Problems in the synthesis of cyclic peptides through use of the Dmab protecting group †

Tony Johnson,* Mark Liley, Timothy J. Cheeseright and Farida Begum

Peptide Therapeutics, Peterhouse Technology Park, 100 Fulbourn Road, Cambridge, UK CB1 9PT

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The preparation of side-chain-to-side-chain-cyclised peptides through lactam bridge formation requires orthogonal protecting groups for side-chain amino and carboxylate functionalities. Use of the 4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino}benzyl ester (Dmab) group for this role in the protection of the glutamyl side-chain resulted in the formation of unexpected side-products. During synthesis of fully protected peptide targets, N^{α}-pyroglutamyl chain-terminated peptides were observed. Pyroglutamyl peptides were not observed in analogous peptides synthesised using the traditional *tert*-butyl ester protecting group. Selective removal of the Dmab group proceeds through a two-stage procedure, hydrazinolytic cleavage of the dimedone moiety followed by 1,6-elimination of the resulting peptide-glutamyl 4-aminobenzyl ester. The latter reaction is sufficiently slow to allow isolation of the transiently stable glutamyl derived 4-aminobenzyl ester peptide. Attempted side-chain-to-side-chain cyclisation (through orthogonally protected Glu and Lys residues) of peptides prepared *via* Glu(ODmab) failed and led to modification of the Lys N^{ε}-amino group when 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was used as carboxy-activating reagent. Analogous peptides prepared utilising allyl side-chain protection for glutamyl residues were successfully cyclised using HBTU.

Introduction

Endogenous peptides display a diverse array of biological interactions, *e.g.* as hormones, neurotransmitters or inhibitors. They generally possess properties ideal for optimal receptor interaction, selective mode of action and clearance from the site of action. However, their use as drugs is severely limited by their proteolytic degradation, low oral bioavailability and poor pharmacodynamics.[1,2]

Cyclic peptides are often prepared as stepping stones in developing pharmaceutical compounds from linear peptide lead molecules and have a number of advantages over their linear counterparts, *e.g.* they are more resistant to protease degradation, and exhibit increased biological specificity and activity. This latter effect often arises from the 'preorganisation' of cyclic peptides into a single conformation, lowering the entropy cost, relative to their linear counterpart, on receptor binding. In recent years many examples from varying fields, *e.g.* Somatostatin,[3,4] Endothelin,[5] CCK,[6,7] HIV[8] and MMP inhibitors,[9,10] have provided evidence as to the general utility of cyclic conformational constraint in pharmaceutical design.

Conventional approaches to peptide cyclisation include formation of disulfide bonds[11] or lactam bridges through joining peptide N- and C-termini (head-to-tail), lactam bridges through amino and carboxy side-chain groups (for example on Lys and Glu) or a combination of these (head-to-side-chain). Because of the speed and convenience of solid-phase synthesis, support-bound cyclisation protocols are ideal for the preparation of numerous cyclopeptide analogues. Obviously for total selectivity during lactam formation, all other amino acid functional groups (other than those destined to form the lactam bridge) must be protected. As *all* side-chain functionalities must be protected during linear assembly, orthogonal side-chain protecting groups are a prerequisite for a successful outcome to such a strategy. Thus, the formation of a lactam bridge as the final coupling event in a solid-phase protocol presents an additional level of difficulty to traditional solid-phase synthesis.

During the solid-phase synthesis of cyclic peptide structures an unanticipated series of side-products related to the use of the orthogonal 4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino}benzyl ester (Dmab) carboxylic acid-protecting group were encountered. In this paper we describe the identification of these side-products and their consequences for efficient peptide cyclisation.

Results

The chemical protocol for the synthesis of a representative sequence from an in-house library of cyclic heptapeptides is shown in Scheme 1. The test peptide was smoothly assembled on NovaSyn TGR using standard Fmoc/*tert*-butyl chemistries [pentafluorophenyl (OPfp) active ester or 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt)-mediated couplings in the presence of *N*-methylmorpholine (NMM)].[12] The residues destined to form the lactam bridge were coupled as their 4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]-amino}benzyl (ODmab)[13] or *tert*-butyl ester (OBu^t) (for Glu) and 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) (for Lys)[14] side-chain-protected derivatives. On completion of the synthesis, the amino terminus was capped with acetic anhydride (Ac_2O) to give peptide-resin assemblies **3a** and **3g** or continued for a further round of amino acid addition to yield assemblies **3b**–**3f** (Dmab protection) and **3h**–**3l** (OBu^t protection). Acidolytic cleavage [mediated by trifluoroacetic acid (TFA)/triethylsilane; 95 : 5, v/v] of assembly **3a** gave the Dmab/Dde side-chain-protected peptide containing two major species as judged by analytical HPLC. Peak 1 (t_R 9.6 min) gave an electrospray mass spectral (ESMS) peak at 1037.8 Da and peak 2 (t_R 14.8 min) gave an ESMS peak at 1408.4 Da (theoretical mass = 1408.7 Da). Likewise, peptide-resin assemblies **3b**–**3f**

† HPLC chromatograms are available as supplementary data. For direct electronic access see http://www.rsc.org/suppdata/p1/b0/b001694m/

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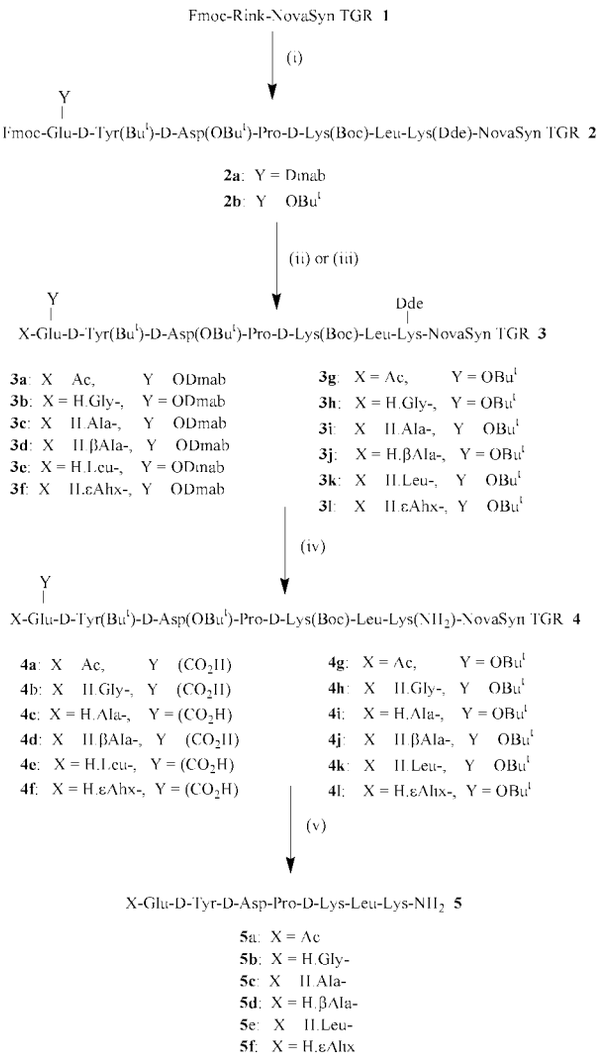
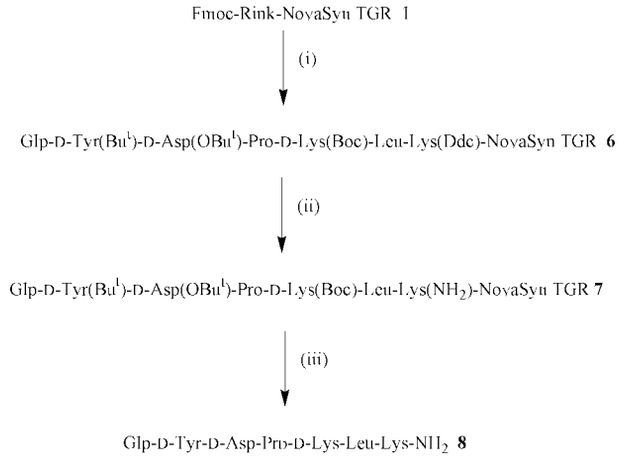


Scheme 1 *Reagents and conditions*: (i) 7 cycles of Fmoc/*tert*-butyl solid-phase peptide synthesis; (ii) 1 further round of Fmoc/*tert*-butyl solid-phase peptide synthesis; (iii) Ac_2O, DMF; (iv) hydrazine hydrate, DMF; (v) TFA, scavengers.

were treated with TFA/triethylsilane and also yielded material containing two products (see Table 1 for collated data). For comparison, acidolytic cleavage of the control peptide-resin, **3g** [prepared *via* Glu(OBu^t)], gave the Dde side-chain-protected peptide containing a single species (t_R 9.5 min) with an ESMS peak at 1097.1 Da (theoretical mass = 1097.3 Da). (See Table 2 for collated HPLC and ESMS data on control peptides **3g**–**3l**.) Similarly, TFA-mediated acidolytic cleavage of peptide-assembly **6** (Scheme 2) yielded the standard Dde-protected pyroglutamyl peptide containing a single major species (t_R 9.6 min) with an ESMS peak at 1037.5 Da (theoretical mass = 1037.2 Da).

