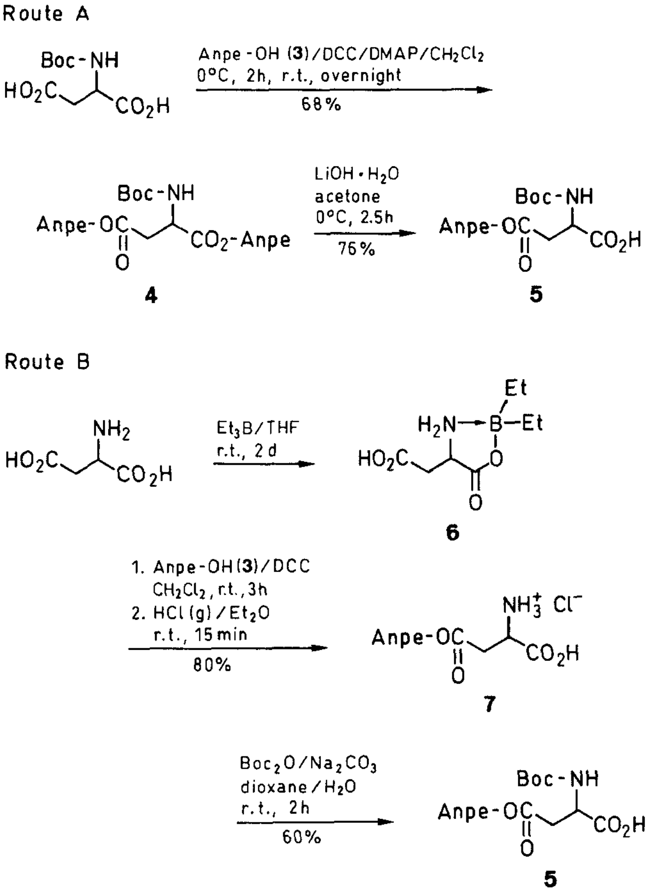
With respect to the alcohol synthons, 2-cyanoethanol and 2-nitroethanol were of commercial origin and 2-(4-nitrophenyl)ethanol and 2-(2,4-dinitrophenyl)ethanol were obtained as described.[15,20] The Anpe synthon 1-[4-(2-hydroxyethyl)-3-nitrophenyl]-1-ethanone was prepared by the subsequent reaction of 2-phenylethanol with acetyl chloride and aluminium trichloride, fuming nitric acid in acetic anhydride and concentrated sulfuric acid, and methanolic hydrogen chloride (Scheme 1).



Scheme 1

Two alternative methods have been used for the synthesis of the corresponding Boc-Asp(X)-OH derivatives: route A) diesterification of Boc-Asp-OH with the alcohol and selective hydrolysis of the α-carboxylic ester with lithium hydroxide;[21] route B) simultaneous protection of both the α-amino and α-carboxyl groups by reaction of aspartic acid with triethylborane and selective esterification of the β-carboxyl,[22] hydrolysis of the oxazaborole complex with hydrochloric acid and α-amino protection with the Boc group.[23] The two methods are illustrated in Scheme 2 for the synthesis of Boc-Asp(Anpe)-OH.

Boc-Asp(Anpe)-OH was prepared by using the two synthetic methods with practically the same overall yield, but a slightly purer product was obtained in the route



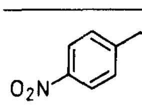
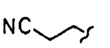
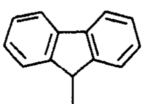
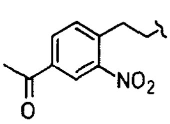
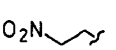
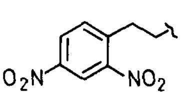
Scheme 2

which uses the oxazaborole complex. Boc-Asp(Npe)-OH was obtained by route B and Boc-Asp(Cne)-OH was synthesised by route A. Boc-Asp(Dnpe)-OH could not be obtained by any of the two methods: on the one hand, Boc-Asp-OH was diesterified to yield Boc-Asp(Dnpe)$_2$, but lithium hydroxide treatment removed both dinitrophenylethyl groups; on the other hand, we were able to obtain H-Asp(Dnpe)-OH via formation of the oxazaborole complex, but during the process of introduction of the Boc group (which has to be effected at a slightly basic pH) the dinitrophenylethyl group was cleaved. Finally, neither were we able to prepare Boc-Asp(Ne)-OH because of the base-lability of the nitroethyl group to even slightly basic media.

These results give a preliminary indication of the relative base-lability of these protecting groups, the 2-nitroethyl and 2-(2,4-dinitrophenyl)ethyl esters being much more labile than the others. It is interesting to note that the lability of the protecting group also depends on the functional group being protected: the 2-(2,4-dinitrophenyl)ethyl group has been successfully used for the protection of α-amino groups[18] and the side chain thiol group of cysteine,[14] whilst, in our hands, Boc-Asp(Dnpe)-OH cannot be prepared. The dinitrophenylethyl ester is much more labile than the corresponding carbamate or thioether.