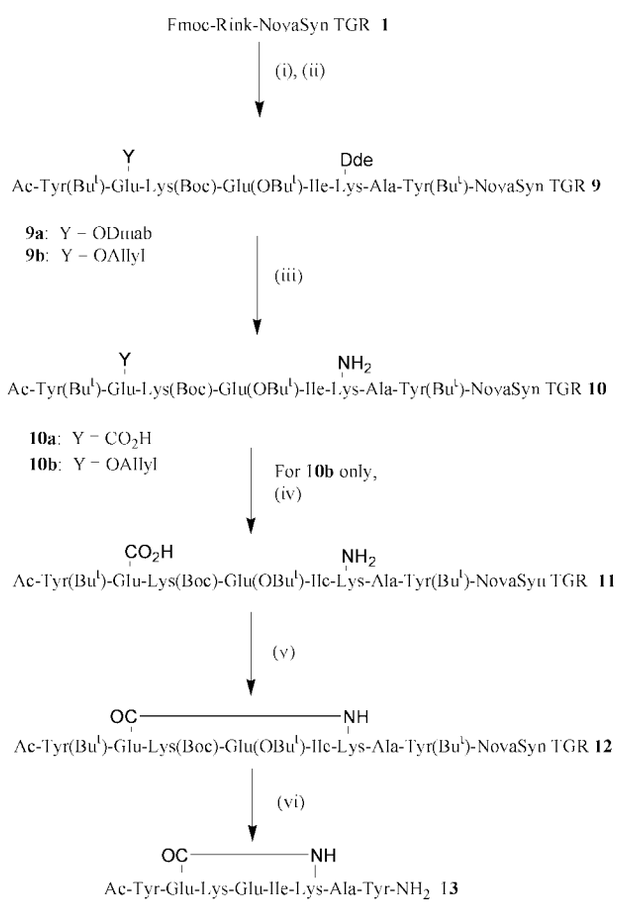
All test and control peptide-resin assemblies (Scheme 1, **3a**–**3l** and Scheme 2, **6**) were treated with hydrazine hydrate in *N*,*N*-dimethylformamide (DMF) to simultaneously remove the Dmab and Dde protecting groups. Acidolytic cleavage of assembly **4a** gave a peptide (**5a**) eluting as two species. Peak 1 (t_R 7.3 min) gave ESMS peaks at 873.0 Da and 933.5 Da and peak 2 (t_R 8.4 min) gave ESMS peaks at 933.5 Da and 1038.5 Da (theoretical mass = 933.1 Da). Assemblies **4b**–**4f** were treated similarly and also yielded material containing 3 products (see Table 3, peptides **5b**–**5f**, for collated data). HPLC re-analysis of peptide **5a** after incubation in solution for 24 h showed that the later eluting material (ESMS 1038.5 Da) had disappeared. Peak 1 remained and gave on analysis by ESMS for peptides of mass 873.0 Da and 933.5 Da.



Scheme 2 *Reagents and conditions*: (i) 7 cycles of Fmoc/*tert*-butyl solid-phase peptide synthesis; (ii) hydrazine hydrate, DMF; (iii) TFA, scavengers.

Acidolytic cleavage of **4g** (Scheme 1) yielded a peptide (**5a**) that eluted as a single component on HPLC (t_R 5.4 min) with an ESMS peak at 933.0 Da (theoretical mass = 933.1 Da). Assemblies **4h**–**4l**, after acidolytic cleavage, yielded material that also eluted as single species on HPLC (see Table 4, peptides **5b**–**5f**, for collated data). Acidolytic cleavage of **7** (Scheme 2) gave a peptide product (**8**) as a single component by HPLC analysis (t_R 5.6 min) with ESMS peak at 873.0 Da (theoretical mass = 873.0 Da).

The protocol for synthesis of the second cyclic target of interest is shown in Scheme 3. The sequence was assembled on NovaSyn TGR using standard machine-driven Fmoc/*tert*-butyl



Scheme 3 *Reagents and conditions*: (i) 8 cycles of Fmoc/*tert*-butyl solid-phase peptide synthesis; (ii) Ac_2O, DMF; (iii) hydrazine hydrate, DMF; (iv) $Pd(PPh_3)$, DMF, $CHCl_3$, AcOH, NMM; (v) HBTU, HOBt, NMM, DMF; (vi) TFA, scavengers.

Table 1 Analytical HPLC and ESMS data for peptides **3a**–**3f** (see Scheme 1)

Peptide	Sequence	Peak	t_R/min [a]	ESMS Found (MH^+) [b]	ESMS Expected (MH^+)
3a	Ac-Glu(ODmab)-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-NH_2	1	9.6	1037.8	1408.7
		2	14.8	1408.4	1408.7
3b	H-Gly-Glu(ODmab)-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-NH_2	1	9.6	1037.8	1423.7
		2	13.7	1423.0	1423.7
3c	H-Ala-Glu(ODmab)-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-NH_2	1	9.6	1037.4	1437.8
		2	13.7	1437.3	1437.8
3d	H-βAla-Glu(ODmab)-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-NH_2	1	9.6	1037.8	1437.8
		2	13.7	1437.2	1437.8
3e	H-Leu-Glu(ODmab)-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-NH_2	1	9.6	1037.8	1479.8
		2	14.1	1479.5	1479.8
3f	H-εAhx-Glu(ODmab)-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-NH_2	1	9.6	1037.4	1479.8
		2	13.8	1479.4	1479.8

[a] HPLC conditions: Phenomenex reversed-phase C_4 column (250 × 4.6 mm), 10–90% B in A linear gradient over a period of 25 min (flow rate 1.5 cm^3 min^{-1}; 215 nm UV detection) where buffer A = 0.1% aq. TFA and buffer B = 90% acetonitrile–10% buffer A. [b] See Experimental section for ESMS machine parameters.

Table 2 Analytical HPLC and ESMS data for peptides **3g**–**3l** (see Scheme 1)

Peptide	Sequence	t_R/min [a]	ESMS Found (MH^+) [b]	ESMS Expected (MH^+)
3g	Ac-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-NH_2	9.5	1097.1	1097.3
3h	H-Gly-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-NH_2	9.1	1112.9	1112.3
3i	H-Ala-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-NH_2	9.1	1126.0	1126.3
3j	H-βAla-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-NH_2	9.2	1125.9	1126.3
3k	H-Leu-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-NH_2	9.4	1168.0	1168.4
3l	H-εAhx-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-NH_2	9.1	1168.0	1168.4
6 [c]	Glp-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-NH_2	9.6	1037.5	1037.2

[a] HPLC conditions: Phenomenex reversed-phase C_4 column (250 × 4.6 mm), 10–90% B in A linear gradient over a period of 25 min (flow rate 1.5 cm^3 min^{-1}; 215 nm UV detection) where buffer A = 0.1% aq. TFA and buffer B = 90% acetonitrile–10% buffer A. [b] See Experimental section for ESMS machine parameters. [c] See Scheme 2.