The comparison between Npe, Anpe, Cne and Fm protecting groups was carried out on the basis of their lability towards a treatment with 0.1 M tetrabutylammonium fluoride in tetrahydrofuran under which they are all removed by β-elimination. We observed that 2 hours were necessary to cleave the 2-(4-nitrophenyl)ethyl ester of Boc-Asp(Npe)-OH, 45 min to quantitatively deprotect Boc-Asp(Cne)-OH, and that the 2-(4-acetyl-2-nitrophenyl)ethyl and 9-fluorenylmethyl groups group could be eliminated in less than 5 minutes. The behaviour of the different protecting groups is summarised in the Table. Among the new carboxyl protecting groups evaluated, the most advantageous seems to be the 2-(4-acetyl-2-nitrophenyl)ethyl group, which has the same order of lability as the 9-fluorenylmethyl group. The 2-(4-nitrophenyl)ethyl and the 2-cyanoethyl groups are too stable and would require very long deprotection times, and the 2-(2,4-dinitrophenyl)ethyl and 2-nitroethyl are too labile and cannot be used for the side chain protection of aspartic acid because their Boc-derivatives cannot even be prepared.

Table. Relative Lability of Aspartic Acid β-Carboxyl Protecting Groups

Carboxyl Protecting Group		Deprotection Time (with 0.1 M TBAF)
	(Npe)	1.5–2 h
	(Cne)	45 min
	(Fm)	< 5 min
	(Anpe)	< 5 min
	(Ne)	–[a]
	(Dnpe)	–[a]

[a] These protecting groups are the most labile, as has been shown by the failure to prepare their N$^\alpha$-Boc derivatives (see text).

The 2-(4-acetyl-2-nitrophenyl)ethyl derivative of aspartic acid was submitted to the reaction conditions used along the different steps of peptide synthesis with Boc-amino acids and it remained intact during all the treatments as well as during the final cleavage with hydrogen fluoride. With respect to the deprotection conditions, the 2-(4-acetyl-2-nitrophenyl)ethyl group can be easily eliminated by the following treatments: a) 0.01 M tetrabutylammonium fluoride in tetrahydrofuran (5 min); b) 0.25 M lithium hydroxide in methanol/dioxane/water (1.5 : 1.5 : 1)[24] (45 min); c) 0.05 M potassium carbonate in methanol/dioxane (1 : 1)[25] (30 min), these conditions remove the 2-(4-acetyl-2-nitrophenyl)ethyl group but yield aspartic acid β-methyl ester. The 2-(4-acetyl-2-nitrophenyl)ethyl group can also be cleaved by treatment with

0.5 M 1,8-diazabicyclo[5.4.0]undec-7-ene in dioxane for 15 hours. Cleavage of the 2-(4-acetyl-2-nitrophenyl)ethyl group yields 4-acetyl-2-nitrophenylethene, which can react with nucleophilic species. The side chain functional groups of the amino acids tryptophane, methionine or cysteine could react with activated olefins such as 4-acetyl-2-nitrophenylethene under acidic conditions in which carbocations are formed,[26,27] but no side reaction has been described which takes place under the basic conditions used to remove the 2-(4-acetyl-2-nitrophenyl)ethyl group.

For an evaluation of the effect on the solubility of a protected peptide, we carried out the synthesis of the tripeptide mentioned above, substituting the fluorenylmethyl group by the 2-(4-acetyl-2-nitrophenyl)ethyl protecting group: peptide Ac-Ser-Gly-Asp(Anpe)-NH_2 was more soluble in tetrahydrofuran than peptide Ac-Ser-Gly-Asp(Fm)-NH_2, and had a shorter retention time when analysed by reversed phase HPLC under the same conditions (see experimental part; Ac-Ser-Gly-Asp(Anpe)-NH_2, retention time, 12.6 min; Ac-Ser-Gly-Asp(Fm)-NH_2, retention time, 17.3 min).

From the comparison of the different aspartic acid derivatives that we have prepared, we conclude that the 2-(4-acetyl-2-nitrophenyl)ethyl protecting group is an alternative to the 9-fluorenylmethyl group, as both have a similar lability to tetrabutylammonium fluoride but the 2-(4-acetyl-2-nitrophenyl)ethyl derivatives are more soluble in organic solvents.