Table 3 Analytical HPLC and ESMS data for peptides **5a**–**5f** prepared *via* Glu(ODmab) (see Scheme 1)

Peptide	Sequence	Peak	t_R/min [a]	ESMS Found (MH^+) [b]	ESMS Expected (MH^+)
5a	Ac-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys-NH_2	1	7.3	873.0 + 933.5	933.1
		2	8.4	933.5 + 1038.5	933.1
5b	H-Gly-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys-NH_2	1	4.7	948.5	948.1
		2	7.0	873.0 + 1053.0	948.1
5c	H-Ala-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys-NH_2	1	4.8	962.5	962.1
		2	7.1	873.2 + 1067.3 + 962.2	962.1
5d	H-βAla-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys-NH_2	1	5.0	962.5	962.1
		2	7.2	873.0 + 1067.0 + 962.4	962.1
5e	H-Leu-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys-NH_2	1	7.1	873.1 + 1004.5	1004.2
		2	8.7	1109.0 + 1004.5	1004.2
5f	H-εAhx-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys-NH_2	1	6.1	1004.5	1004.2
		2	7.2	873.0	1004.2
		3	8.0	1109.0 + 1004.8	1004.2

[a] HPLC conditions: Phenomenex reversed-phase C_4 column (250 × 4.6 mm), 10–40% B in A linear gradient over a period of 25 min (flow rate 1.5 cm^3 min^{-1}; 215 nm UV detection) where buffer A = 0.1% aq. TFA and buffer B = 90% acetonitrile–10% buffer A. [b] See Experimental section for ESMS machine parameters.

Table 4 Analytical HPLC and ESMS data for peptides **5a**–**5f** prepared *via* Glu(OBu^t) (see Scheme 1)

Peptide	Sequence	t_R/min [a]	t_R/min [b]	ESMS Found (MH^+) [c]	ESMS Expected (MH^+)
5a	Ac-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys-NH_2	5.4	7.2	933.0	933.1
5b	H-Gly-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys-NH_2	4.4	4.9	948.0	948.1
5c	H-Ala-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys-NH_2	4.3	4.8	962.1	962.1
5d	H-βAla-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys-NH_2	4.3	5.0	962.0	962.1
5e	H-Leu-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys-NH_2	5.5	7.2	1004.5	1004.2
5f	H-εAhx-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys-NH_2	5.0	6.3	1004.9	1004.2
8 [d]	Glp-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys-NH_2	5.6	7.5	873.0	873.0

[a] HPLC conditions: Phenomenex reversed-phase C_4 column (250 × 4.6 mm), 10–90% B in A linear gradient over a period of 25 min (flow rate 1.5 cm^3 min^{-1}; 215 nm UV detection) where buffer A = 0.1% aq. TFA and buffer B = 90% acetonitrile–10% buffer A. [b] HPLC conditions as above except a linear gradient of 10–40% B in A over 25 min was used. [c] See Experimental section for ESMS machine parameters. [d] See Scheme 2.

protocols (coupling reactions used OPfp active ester or HBTU/HOBt chemistries) to yield peptide-resins **9a** [*via* Glu(ODmab)] and **9b** [*via* Glu(OAllyl)]. TFA-mediated cleavage of assembly **9a** gave crude side-chain Dmab/Dde-protected peptide as a single major species (t_R 18.5 min) with an ESMS peak of 1559.5 Da (theoretical mass = 1559.9 Da). Assembly **9a** was treated with hydrazine hydrate in DMF to remove the Dmab and Dde protecting groups. Small-scale cleavage of the hydrazine-treated peptide-resin assembly gave material that appeared to elute as a single, broad HPLC species. Careful inspection of the chromatogram revealed a shoulder on the leading edge of the peak. ESMS analysis confirmed the inhomogeneity of the crude peptide, giving rise to components with mass 1084.5 Da (theoretical mass = 1084.3) and 1188.8 Da (+104.5 Da). Cyclisation (lactam-bridge formation) of the selected Glu and Lys side-chain moieties in assembly **10a** was performed using HBTU/HOBt/NMM in DMF for 6 h to yield assembly **12**. Acidolytic cleavage of assembly **12** gave crude peptide (**13**) eluting as two species on HPLC analysis. ESMS analysis of peak 1 (t_R 10.4 min) showed peaks at 1084.1 Da and 1189.6 Da (theoretical mass for cyclised peptide, 1066.3 Da); peak 2 (t_R 11.0 min) gave ESMS peaks at 1182.6 Da and 1288.2 Da.

As a comparison, the Glu(OAllyl) analogue was treated as follows: peptide-resin assembly **9b** was incubated with hydrazine hydrate in DMF to remove the Dde protecting group. TFA-mediated acidolytic cleavage of a small quantity of assembly **10b** gave peptidic material that eluted as a single species on HPLC analysis (t_R 12.0 min) with an ESMS peak at 1125.2 Da (theoretical mass 1124.3 Da). Peptide-resin **10b** was subjected to palladium(0)-mediated removal of the Glu side-chain allyl ester-protecting group as described by Kates *et al.*[15] Analytical HPLC analysis of a small-scale acidolytic cleavage of assembly **11** gave a single species of excellent quality with ESMS at 1084.4 Da (theoretical mass 1084.3 Da). Lactam-bridge formation was mediated through HBTU/HOBt/NMM in DMF for 6 h. TFA-mediated cleavage of assembly **12** gave a product (**13**) containing two peptides (1 : 4 ratio) with ESMS data as follows: peak 1 at 1084.4 Da and peak 2 at 1066.3 Da (theoretical mass = 1066.3 Da). Lactam-bridge formation mediated through HATU/HOAt/NMM in DMF for 6 h gave a single peak on analysis by HPLC (t_R 10.2 min) with ESMS peak at 1066.4 Da (theoretical mass = 1066.3 Da).

Discussion

Choice of orthogonal protection strategy

Scheme 1 shows the synthesis of a peptide chosen as a test system for synthesis on Multipin Crowns™ prior to preparation of a cyclic peptide compound screening library for an in-house project. Our own experience, and that of others,[16–18] has shown that Multipin Crowns™ are excellent supports for peptide synthesis. Combined with the ability to perform parallel synthesis in an 8 × 10 format, multipin syntheses are ideal for peptide library preparation. Choice of orthogonal Lys/Glu side-chain-protecting groups for use in combination with the Fmoc/*tert*-butyl peptide-synthesis strategy[12] must also be compatible with the multipin support medium and the required synthesis protocols (deprotection, washing, coupling steps, *etc.*). Appropriate side-chain protecting groups such as the commercially available 4-methyltrityl (Mmt) and 2-phenylpropoxycarbonyl (Ppoc) (weakly acid labile), allyl/alloxycarbonyl Alloc (Pd-catalysed removal) and Dde/Dmab (hydrazine labile) were all considered for orthogonal protection. For routine peptide synthesis, parallel deprotection (Fmoc removal) and washing steps (post deprotection and coupling) of 8 × 10 layouts of Multipin Crowns™ are carried out in a plastic bath containing piperidine–DMF (1 : 4, v/v). Manual agitation of the pins (held in an 8 × 10 format by stems fixed into a PTFE 'cap') is required during these operations. Thus, the Pd-catalysed removal of allyl-based protecting groups was considered unworkable because of the heterogeneous nature of the catalyst (requiring continual agitation) and the bath, containing the reagents, allowing air incursion, rendering the catalyst useless. This latter consideration also weighed against use of the commercially available, weakly acid-labile protecting groups, Mmt and Ppoc. Weakly acidic media (1–2% TFA in DCM) would be prone to evaporation of the diluent, increasing the acidity of the remaining solvent and potentially leading to deprotection of other sensitive side-chain-protecting groups, *e.g.* His(Trt) and Cys(Trt). On the other hand, the Dmab group is removed using brief treatment with hydrazine in DMF. This protocol requires minimal agitation, does not suffer evaporative loss and it is unlikely that air incursion will prevent deprotection.

Initial observations

Peptide synthesis of **3a** (Scheme 1) on Multipin Crowns™ was followed by Dmab/Dde removal mediated through hydrazine hydrate in DMF. The selectively deprotected peptide was subjected to HBTU/HOBt-mediated cyclisation (previous work in these laboratories had shown this side-chain-to-side-chain coupling protocol to be one of the most convenient and reliable). On cleavage of the peptide from the crowns, analysis by HPLC and ESMS showed that none of the expected cyclic material was present. The cleaved material appeared to contain uncyclised linear peptide and (from ESMS) uncyclised N^{α}-pyroglutamyl peptide (data not shown). At this stage we were unsure as to why cyclisation had failed and why N^{α}-pyroglutamyl formation had occurred. A thorough investigation of the entire procedure was carried out using polymer-supported peptide, as this would allow for checks at intermediate stages of peptide manipulations.

Sequence 1. Dmab-derived side-reactions. Synthesis of the test peptide on NovaSyn TGR proceeded smoothly. Cleavage

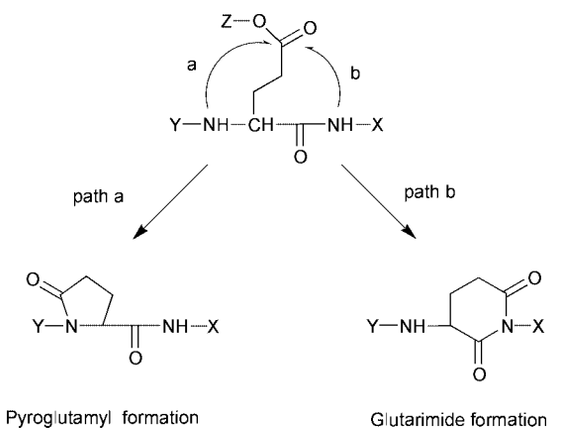
Table 5 Analytical HPLC and ESMS data for peptides **5b**–**5f** prepared *via* Glu(ODmab) and the transient side-product (see Scheme 1) (*cf*. data for the same compounds in Tables 3 and 4)

Peptide[a]	Sequence	Peak	t_R/min[b]	ESMS Found (MH^+)[c]	ESMS Expected (MH^+)
5b	H-Gly-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys-NH_2	1	4.8 (86%)	948.5	948.1
		2	7.3 (14%)	873.1	948.1
5c	H-Ala-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys-NH_2	1	4.8 (84.5%)	962.5	962.1
		2	7.2 (15.5%)	873.0	962.1
5d	H-βAla-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys-NH_2	1	5.2 (84%)	962.5	962.1
		2	7.3 (16%)	873.0	962.1
5e	H-Leu-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys-NH_2		7.1	873.1 + 1004.1	1004.2
5f	H-εAhx-Glu-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys-NH_2	1	6.1 (86%)	1004.0	1004.2
		2	7.2 (14%)	873.1	1004.2

[a] Data acquired post 24 h incubation of peptide in acetonitrile–0.1% aq. TFA solution. [b] HPLC conditions: Phenomenex reversed-phase C_4 column (250 × 4.6 mm), 10–40% B in A linear gradient over a period of 25 min (flow rate 1.5 cm^3 min^{-1}; 215 nm UV detection) where buffer A = 0.1% aq. TFA and buffer B = 90% acetonitrile–10% buffer A. [c] See Experimental section for ESMS machine parameters.

of assembly **3a** yielded the partially protected peptide sequence containing two species. ESMS analysis indicated that the earlier eluting species appeared to be the N^{α}-pyroglutamyl peptide (≈15%) and the later eluting species the target structure. The earlier eluting species was shown to be the N^{α}-pyroglutamyl peptide by comparison to the standard peptide prepared as shown in Scheme 2. The standard N^{α}-pyroglutamyl peptide gave an identical HPLC retention time and ESMS analysis and co-eluted with peak 1 obtained from the Dmab synthesis. Analysis of the peptide prepared using Glu(OBu^t) (Scheme 1) gave only a single species, with the expected mass analysis. This confirms that with the traditional *tert*-butyl side-chain protecting group, used in conjunction with N^{α}-Fmoc protection, pyroglutamyl formation is not a problem. Alternative amino acids coupled to the N^{α} of Glu(ODmab) peptide (**3b**–**3f**), varying in steric bulk from Gly to Leu, appeared to make little difference to the formation of N^{α}-pyroglutamyl peptide (see Table 1). Thus it would appear that the Glu(ODmab) derivative itself was responsible for the formation of N^{α}-pyroglutamyl peptide. ^{13}C and ^{1}H NMR analysis of the commercially available Glu(Dmab), used during synthesis, and commercially available pyroglutamic acid (Glp-OH) showed that the latter possessed diagnostic ^{13}C NMR shifts that could be used to detect its presence in the former. The Glu(ODmab) sample showed none of these diagnostic shifts. The only conclusion is that the Dmab protecting group is at least partially labile under solid-phase conditions designed to manipulate peptide-resin constructs (amino acid coupling, peptide-resin deprotection or acidolytic peptide-resin cleavage).

It is well known that glutamic acid can undergo cyclisation to form both five (pyroglutamyl)- and six-membered (glutarimide) rings (see Scheme 4).[19] The former occurs particularly readily if an N-terminal glutamyl residue possesses an activated γ-carboxy group and takes place mainly under the influence of bases.[20] Such intramolecular displacements under basic conditions are not uncommon in peptide synthesis. It is possible for even the sterically hindered *tert*-butyl ester-protecting group to be displaced from the side-chain of peptide aspartyl residues during succinimide formation[20–22] and a recent report[23] showed that the *tert*-butyl residue could be displaced from glutamyl side-chains to yield the glutarimide and ultimately the γ-peptide. Benzyl-based protecting groups (particularly when used for aspartyl side-chain protection) seem to be particularly prone to such intramolecular nucleophilic displacement reactions.[24] The side-reaction observed with the benzyl-based Dmab protecting group would appear to be that of pyroglutamyl formation rather than the alternative cyclisation to glutarimide. This is supported by the inability to further acylate the Glu(Dmab)-derived side-product during subsequent peptide-chain-extension cycles. This would obviously not be the case where glutarimide formation was the side-reaction. In addition, the side-product generated from the Glu(Dmab) residue co-eluted with the pyroglutamyl control peptide (data not shown). However, the extent to which this side-reaction is sequence dependent is as yet unknown.



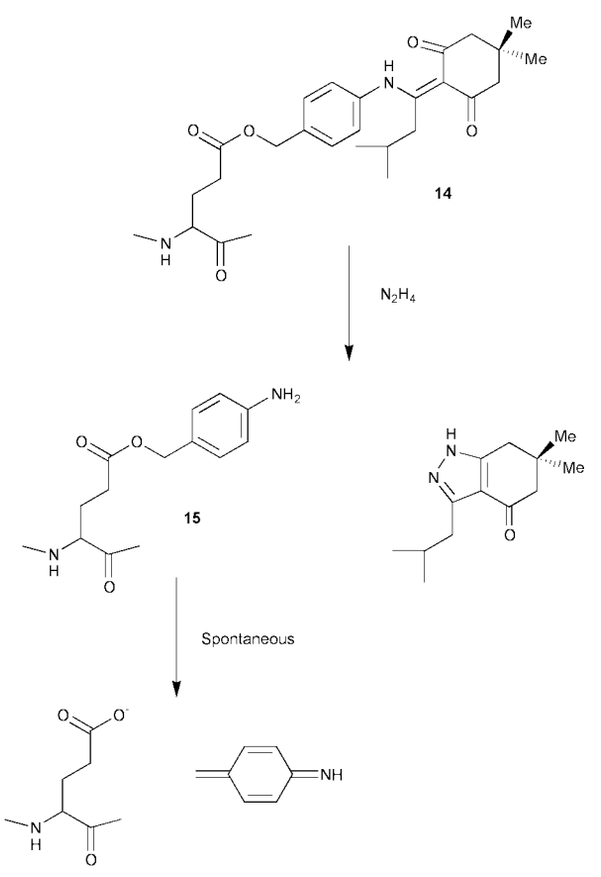
Scheme 4 Side-reactions exhibited by glutamic acid: pathway a, pyroglutamyl formation and pathway b, glutarimide formation.

A second side-product was evident in the analysis of crude peptides **5a**–**5f** prepared *via* Glu(ODmab) (see Table 3). This second side-product had a molecular mass 105 Da greater than that expected for the parent ion. Only for the εAhx (**5f**)-derived peptide did it prove possible to find HPLC conditions that effectively separated the three peptides formed during solid-phase synthesis. This additional side-product had a finite lifetime in aqueous solution (0.1% TFA–MeCN), making its identification difficult. All peptides synthesised from the core sequence **2a** showed the presence of this transient peptide species that, on incubation in aqueous solution, decomposed to yield the parent peptide (Table 5). Control peptides prepared using Glu(OBu^t) did not show this side-product, again pointing to Glu(ODmab) being responsible. The tentative identification of this second side-product and its effect on peptide side-chain-to-side-chain cyclisation reactions are addressed below.