The γ-[2-(4-acetyl-2-nitrophenyl)ethyl] ester of glutamic acid has also been obtained by temporary protection of the α-amino and α-carboxyl groups as an oxazaborole complex and esterification of the side chain carboxyl group with 1-[4-(2-hydroxyethyl)-3-nitrophenyl]-1-ethanone, as we have found for aspartic acid that this synthetic method yields a purer product than diesterification of the amino acid and selective hydrolysis of the α-carboxyester. These new amino acid derivatives are fully compatible with all the reagents used in solid phase peptide synthesis with N^{α}-Boc-amino acids. The 2-(4-acetyl-2-nitrophenyl)ethyl group adds a new alternative to the range of carboxyl protecting groups, thus allowing different combinations of protecting groups to be used for the selective removal of one of them in the presence of the others.

^1H and ^{13}C nuclear magnetic resonance spectra were recorded either on a Varian XL-200 or on a Gemini-200 instrument. ^1H and ^{13}C chemical shifts (δ) are given in ppm relative to TMS as internal standard. Mass spectra were obtained using a Hewlett-Packard 5988 A mass spectrometer. Optical rotations were measured with a Perkin-Elmer 241-MC polarimeter. Melting points were recorded using a Büchi apparatus and are uncorrected. IR spectra were registered on a Perkin-Elmer 681 and UV-visible absorption was detected on a Perkin-Elmer Lambda 5 spectrophotometer. Elemental analyses were determined on a Perkin-Elmer 240 apparatus.

TLC was carried out on aluminum-backed sheets (silica gel 60 F, 0.2 mm, Merck), with the following solvent systems: A, $CHCl_3$/MeOH/AcOH (73:25:2); B, EtOAc/hexane (50:50); C, CH_2Cl_2/MeOH (98:2); D, $CHCl_3$/MeOH/AcOH (90:10:2). Column chromatography was performed on silica gel (Chromagel 60 A CC, 230–400 mesh, SDS).

CH_2Cl_2 was neutralised and dried by passing it through basic alumina and storage over CaH_2. Anhydr. THF was obtained by distillation over Na metal in the presence of benzophenone. Et_2O was dried over Na wire. DMF was bubbled with N_2 to remove volatile contaminants and kept stored over activated 4 A molecular sieves. All other solvents and reagents were analytical grade or distilled before use.

HPLC was carried out using either Shimadzu, Beckman or Waters systems. Analyses were performed on either C-18 Spherisorb or Nucleosil columns (25 × 0.5 cm, 10 μm), using linear gradients of 0.045 % CF_3CO_2H in water (A) and 0.36 % CF_3CO_2H in MeCN (B) at 1 mL/min. Gel filtration was carried out on Sephadex LH-20 from Pharmacia.

Boc-L-amino acids (except Boc-Asp(Anpe)-OH) and peptide resins were obtained from Bachem Feinchemikalien AG and Novabiochem AG. Peptide syntheses were done manually in a polypropylene syringe fitted with a polyethylene disk. Amino acid analyses were performed on a Beckmann System 6300 analyser. Hydrolyses were carried out in 12 M HCl/propionic acid (1:1) at 150 °C for 60–90 min.

2-Cyanoethanol and 2-nitroethanol were of commercial origin (Aldrich, Merck) and 2-(4-nitrophenyl)ethanol and 2-(2,4-dinitrophenyl)ethanol were prepared as described.[11,15] Satisfactory microanalyses (C ± 0.66, H ± 0.55, N ± 0.58) were obtained for all new compounds.

1-[4-(2-Hydroxyethyl)-3-nitrophenyl]-1-ethanone (Anpe-OH) 3:

2-(4-Acetylphenyl)ethyl Acetate (**1**): A solution of 2-phenylethanol (20 g, 164 mmol) in CH_2Cl_2 (25 mL) was added dropwise to a suspension of $AlCl_3$ (68.3 g, 512 mmol) in CH_2Cl_2 cooled in an ice bath. When the solid was completely dissolved, AcCl (23 mL, 325 mmol) was added. After 30 min, the ice bath was removed and the mixture stirred for 1 h at r.t. After cooling the mixture at −20 °C, 10 % aq HCl in ice (100 mL) was added to destroy the excess of $AlCl_3$. The solid formed was filtered and Et_2O (100 mL) was added to the filtrate. The organic phase was washed with 10 % aq HCl (3 × 90 mL) and 10 % aq $NaHCO_3$ (3 × 90 mL), dried (Na_2SO_4) and evaporated to dryness. Purification of the residue by column chromatography on silica gel eluting with a gradient of 0–2 % MeOH in CH_2Cl_2 afforded 20 g of a yellow oil; yield: 20 g (60 %); TLC: R_f (system B): 0.4, (system C): 0.6.

^1H NMR ($CDCl_3$, 200 MHz): δ = 7.90 (2 H, d, J = 8 Hz, Ar), 7.32 (2 H, d, J = 8 Hz, Ar), 4.30 (2 H, t, J = 7 Hz, CH_2O), 3.00 (2 H, t, J = 7 Hz, $C\underline{H}_2Ar$), 2.58 (3 H, s, CH_3CO).