Sequence 2. Dmab-derived side reactions. The second test sequence was studied as part of a programme to devise a strategy, based around orthogonal Fmoc/*tert*-butyl protocols, that would lead readily to the synthesis of short, constrained peptides that possess some α-helical structure. For our initial studies, the peptide shown in Scheme 3 (**13**) was chosen as this had previously shown a tendency to form an α-helical structure.[25] The structure differed from the original through inclusion of tyrosine residues at the N- and C-termini to facilitate

HPLC monitoring during linear peptide synthesis and subsequent formation of the lactam bridge. The solid-phase strategy (Scheme 3) was designed to allow the selective formation of a side-chain-to-side-chain (Lys to Glu) amide bond from an *i* to *i* + 4 position.

Peptide-resin assembly **9a** was readily prepared as shown in Scheme 3. Analysis of a test cleavage yielded the partially protected species of good quality with no evidence of pyroglutamyl formation. These data were a little surprising considering the evidence presented earlier. However, as suggested above, the extent to which pyroglutamyl formation is sequence dependent is unknown. The hydrazine-treated peptide, however, yielded material that contained two moieties, the target peptide and a species of 105 Da-higher molecular mass. This latter species had also been observed in all post-hydrazinolysis test peptides (**5a**–**5f**) synthesised *via* Scheme 2 (see Table 3). This species was observed in the initial post-hydrazinolysis cleavage products, but over time (24 h in aq. MeCN–0.1% TFA solution) decomposed to yield the expected peptide. It should be noted that ESMS analysis of this transient peptide species was always accompanied by observation of the expected parent ion. That two completely dissimilar peptide sequences should reveal a common side-product must be attributable to a common substituent. Other than Glu(ODmab), all other residues [including Lys(Dde)] were present in test and control peptides that did not exhibit the transient peptide species (see Table 4). Hydrazinolytic removal of the Dmab protecting group proceeds through two stages (Scheme 5): (i) cleavage of the dimedone entity from



Scheme 5 Hydrazine-mediated cleavage of Glu(ODmab).

the fully protected side-chain (**14**) followed by (ii) spontaneous 1,6-elimination of the resulting 4-aminobenzyl ester (**15**) to release free glutamyl side-chain (**16**) and 4-methylenecyclohexa-2,5-dienylideneimine (**17**). Consideration of the mechanism indicates that if the 1,6-elimination is slow, peptides containing the transient 4-aminobenzyl ester should be observed 105 Da higher in the ESMS data (as seen, *e.g.*, in Table 3), although ultimately this transient species should yield the desired peptide.

The relatively slow elimination of the 4-aminobenzyl intermediate derived from Glu(ODmab) will inevitably influence subsequent attempts at side-chain-to-side-chain cyclisation. HBTU/HOBt-mediated cyclisation of **11** [*via* Glu(ODmab)] (Scheme 3) yielded peptidic material that appeared to contain two species. ESMS analysis of the earlier eluting peak from the analytical HPLC chromatogram (t_R 10.4 min) indicated that the 4-aminobenzyl ester intermediate, from hydrazine treatment of Dmab-protected peptide, was present along with a mass ion consistent with the parent uncyclised peptide. However, as stated above, the ESMS data obtained thus far on the Dmab-intermediate-peptide constructs show the presence of the expected, fully deprotected parent peptide and is probably a mass spectroscopic artefact. ESMS analysis of the second HPLC component (t_R 11.0 min) indicated the presence of two species differing by 105 Da [presence of Dmab-derived Glu(4-aminobenzyl) residue] with an additional mass increase of 99 Da. Amino acid analysis of this isolated material indicated the presence of a single Lys residue rather than the expected two. This implies irreversible modification of the Lys side-chain leading to a derivatised amino acid, not susceptible to acid hydrolysis. Both pieces of evidence suggested that the tetramethylguanidinium group from HBTU had been transferred to the side-chain amino group of the Lys residue. Such a side-reaction has been observed previously in the attempted synthesis of cyclic [Leu^5]-enkephalin analogues.[26] Two explanations are possible for our observations regarding the test peptide: (i) the Glu side-chain carboxylic acid is unavailable for reaction with HBTU (due to the presence of the transient 4-aminobenzyl ester intermediate), allowing for Lys ε-NH_2 reaction instead, or (ii) the test peptide represents a sequence that is difficult to cyclise and would result in the HBTU-mediated tetramethylguanidinium side-product regardless of the orthogonal protecting-group strategy employed.

To test the latter hypothesis, the protecting-group strategy was changed and the allyl ester protecting group was employed for the side-chain of glutamic acid. The peptide was synthesised as shown in Scheme 3. Analytical analysis of the partially protected peptide obtained from cleavage of **10b** gave a peptide of excellent quality and anticipated mass analysis. Palladium-mediated removal of the remaining allyl ester protecting group proceeded smoothly to completion and lactam-bridge formation utilising HBTU/HOBt [identical conditions with those employed during attempted cyclisation of peptide **10a** prepared using Glu(ODmab)] yielded ≈80% of cyclised peptide. The cyclisation reaction could be forced to completion using the highly activated HATU reagent. These results indicate that the test peptide itself does not present a barrier to the cyclisation reaction. Thus, the lack of cyclisation of the peptide obtained from the Glu(ODmab) synthesis, and the concomitant formation of N^{ε}-tetramethylguanidinyl-substituted Lys, point to the problem being the long half-life of the transient Glu(4-aminobenzyl) peptide.

Conclusions

We have shown that the Dmab group, when used for temporary protection of the glutamyl side-chain acid functionality, is prone to two deleterious side-reactions. The first generates truncated N^{α}-pyroglutamyl peptides *via* intramolecular cyclisation either during N^{α}-Fmoc removal or subsequent acylation (coupling) reactions. The extent to which this intramolecular side-reaction is sequence dependent is unknown but would be expected to be determined to some degree by the rate of acylation of the incoming amino acid during coupling to N^{α}-Glu(Dmab) peptide. However, from our observations there would appear to be little correlation between incoming amino acid and amount of pyroglutamyl peptide formed. Any trun-

cated peptide formed will remain to contaminate the final product where it may prove difficult or impossible to remove, for example peptide **5e** and N^{α}-pyroglutamyl peptide **8** co-elute on HPLC and are distinguished only by ESMS analysis.

The second side-reaction is a result of the slow 1,6-elimination reaction of the 4-aminobenzyl group (Scheme 5, **15**) derived from hydrazine treatment of Glu(ODmab). Although this is a transient species, it is sufficiently long-lived to be isolated from HPLC eluents and analysed by ESMS. The presence of the 4-aminobenzyl ester precludes activation of the glutamyl side-chain functionality. In our case this led to a coupling failure when cyclisation to a free Lys side-chain amino group was attempted and resulted in modification of the lysine side-chain amino group by the carboxy-activating reagent used.

As more ambitious peptidic targets are attempted, there is a need for a toolbox of amino acid derivatives that allow for orthogonal deprotection strategies and subsequent modification (reaction) of selectively unmasked functionality. The semi-orthogonal (with respect to Fmoc-based strategies) Dmab protecting group held promise in this area but has now been shown to be less than ideal.

Experimental

Equipment, materials and methods

Continuous-flow Fmoc-polyamide methods, reviewed by Atherton and Sheppard,[12] were used exclusively. Fmoc amino acid pentafluorophenyl activated esters (Novabiochem, UK and Perseptive Biosystems, UK) were used exclusively except for Lys(Dde), Glu(ODmab), Glp, D-Lys(Boc), D-Asp(OBu^t), D-Tyr(Bu^t), βAla and εAhx which were coupled through the free acid activated with HBTU reagent in the presence of HOBt and NMM. NovaSyn TGR (Rink amide linker, 0.2 mmol g^{-1} loading; Novabiochem, UK) was used exclusively as the base support.

DMF, methanol, *tert*-butyl methyl ether, dichloromethane, acetonitrile, piperidine and TFA (super purity solvent) were obtained from Romil Scientific (UK) and used without further purification. Triethylsilane was obtained from Aldrich (UK) and used without further purification.