^{13}C NMR ($CDCl_3$, 50 MHz): δ = 195.0 ($\underline{C}OAr$), 170.7 (CO_2), 143.4, 136.7 (C_{arom}), 128.9, 128.4 (CH_{arom}), 64.0 (CH_2O), 34.8 ($\underline{C}H_2Ar$), 26.4 ($\underline{C}H_3COAr$), 20.7 ($\underline{C}H_3CO_2$).

IR (film): ν = 1735, 1680, 1240 cm^{-1}.

2-(4-Acetyl-2-nitrophenyl)ethyl Acetate (**2**): 2-(4-acetylphenyl)ethyl acetate (**1**; 7 g, 34 mmol) was dissolved in Ac_2O (20 mL) and cooled to −20 °C. A solution of 96 % H_2SO_4 (1.7 mL) in Ac_2O (10 mL) was added dropwise, followed by fuming HNO_3 (1.7 mL, 41 mmol), at −20 °C. After 1 h, the solution was poured over ice (100 mL). The product was extracted with Et_2O (2 × 100 mL). The organic phase was washed with 10 % aq $NaHCO_3$ (3 × 100 mL) and water (100 mL), dried (Na_2SO_4) and evaporated to dryness. The residue was purified by column chromatography on silica gel with a gradient of 0–2 % MeOH in CH_2Cl_2; a yellow oil was obtained; yield: 5.1 g (60 %); TLC: R_f (system B): 0.30, (system C): 0.65.

^1H NMR ($CDCl_3$, 200 MHz): δ = 8.48 (1 H, d, J = 2 Hz, Ar), 8.12 (1 H, dd, J = 2, 8 Hz, Ar), 7.52 (1 H, d, J = 8 Hz, CH_2O), 4.38 (2 H, t, J = 7 Hz, $C\underline{H}_2Ar$), 2.66 (3 H, s, $C\underline{H}_3COAr$), 2.02 (3 H, s, CH_3CO_2).

^{13}C NMR ($CDCl_3$, 50 MHz): δ = 195.3 ($\underline{C}OAr$), 170.5 (CO_2), 137.7, 137.0, 134.3 (C_{arom}), 133.0, 131.8 (CH_{arom}), 63.2 (CH_2O), 32.1 (CH_2Ar), 26.5 ($\underline{C}H_3COAr$), 20.6 ($\underline{C}H_3CO$).

IR (film): ν = 1735, 1685, 1525, 1355, 1240 cm^{-1}.

1-[4-(2-Hydroxyethyl)-3-nitrophenyl]-1-ethanone (Anpe-OH) (**3**): 2-(4-acetyl-2-nitrophenyl)ethyl acetate (**2**; 5.1 g, 20 mmol) was refluxed in conc. aq HCl/MeOH (2:8, 100 mL) for 2 h at 50–60 °C, and then the solution was concentrated to dryness. The residue was purified by column chromatography on silica gel eluting with EtOAc/hexane (1 : 1) to afford a pale yellow oil; yield: 3.2 g (77 %); TLC: R_f (system B): 0.20, (system C): 0.25, (system D): 0.50.

^{1}H NMR ($CDCl_3$, 200 MHz): δ = 8.43 (1 H, d, J = 2 Hz, Ar), 8.20 (1 H, dd, J = 2, 8 Hz, Ar), 7.55 (1 H, d, J = 8 Hz, Ar), 3.96 (2 H, t, J = 7 Hz, $C\underline{H}_2OH$), 3.22 (2 H, t, J = 7 Hz, $C\underline{H}_2Ar$), 2.64 (3 H, s, $C\underline{H}_3COAr$), 2.20 (1 H, OH).

^{13}C NMR ($CDCl_3$, 50 MHz): δ = 195.3 (CO), 138.8, 136.1, 134.5 (C_{arom}), 133.2, 131.7, 124.4 (CH_{arom}), 61.9 (CH_2OH), 35.9 ($\underline{C}H_2Ar$), 26.5 ($\underline{C}H_3CO$).

IR (film): ν = 3420, 1695, 1535, 1360 cm^{-1}.

MS (EI): m/z = 210 ($M^+ + 1$), 194, 179, 162, 120, 77, 43.