Solid-phase peptide synthesis was performed on a Milligen 9050 automated synthesiser programmed to perform acylation reactions (in DMF) for 60 min and Fmoc deprotection reactions (in 20% piperidine–DMF v/v) for 1 min and 5 min (5 cm^3 min^{-1} and 3 cm^3 min^{-1} flow rates, respectively). All chiral amino acids used were of the L-configuration except where noted. Amino acid side-chain protection was as follows: aspartic and glutamic acid (*tert*-butyl ester, OBu^t); lysine (N^{ε}-*tert*-butoxycarbonyl, Boc); tyrosine (*tert*-butyl ether, Bu^t).

Peptide hydrolyses were performed at 110 °C for 24 h in 6 M HCl containing a trace of phenol in evacuated, sealed tubes. Analysis of hydrolysis products was performed on a Pharmacia 'Biochrom 20' analyser. Separation was obtained using ion-exchange resin with manufacturer's buffer solutions and post-column detection by ninhydrin.

Electrospray mass spectra (ESMS) were obtained on a Fisons VG Platform. Acetonitrile–0.1% aq. TFA was used as the carrier solvent, cone voltage was set at 15 kV and 170 °C. Data were acquired in open access mode.

General manual synthesis procedures

Manual deprotection (N^{α}-Fmoc removal) of peptide-resins was performed as follows: 100 mg of peptide-resin was placed on a frit in a syringe barrel. The peptide-resin was suspended in DMF (2 cm^3) for 5 min and then the solvent removed by filtration under vacuum. The Fmoc group was removed from the peptide assembly with two treatments of piperidine–DMF (1 : 4, v/v) for 3 + 7 min. Following removal of the deprotection solvent by filtration, the peptide-resin was thoroughly washed with DMF (5 cm^3 × 12).

One cycle of manual coupling of amino acids to N^{α}-deprotected peptide-resin assemblies was performed as follows. Method A: Following deprotection (as described above) the DMF-swollen resin was then resuspended in DMF (0.5 cm^3) containing N^{α}-Fmoc-amino acid-OPfp (0.08 mmol) and HOBt (0.08 mmol, 12.2 mg). Reaction was allowed to proceed with gentle agitation for 1 h. The peptide-resin was washed thoroughly with DMF (5 cm^3 × 8) and the DMF-swollen resin suspended in piperidine–DMF (1 : 4, v/v; 4 cm^3) for 3 min. The solvent was removed by filtration under vacuum and the piperidine–DMF treatment repeated for a further 7 min. Following deprotection, the resin was washed successively with DMF, methanol and *tert*-butyl methyl ether and dried *in vacuo* (over KOH pellets).

Alternatively, the following method was used. Method B: Following deprotection (as described above) the DMF-swollen resin was then resuspended in DMF (0.5 cm^3) containing N^{α}-Fmoc-amino acid (0.08 mmol), HBTU (0.08 mmol, 30 mg), HOBt (0.08 mmol, 12.2 mg) and NMM (0.156 mmol, 15.8 mg). Reaction was allowed to proceed with gentle agitation for 1 h. The peptide-resin was washed thoroughly with DMF (5 cm^3 × 8) and the DMF-swollen resin suspended in piperidine–DMF (1 : 4, v/v; 4 cm^3) for 3 min. The solvent was removed by filtration under vacuum and the piperidine–DMF treatment repeated for a further 7 min. Following deprotection, the resin was washed successively with DMF, methanol and *tert*-butyl methyl ether and dried *in vacuo* (over KOH pellets).

The Dmab and Dde protecting groups were removed as follows: peptide-resin assembly (≈100 mg) was placed on a frit in a syringe barrel and allowed to equilibrate for 5 min in DMF (2 cm^3). The solvent was removed from the resin under vacuum and the residue was resuspended in DMF–hydrazine hydrate (98 : 2, v/v; 2 cm^3). Reaction was allowed to proceed for 5 min with gentle agitation and the hydrazine treatment repeated a further 3 times to ensure complete reaction. The peptide-resin was washed with DMF (2 cm^3 × 5) and resuspended in DMF–diisopropylethylamine (9 : 1, v/v; 2 cm^3) for 10 min. Finally, the peptide-resin construct was washed successively with DMF, methanol, *tert*-butyl methyl ether and dried *in vacuo* (over KOH pellets).

Following preparation and appropriate post-synthesis procedures, peptide was released from the support as follows (small-scale cleavage): Peptide-resin assembly (20 mg) was cleaved using TFA–triethylsilane (95 : 5, v/v; 1 cm^3) for 2 h. The cleaved resin was removed by filtration, washed with a little neat TFA and the combined filtrates were sparged with N_2 to remove the bulk of the TFA. Ice-cooled *tert*-butyl methyl ether (12 cm^3) was added, precipitating the peptide; the suspension was cooled in acetone–solid CO_2 for 5 min and centrifuged at 3000 rpm for 5 min. The ethereal solution was decanted from the peptide and further *tert*-butyl methyl ether extractions (4 × 12 cm^3) were performed. The residue was dried *in vacuo* over KOH pellets for 1 h, following which it was dissolved in 0.1% aq. TFA–acetonitrile (1 : 1 v/v; 1 cm^3).

Analytical HPLC was performed on a Phenomenex Jupiter C_4 column (250 × 4.6 mm). A linear gradient of %B in A over a period of 25 min (1.5 cm^3 min^{-1}) was used (see individual compounds for gradient data), where A = 0.1% aq. TFA and B = 90% acetonitrile–10% A. HPLC systems were based on Gilson 306 pumps and a Gilson 119 UV/vis detector operating at 215 and 230 nm and utilised Gilson UniPoint software for data acquisition, display and analysis.

Preparation of Fmoc-Glu(ODmab)-D-Tyr(Bu^t)-D-Asp(OBu^t)-Pro-D-Lys(Boc)-Leu-Lys(Dde)-NovaSyn TGR (peptide-resin 2a; Scheme 1)

Peptide-resin assembly **2a** was synthesised by standard Fmoc/*tert*-butyl solid-phase methods on NovaSyn TGR resin (Scheme 1). Fmoc-Rink-NovaSyn TGR **1** (1.0 g, 0.2 mmol)

Table 6 Amino acid analysis data for resin-bound peptides **3a**–**3l** (Scheme 1)

Peptide	Amino acid analysis Found (Expected)
Ac-Glu(ODmab)-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-R	Asp, 1.05 (1); Glu, 1.00 (1); Leu, 0.93 (1); Tyr, 1.06 (1); Lys, 1.92 (2); Pro, 1.02 (1)
H-Gly-Glu(ODmab)-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-R [a]	Asp, 0.99 (1); Glu, 1.02 (1); Gly, 0.80 (1); Leu, 1.03 (1); Tyr, 0.98 (1); Lys, 1.95 (2); Pro, 1.06 (1)
H-Ala-Glu(ODmab)-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-R [a]	Asp, 1.02 (1); Glu, 0.98 (1); Ala, 0.82 (1); Leu, 0.98 (1); Tyr, 0.95 (1); Lys, 2.00 (2); Pro, 1.05 (1)
H-βAla-Glu(ODmab)-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-R [b]	Asp, 1.01 (1); Glu, 0.95 (1); Leu, 1.03 (1); Tyr, 1.03 (1); Lys, 2.04 (2); Pro, 0.98 (1)
H-Leu-Glu(ODmab)-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-R [a]	Asp, 0.98 (1); Glu, 0.97 (1); Leu, 2.05 (2); Tyr, 1.01 (1); Lys, 1.98 (2); Pro, 0.96 (1)
H-εAhx-Glu(Dmab)-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-R [b]	Asp, 1.02 (1); Glu, 1.04 (1); Leu, 0.96 (1); Tyr, 0.94 (1); Lys, 1.94 (2); Pro, 1.03 (1)
Ac-Glu(OBu^t)-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-R	Asp, 1.01 (1); Glu, 0.98 (1); Leu, 0.99 (1); Tyr, 1.05 (1); Lys, 1.93 (2); Pro, 0.98 (1)
H-Gly-Glu(OBu^t)-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-R [a]	Asp, 0.96 (1); Glu, 1.03 (1); Gly, 0.97 (1); Leu, 1.03 (1); Tyr, 0.97 (1); Lys, 1.94 (2); Pro, 1.01 (1)
H-Ala-Glu(OBu^t)-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-R [a]	Asp, 0.97 (1); Glu, 1.02 (1); Ala, 0.97 (1); Leu, 1.07 (1); Tyr, 0.99 (1); Lys, 1.93 (2); Pro, 1.01 (1)
H-βAla-Glu(OBu^t)-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-R [b]	Asp, 1.01 (1); Glu, 1.05 (1); Leu, 0.98 (1); Tyr, 0.96 (1); Lys, 1.93 (2); Pro, 1.05 (1)
H-Leu-Glu(OBu^t)-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-R [a]	Asp, 0.97 (1); Glu, 1.00 (1); Leu, 2.10 (2); Tyr, 0.98 (1); Lys, 1.95 (2); Pro, 0.97 (1)
H-εAhx-Glu(OBu^t)-D-Tyr-D-Asp-Pro-D-Lys-Leu-Lys(Dde)-R [b]	Asp, 1.00 (1); Glu, 1.00 (1); Leu, 1.05 (1); Tyr, 0.99 (1); Lys, 1.94 (2); Pro, 0.98 (1)

[a] Coupling of final residue *via* -OPfp/HOBt activation in DMF solvent for 2 h. [b] Coupling of final residue *via* HBTU/HOBt activation in DMF solvent for 2 h.

was suspended in DMF for 10 min prior to loading onto the Milligen 9050 synthesis column. The peptide was synthesised in a stepwise manner with activated residues coupled (0.8 mmol vials, 4 equivalents) (amino acid Pfp esters, HOBt-catalysed) under standard conditions. Fmoc-Lys(Dde)-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-D-Asp(OBu^t)-OH, Fmoc-D-Tyr(Bu^t)-OH and Fmoc-Glu(ODmab)-OH (0.8 mmol vials, 4 equivalents) were coupled using HBTU (0.8 mmol, 303 mg), HOBt (0.8 mmol, 122 mg) and NMM (1.52 mmol, 154 mg) for 1 h. On completion of the synthesis, N^{α}-Fmoc-protected resin **2a** was removed from the synthesiser, washed successively with DMF, methanol and *tert*-butyl methyl ether and dried *in vacuo* (over KOH pellets).

Preparation of Ac-Glu(ODmab)-D-Tyr(Bu^t)-D-Asp(OBu^t)-Pro-D-Lys(Boc)-Leu-Lys(Dde)-NovaSyn TGR (peptide-resin 3a; Scheme 1)

100 mg of peptide-resin **2a** were placed on a frit in a syringe barrel, deprotected and washed as described above. The DMF-swollen resin was then resuspended in DMF (0.5 cm^3) and Ac_2O (20.5 mg, 0.2 mmol, 10 equivalents) was added followed by *N*,*N*-diisopropylethylamine (DIEA) (13 mg, 0.1 mmol, 5 equivalents). Reaction was allowed to proceed with gentle agitation for 1 h. The peptide-resin, **3a**, was washed successively with DMF, methanol and *tert*-butyl methyl ether and dried *in vacuo* (over KOH pellets).

In order to check on the progress of the assembly, a small-scale cleavage was performed as described above and the crude product was examined by analytical HPLC and ESMS (Table 1). Amino acid analysis data are shown in Table 6.

Preparation of H-Xaa-Glu(ODmab)-D-Tyr(Bu^t)-D-Asp(OBu^t)-Pro-D-Lys(Boc)-Leu-Lys(Dde)-NovaSyn TGR (where Xaa = Gly, Ala, βAla, Leu and εAhx; peptide-resins 3b–3f; Scheme 1)

Starting with 100 mg quantities of peptide-resin **2a**, peptide-resins **3b**–**3f** (Scheme 1) were synthesised using the manual procedures described above. Analytical HPLC and ESMS data are shown in Table 1. Amino acid analysis data are shown in Table 6 and the method of amino acid coupling also indicated.

Preparation of Fmoc-Glu(OBu^t)-D-Tyr(Bu^t)-D-Asp(OBu^t)-Pro-D-Lys(Boc)-Leu-Lys(Dde)-NovaSyn TGR (peptide-resin 2b; Scheme 1)

Peptide-resin assembly **2b** was synthesised by standard Fmoc/*tert*-butyl solid-phase methods on NovaSyn TGR resin (Scheme 1). Fmoc-Rink-NovaSyn TGR **1** (1.0 g, 0.2 mmol) was suspended in DMF for 10 min prior to loading onto the Milligen 9050 synthesis column. The peptide was synthesised in a stepwise manner with activated residues coupled (0.8 mmol vials, 4 equivalents) (amino acid Pfp esters, HOBt-catalysed) under standard conditions. Fmoc-Lys(Dde)-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-D-Asp(OBu^t)-OH and Fmoc-D-Tyr(Bu^t)-OH (0.8 mmol vials, 4 equivalents) were coupled using HBTU (0.8 mmol, 303 mg), HOBt (0.8 mmol, 122 mg) and NMM (1.52 mmol, 154 mg) for 1 h.

On completion of the synthesis, N^{α}-Fmoc-protected resin **2b** was removed from the synthesiser, washed successively with DMF, methanol and *tert*-butyl methyl ether and dried *in vacuo* (over KOH pellets).

In order to check on the progress of the assembly, a small-scale cleavage was performed and the crude product examined by analytical HPLC and ESMS. The peptide-resin (20 mg) was deprotected, washed and dried as described in the manual procedures above. The peptide-resin (20 mg) was cleaved using TFA–triethylsilane (95 : 5, v/v; 1 cm^3) for 2 h, and the peptide isolated as described above. Analytical HPLC and ESMS data are shown in Table 1.

Preparation of Ac-Glu(OBu^t)-D-Tyr(Bu^t)-D-Asp(OBu^t)-Pro-D-Lys(Boc)-Leu-Lys(Dde)-NovaSyn TGR (peptide-resin 3g; Scheme 1)

100 mg of peptide-resin **2b** were placed on a frit in a syringe barrel, deprotected and washed as described above. The DMF-swollen resin was then resuspended in DMF (0.5 cm^3) and Ac_2O

(20.5 mg, 0.2 mmol, 10 equivalents) was added followed by DIEA (13 mg, 0.1 mmol, 5 equivalents). Reaction was allowed to proceed with gentle agitation for 1 h. The peptide-resin, **3g**, was washed successively with DMF, methanol and *tert*-butyl methyl ether and dried *in vacuo* (over KOH pellets).

Peptide-resin **3g** (20 mg) was treated with TFA–triethylsilane (95 : 5, v/v; 1 cm^3) for 2 h and the crude peptide isolated as described above. Analytical HPLC and ESMS data are shown in Table 2. Amino acid analysis data are shown in Table 6.

Preparation of H-Xaa-Glu(OBu′)-D-Tyr(Bu′)-D-Asp(OBu′)-Pro-D-Lys(Boc)-Leu-Lys(Dde)-NovaSyn TGR (where Xaa = Gly, Ala, βAla, Leu and εAhx; peptide-resins 3h–3l; Scheme 1)

Starting with 100 mg quantities of peptide-resin **2b**, peptide-resins **3b**–**3f** (Scheme 1) were synthesised using the manual procedures described above. Analytical HPLC and ESMS data are shown in Table 2. Amino acid analysis data are shown in Table 6 and the method of amino acid coupling also indicated.

Preparation of Glp-D-Tyr(Bu′)-D-Asp(OBu′)-Pro-D-Lys(Boc)-Leu-Lys(Dde)-NovaSyn TGR (peptide-resin 6; Scheme 2)

Peptide-resin assembly **6** was synthesised by standard Fmoc/*tert*-butyl solid-phase methods on NovaSyn TGR resin (Scheme 2). Fmoc-Rink-NovaSyn TGR **1** (0.5 g, 0.1 mmol) was suspended in DMF for 10 min prior to loading onto the Milligen 9050 synthesis column. The peptide was synthesised in a stepwise manner with activated residues coupled (0.4 mmol vials, 4 equivalents) (amino acid Pfp esters, HOBt-catalysed) under standard conditions. Fmoc-Lys(Dde)-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-D-Asp(OBu′)-OH, Fmoc-D-Tyr(Bu′)-OH and Glp-OH (0.4 mmol vials, 4 equivalents) were coupled using HBTU (151 mg, 0.4 mmol), HOBt (61 mg, 0.4 mmol) and NMM (77 mg, 0.76 mmol) for 1 h.