Preparation of N^α-*tert*-butoxycarbonyl-L-aspartic Acid β-esters, Boc-Asp(X)-OH:

Route A:

a) Boc-Asp(X)$_2$:

Boc-Asp-OH and the corresponding alcohol (2 equiv) were suspended in CH_2Cl_2 and cooled in an ice bath. Dicyclohexylcarbodiimide (DCC) (3 equiv) dissolved in CH_2Cl_2 was added dropwise for 2 h at 0 °C followed by 4-dimethylaminopyridine (0.1 equiv). Then the bath was removed and the reaction continued overnight. The mixture was chilled for 1 h to promote the precipitation of dicyclohexylurea, which was separated by filtration. The filtrate was diluted with CH_2Cl_2 and washed twice with 5 % aq $NaHCO_3$ and 1 M aq $KHSO_4$, dried (Na_2SO_4) and evaporated to dryness. The residue was purified by column chromatography on silica gel eluting with a gradient of MeOH (0–5 %) in CH_2Cl_2.

Boc-Asp(Cne)$_2$: white solid; yield: (85 %); TLC; R_f (system D): 0.75.

^{1}H NMR ($CDCl_3$, 200 MHz): δ = 5.65 (1 H, NH), 4.30–3.90 (5 H, m, CHα, CH_2O), 3.10–2.90 (6 H, m, CH_2CN, $CH_2\beta$), 1.46 (9 H, s, t-C_4H_9).

Boc-Asp(Dnpe)$_2$: orange foam; yield: (75 %); TLC: R_f (system A): 0.75.

^{1}H NMR ($CDCl_3$, 200 MHz): δ = 8.72 (2 H, d, J = 2 Hz, Ar); 8.43 (2 H, dd, J = 2,8 Hz, Ar), 7.60 (2 H, d, J = 8 Hz, Ar), 5.54 (1 H, m, NH), 4.45–4.00 (5 H, m, CHα, CH_2O), 3.30–3.00 (6 H, m,$C\underline{H}_2Ar$, $CH_2\beta$), 1.47 (9 H, s, t-C_4H_9).

Boc-Asp(Ne)$_2$: white foam; yield: (60 %); TLC: R_f (system A): 0.70.

^{1}H NMR ($CDCl_3$, 200 MHz): δ = 5.62 (1 H, NH), 4.30–3.95 (7 H, m, CH_2NO_2, CH_2O, CHα), 3.10–2.90 (2 H, m, $CH_2\beta$), 1.48 (9 H, s, t-C_4H_9).

Boc-Asp(Anpe)$_2$ (**4**): yellow foam; yield: (68 %); TLC: R_f (system D): 0.70.

^{1}H NMR ($CDCl_3$, 200 MHz): δ = 8.48 (2 H, s, Ar), 8.12 (2 H, dd, Ar), 7.54 (2 H, m, Ar), 4.52–4.24 (3 H, m, CHα, CH_2O), 3.30 (2 H, m, $\underline{C}\underline{H}_2Ar$), 3.10–2.80 (2 H, m, $CH_2\beta$), 2.66 (3 H, s, CH_3CO), 1.43 (9 H, s, t-C_4H_9).

IR (film): ν = 3340, 2950–2875, 1720–1680, 1530, 1350, 1250, 1220, 1150 cm^{-1}.

b) Boc-Asp(X)-OH:

To a solution of Boc-Asp(X)$_2$ in acetone, cooled in an ice bath, was added a solution of LiOH · H_2O (0.8 equiv) in water during 1.5 h at 0 °C.[21] The reaction mixture was allowed to warm up to r. t. and the stirring was maintained for an additional 0.5 h, after which time the acetone was removed. The aqueous solution was diluted and washed with EtOAc (2 × 25 mL) and after cooling in an ice bath, it was acidified to pH 2 with 10 % aq HCl. The desired product, Boc-Asp(X)-OH, was then extracted with EtOAc (2 × 25 mL) and the organic phase was dried (Na_2SO_4) and evaporated to dryness.

Boc-Asp(Cne)-OH: white solid, crystallised from EtOAc/hexane; yield: (85 %); TLC: R_f (system A): 0.80; mp 78–80 °C; $[\alpha]_D$: + 33.5° (c = 1.00, $CHCl_3$).

^{1}H NMR ($CDCl_3$, 200 MHz): δ = 5.36 (1 H, NH), 4.30–4.10 (3 H, m, CH_2O, CHα), 3.10 (2 H, m, CH_2CN), 2.90 (2 H, m, $CH_2\beta$), 1.45 (9 H, s, t-C_4H_9).

^{13}C NMR ($CDCl_3$, 50 MHz): δ = 175.0 (CO_2H), 170.6 (CO_2R), 155.4 (OCONH), 80.9 [$\underline{C}(CH_3)_3$], 50.7 (CHα), 50.8 (CH_2O), 37.7 ($CH_2\beta$), 32.8 (CH_2CN), 28.7 [$C(\underline{C}H_3)_3$].

IR (film): 3350, 2945, 2860, 2275, 1715, 1650, 1175 cm^{-1}.

Analytical HPLC (linear gradient of 30 to 100 % B in 20 min, 220 nm) retention time: 14.6 min.

Boc-Asp(Anpe)-OH (**5**): yellow foam yield: (76 %); For characterisation, see method B.

Route B:

a) HCl · H-Asp(X)-OH:

The aspartic acid 2,2-diethyl-5-oxotetrahydro-1,3,2-oxazaborole derivative[28] **6** was dissolved in CH_2Cl_2 and 1.2 equiv of both the alcohol (X–OH) and DCC were added. The mixture was stirred for 3 h at r. t. The suspension was cooled in an ice bath to promote the precipitation of dicyclohexylurea and then filtered. The filtrate was evaporated to dryness, redissolved in anhydr. Et_2O, cooled in an ice bath and gaseous HCl was passed through the solution for about 10 min. The solid that separated was decanted, washed twice with Et_2O and dried.

HCl·H-Asp(Npe)-OH: yellow foam; yield: (80 %); TLC: R_f (system A): 0.30.

^{1}H NMR (CD_3OD, 200 MHz): δ = 8.14 (2 H, d, Ar), 7.46 (2 H, d, J = 9 Hz, Ar), 4.42 (2 H, t, J = 7 Hz, CH_2O), 3.90 (1 H, m, CHα), 3.10–2.80 (4 H, m, $C\underline{H}_2Ar$, $CH_2\beta$).

IR (film): ν = 3500–2500, 1740, 1520, 1345 cm^{-1}.

HCl·H-Asp(Dnpe)-OH: orange foam; yield: (75 %); TLC: R_f (system A): 0.35.

^{1}H NMR (CD_3OD, 200 MHz): δ = 8.35 (1 H, d, J = 2 Hz, Ar), 8.12 (1 H, dd, J = 2,8 Hz, Ar), 7.46 (1 H, d, J = 8 Hz, Ar), 4.25–3.80 (3 H, m, CH_2O, CHα), 3.15–2.80 (4 H, m, CH_2Ar, CHβ).

IR (film): ν = 3500–2600, 1750, 1540, 1350 cm^{-1}.

HCl·H-Asp(Anpe)-OH (**7**): yellow foam; yield: (75 %); TLC: R_f (system A): 0.15.

^{1}H NMR (CD_3OD, 200 MHz): δ = 8.50 (1 H, s, Ar), 8.23 (1 H, d, J = 7 Hz, Ar), 7.68 (1 H, d, J = 7 Hz, Ar), 4.45 (3 H, m, CHα, CH_2O), 3.30 (2 H, m, CH_2Ar), 3.00 (2 H, m, $CH_2\beta$), 2.64 (3 H, s, CH_3CO).

IR (film): ν = 3650–2300, 1740, 1685, 1610, 1535, 1350 cm^{-1}.

b) Boc-Asp(X)-OH:

HCl · H-Asp(X)-OH was dissolved in dioxane/water (2 : 1) and brought to pH 9 by adding 10 % aq Na_2CO_3. The solution was cooled with an ice bath and di-*tert*-butyl dicarbonate (1.5 equiv) was slowly added.[23] The reaction was continued for about 2 h keeping the pH at 9 by adding further Na_2CO_3 solution. When TLC analyses indicated that the reaction was complete, the aqueous solution was washed with Et_2O (2 × 25 mL), chilled, acidified with 10 % aq HCl to pH 2 and extracted with Et_2O (3 × 25 mL). The organic phase was dried (Na_2SO_4) and concentrated to dryness. The residue was purified by column chromatography eluting with mixtures of EtOAc/MeOH/AcOH.

Boc-Asp(Npe)-OH: yellowish solid after precipitation from Et_2O/hexane; yield: (60 %); TLC: R_f (system A): 0.85; mp: 62–64 °C; $[\alpha]_D$: + 25.0° (c = 1.02, $CHCl_3$).

^{1}H NMR ($CDCl_3$, 200 MHz): δ = 8.22 (2 H, d, J = 9 Hz, Ar), 7.42 (2 H, d, J = 9 Hz, Ar), 5.50 (1 H, d, J = 8 Hz, NH), 4.39 (2 H, m, CH_2O), 3.76 (1 H, m, CHα), 3.10–2.75 (4 H, m, $CH_2\beta$, CH_2Ar), 1.45 (9 H, s, t-C_4H_9).

^{13}C NMR ($CDCl_3$, 50 MHz): δ = 174.4 (CO_2H), 171.4 (CO_2R), 156.1 (OCONH), 147.0, 146.1 (C_{arom}), 130.3, 124.2 (CH_{arom}), 80.8 [$\underline{C}(CH_3)_3$], 64.9 (CH_2O), 50.2 (CHα), 28.6 [$C(\underline{C}H_3)_3$].

IR (film): ν = 3340, 2980–2975, 1740–1710, 1520, 1350, 1160 cm^{-1}.

MS (CI, NH_3) m/z: 400 ($M + NH_4^+$).

Analytical HPLC (linear gradient of 30 to 100 % B in 20 min, 220 nm) retention time: 12.3 min.

Boc-Asp(Anpe)-OH **(5)**: yellow foam; yield: (64 %); TLC: R_f (system A): 0.65; $[\alpha]_D$: + 22.6° ($c = 1.97$, $CHCl_3$).

UV (EtOH): $\lambda_{max} = 234$ nm ($\varepsilon = 35500$).

^1H NMR ($CDCl_3$, 200 MHz): δ = 8.46 (1 H, d, J = 8 Hz, Ar), 8.10 (1 H, dd, J = 1.5, 8 Hz), 7.50 (1 H, d, J = 8 Hz, Ar), 5.55 (1 H, d, J = 7.5 Hz, NH), 4.45 (1 H, m, CHα), 4.40 (2 H, m, CH_2O), 3.28 (2 H, m, $C\underline{H}_2Ar$), 3.10–2.80 (2 H, m, $CH_2\beta$), 2.63 (3 H, s, CH_3CO), 1.40 (9 H, s, t-C_4H_9).

^{13}C NMR ($CDCl_3$, 50 MHz): δ = 196.7 ($COCH_3$), 174.8 (CO_2H), 170.7 (CO_2R), 155.5 (OCONH), 137.5, 136.7, 134.2 (C_{arom}), 133.2, 132.1, 124.7 (CH_{arom}), 80.4 [$C(CH_3)_3$], 63.9 (CH_2O), 49.6 (CHα), 36.4 ($CH_2\beta$), 32.3 ($\underline{C}H_2Ar$), 28.1 [$C(\underline{C}H_3)_3$], 26.5 (CH_3CO).

IR (film) ν = 3500–2700, 1745, 1720, 1710, 1695, 1530, 1370, 1230, 1180 cm^{-1}.

MS (CI, CH_4): m/z = 451 ($M^+ + C_2H_5$), 441 ($M^+ + CH_5$), 425 ($M^+ + 1$).

Analytical HPLC (linear gradient of 30 to 100 % B in 20 min, 220 nm) retention time: 11.4 min.

Solid material was obtained by formation of the dicyclohexylammonium salt: to a solution of Boc-Asp(Anpe)-OH in Et_2O cooled in an ice bath, dicyclohexylamine (1 equiv) was added. The solution was concentrated to dryness and a yellowish solid was obtained; mp 60–62 °C.

Boc-Glu(Anpe)-OH:

This was prepared as described above for aspartic acid, route B.

HCl.H-Glu(Anpe)-OH: orange foam; yield: (70 %); TLC: R_f (system A): 0.25.

^1H NMR (CD_3OD, 200 MHz): δ = 8.4 (1 H, d, J = 2 Hz, Ar), 8.15 (1 H, dd, J = 2,7 Hz, Ar), 7.62 (1 H, d, J = 7 Hz, Ar), 3.90–3.60 (3 H, m, CHα, CH_2O), 3.30–3.00 (4 H, m, $C\underline{H}_2Ar$, $CH_2\beta$), 2.62 (3 H, s, CH_3CO), 2.35–2.10 (2 H, m, $CH_2\gamma$).

IR (film): ν = 3375, 2980, 2875, 2600–2000, 1740, 1690, 1540, 1350, 1130 cm^{-1}.

Boc-Glu(Anpe)-OH: yellowish solid after precipitation from Et_2O/hexane; yield: (40 %); TLC: R_f (system A): 0.65; $[\alpha]_D$: + 2.2° ($c = 0.92$, $CHCl_3$); mp 52–54 °C.

^1H NMR ($CDCl_3$/200 MHz): δ = 8.50 (1 H, d, J = 2 Hz, Ar), 8.08 (1 H, dd, J = 2,7 Hz, Ar), 7.52 (1 H, d, J = 7 Hz, Ar), 5.42 (1 H, NH), 4.36 (2 H, m, CH_2O), 3.80–3.70 (1 H, m, CHα), 3.26 (2 H, t, J = 7 Hz, $C\underline{H}_2Ar$), 2.97 (2 H, m, $CH_2\beta$), 2.62 (3 H, s, CH_3CO), 2.23 (2 H, m, $CH_2\gamma$), 1.40 (9 H, s, t-C_4H_9).

^{13}C NMR ($CDCl_3$, 50 MHz): δ = 194.0 ($COCH_3$), 173.2 (CO_2H), 171.3 (CO_2R), 154.0 (OCONH), 138.2 (C_{arom}), 131.5, 122.9 (CH_{arom}), 79.2 [$\underline{C}(CH_3)_3$], 61.8 (CH_2O), 50.8 (CHα), 30.5 ($CH_2\gamma$), 28.6, 28.3 ($CH_2\beta$, $\underline{C}H_2Ar$), 26.4 ($C(CH_3)_3$], 25.6 (CH_3CO).

IR (film): ν = 3340, 2980–2800, 1740, 1720, 1695, 1540, 1370, 1160 cm^{-1}.

MS (CI NH_3) m/z: 456 ($M + NH_4^+$).

Stability of Boc-Asp(X)-OH Under Different Conditions

The lability of Boc-Asp(X)-OH derivatives to basic deprotecting conditions was determined by checking the disappearance of Boc-Asp(X)-OH and the appearance of Boc-Asp-OH by either TLC (system A) or HPLC analysis (linear gradient of 30 to 100 % B in 20 min, 220 nm). The amino acid derivatives were treated with 10 equiv of 0.1 M Bu_4NF ($TBAF.3H_2O$) in THF (see results in the Table). Boc-Asp(Anpe)-OH was also treated with 10 equiv of either a solution of 0.05 M K_2CO_3 in MeOH/dioxane (1:1), a solution of 0.25 M $LiOH.H_2O$ in MeOH/dioxane/H_2O (1.5:1.5:1) or a 0.5 M solution of 1,8-diazabicyclo[5.4.0]undec-7-ene in dioxane, the Anpe protecting group being completely eliminated in 30 min, 45 min and 15 h, respectively.

The stability of Boc-Asp(Anpe)-OH to the different reagents used in peptide synthesis was checked by preparing a solution of the product (approx. 10 mM) in either 5 % ethyldiisopropylamine (EDIA) in CH_2Cl_2 on 30 % CF_3CO_2H/CH_2Cl_2. No loss of Anpe protecting group was observed after 24 h under any of the two conditions by TLC analysis [system A or butanol/H_2O/pyridine/AcOH (15:15:12:3), respectively]. The Anpe protecting group also showed to be stable to a 1 h treatment with HF/anisole 9:1 at 0 °C (butanol/H_2O/pyridine/AcOH, 15:15:12:3).

Ac-Ser-Gly-Asp(Anpe)-NH$_2$:

Boc-Asp(Anpe)-OH, Boc-Gly-OH and Boc-Ser(Bzl)-OH were subsequently coupled onto a p-methylbenzhydrylamine resin (0.5–1 mmol scale) using standard protocols which comprise acidolytic removal of the Boc protecting group with 30 % TFA/CH_2Cl_2 (5 + 25 min), neutralisation of the α-amino group with 5 % $EDIA/CH_2Cl_2$ and a DCC-mediated coupling (2–3 equivs of both the Boc-amino acid and DCC, in CH_2Cl_2 or mixtures CH_2Cl_2/DMF depending on the solubility of the amino acids, for 1 h). A qualitative ninhydrin test was used to monitor the completeness of couplings. In the last step, the N-terminus was blocked by reaction with 10 equiv of Ac_2O and EDIA in DMF. The protected peptide was detached from the resin by treatment with HF/anisole (9:1) at 0 °C for 1 h, and after evaporation of HF the resin was washed with Et_2O, THF and DMF. Some peptide was present in the THF filtrate (~ 25 %), and the rest in the DMF solution. Purification of the peptide was carried out by gel filtration on Sephadex LH-20 (100 × 1 cm) eluting with DMF (0.5 mL/min, detection of the elution profile at 254 nm). Ac-Ser-Gly-Asp(Anpe)-NH_2 was obtained as a white solid after evaporation of the corresponding fractions and trituration with anhydr. Et_2O; yield: 53 % (synthesis and purification); amino acid analysis: Ser: 0.7, Gly: 1.1, Asp: 1.0.

^1H NMR (DMSO-d_6, 200 MHz): δ = 8.43 (1 H, d, J = 1.5 Hz, Ar), 8.25 (3 H, m, Ar, NH Gly), 8.05 (2 H, m, NH Asp, NH Ser), 7.76 (1 H, d, J = 8 Hz, Ar), 7.24, 7.19 (2 H, s, NH_2), 4.55 (1 H, m, CHα Asp), 4.25 (3 H, m, CHα Ser, CH_2O), 3.72 (2 H, m, $CH_2\alpha$ Gly), 3.58 (2 H, m, $CH_2\beta$ Ser), 3.23 (2 H, m, $C\underline{H}_2Ar$), 2.65 (3 H, s, $\underline{C}\underline{H}_3COAr$), 1.87 (3 H, s, CH_3CONH).

MS (FAB, nitrobenzylalcohol, Xe, positive model): m/z = 510 ($M + H^+$), 532 ($M + Na^+$).

Analytical HPLC (linear gradient of 10 to 55 % B in 20 min, 260 nm), retention time: 12.6 min.

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