On completion of the synthesis, N^{α}-Fmoc-protected resin **6** was removed from the synthesiser, washed successively with DMF, methanol and *tert*-butyl methyl ether and dried *in vacuo* (over KOH pellets). Amino acid analysis is shown in Table 6.

In order to check on the quality of the synthesis, peptide-resin **6** (20 mg) was treated with TFA–triethylsilane (95 : 5, v/v; 1 cm^3) for 2 h and the crude peptide isolated as described above. The residue was dried *in vacuo* over KOH pellets for 1 h, following which it was dissolved in 0.1% aq. TFA–acetonitrile (1 : 1 v/v; 1 cm^3). Analytical HPLC and ESMS data are shown in Table 1.

Preparation of peptide-resins 4a–4l (Scheme 1) and 7 (Scheme 2)

Starting with ≈75 mg quantities, peptide-resins **3a**–**3l** and **6** were subjected to DMF–hydrazine treatment to remove Dmab and Dde side-chain-protecting groups as described in the general manual synthesis protocols above. All peptide-resin assemblies (**4a**–**l**, **7**) were subsequently treated with TFA–triethylsilane and the peptides (**5a**–**5f**, Scheme 1 and **8**, Scheme 2) were isolated as described above. Analytical HPLC and ESMS data are shown in Tables 3 and 4.

Preparation of Ac-Tyr(Bu′)-Glu(ODmab)-Lys(Boc)-Glu(OBu′)-Ile-Lys(Dde)-Ala-Tyr(Bu′)-NovaSyn TGR (peptide-resin 9a; Scheme 3)

Peptide-resin assembly **9a** was synthesised by standard Fmoc/*tert*-butyl solid-phase methods on NovaSyn TGR resin (Scheme 3). Fmoc-Rink-NovaSyn TGR **1** (1.0 g, 0.2 mmol) was suspended in DMF for 10 min prior to loading onto the Milligen 9050 synthesis column. The peptide was synthesised in a stepwise manner with activated residues coupled (0.8 mmol vials, 4 equivalents) (amino acid Pfp esters, HOBt-catalysed) under standard conditions. Fmoc-Lys(Dde)-OH and Fmoc-Glu(ODmab)-OH (0.8 mmol vials, 4 equivalents) were coupled using HBTU (0.8 mmol, 303 mg), HOBt (0.8 mmol, 122 mg) and NMM (1.52 mmol, 154 mg) for 1 h. On completion of the synthesis the deprotected resin was removed from the synthesiser and placed in a syringe barrel containing a frit. The peptide-resin was treated with Ac_2O (205 mg, 2.0 mmol, 10 equivalents) and DIEA (129 mg, 1.0 mmol, 5 equiv.) in DMF (5 cm^3) for 1 h. On completion of the reaction, peptide-resin **9a** was washed successively with DMF, methanol and *tert*-butyl methyl ether and dried *in vacuo* (over KOH pellets). Amino acid analysis [Found (expected): Glu, 2.05 (2); Ala, 1.05 (1); Ile, 1.02 (1); Tyr, 2.03 (2); Lys, 1.90 (2)].

In order to check on the progress of the assembly, a small-scale cleavage was performed. Peptide-resin (20 mg) was cleaved using TFA–triethylsilane (95 : 5, v/v; 1 cm^3) for 2 h and the peptide isolated as described above. The residue was dried in vacuum over KOH (1 h) following which it was dissolved in MeCN–0.1% aq. TFA (1 : 2, v/v; 1 cm^3). Analytical HPLC [gradient 10–90%B] showed the presence of a single major peak of retention time 18.5 min with ESMS at 1559.5 Da (theoretical molecular mass = 1559.9 Da).

Preparation of Ac-Tyr(Bu′)-Glu-Lys(Boc)-Glu(OBu′)-Ile-Lys-Ala-Tyr(Bu′)-NovaSyn TGR (peptide-resin 10a; Scheme 3)

Peptide-resin assembly **9a** was subjected to treatment with DMF–hydrazine, to remove Dmab and Dde side-chain protecting groups, as described in the general manual-synthesis protocols above. Following Dmab/Dde removal, a small quantity of resin (≈20 mg) was treated with TFA–triethylsilane (95 : 5, v/v; 1 cm^3) for 2 h and the peptide isolated as described above. The residue was dried in vacuum over KOH (1 h) and then dissolved in MeCN–0.1% aq. TFA (1 : 2, v/v; 1 cm^3). Analytical HPLC [gradient 10–90%B] showed the presence of a single broad peak of retention time 10.4 min with ESMS at 1084.5 and 1188.8 Da (theoretical molecular mass = 1084.3 Da).

Preparation of Ac-Tyr(Bu′)-Glu(OAllyl)-Lys(Boc)-Glu(OBu′)-Ile-Lys(Dde)-Ala-Tyr(Bu′)-NovaSyn TGR (peptide-resin 9b; Scheme 3)

Peptide-resin assembly **9b** was synthesised by standard Fmoc/*tert*-butyl solid-phase methods on NovaSyn TGR resin (Scheme 3). Fmoc-Rink-NovaSyn TGR **1** (1.0 g, 0.2 mmol) was suspended in DMF for 10 min prior to loading onto the Milligen 9050 synthesis column. The peptide was synthesised in a stepwise manner with activated residues coupled (0.8 mmol vials, 4 equivalents) (amino acid Pfp esters, HOBt-catalysed) under standard conditions. Fmoc-Lys(Dde)-OH and Fmoc-Glu(OAllyl)-OH (0.8 mmol vials, 4 equivalents) were coupled using HBTU (0.8 mmol, 303 mg), HOBt (0.8 mmol, 122 mg) and NMM (1.52 mmol, 154 mg) for 1 h. On completion of the synthesis the deprotected resin was removed from the synthesiser and placed in a syringe barrel containing a frit. The peptide-resin was treated with Ac_2O (205 mg, 2.0 mmol, 10 equivalents) and DIEA (129 mg, 1.0 mmol, 5 equiv.) in DMF (5 cm^3) for 1 h. On completion of the reaction, peptide-resin **9b** was washed successively with DMF, methanol and *tert*-butyl methyl ether and dried *in vacuo* (over KOH pellets). Amino acid analysis [Found (expected): Glu, 2.06 (2); Ala, 1.02 (1); Ile 1.05 (1); Tyr, 1.98 (2); Lys, 1.90 (2)].

In order to check on the progress of the assembly, a small-scale cleavage was performed. Peptide-resin (20 mg) was cleaved using TFA–triethylsilane (95 : 5, v/v; 1 cm^3) for 2 h and the peptide isolated as described above. The residue was dried in vacuum over KOH (1 h) following which it was dissolved in MeCN–0.1% aq. TFA (1 : 2, v/v; 1 cm^3). Analytical HPLC [gradient 10–90%B] showed the presence of a single major peak of retention time 15.2 min with ESMS at 1289.1 Da (theoretical molecular mass = 1288.5 Da).

Preparation of Ac-Tyr(Bu^t)-Glu(OAllyl)-Lys(Boc)-Glu(OBu^t)-Ile-Lys-Ala-Tyr(Bu^t)-NovaSyn TGR (peptide-resin 10b; Scheme 3)

Peptide-resin assembly **9b** was subjected to treatment with DMF–hydrazine, to the Dde side-chain-protecting group, as described in the general manual-synthesis protocols above. Following Dmab/Dde removal, a small quantity of resin (≈20 mg) was treated with TFA–triethylsilane (95 : 5, v/v; 1 cm^3) for 2 h and the peptide isolated as described above. The residue was dried *in vacuo* over KOH (1 h) and then dissolved in MeCN–0.1% aq. TFA (1 : 2, v/v; 1 cm^3). Analytical HPLC [gradient 10–90%B] showed the presence of a single species of retention time 12.0 min with ESMS at 1125.2 Da (theoretical molecular mass = 1124.3 Da).

Preparation of Ac-Tyr(Bu^t)-Glu-Lys(Boc)-Glu(OBu^t)-Ile-Lys-Ala-Tyr(Bu^t)-NovaSyn TGR (peptide-resin 11; Scheme 3)

Peptide-resin assembly **10b** (500 mg, 0.1 mmol) was treated with tetrakis(triphenylphosphine)palladium(0) (350 mg, 0.3 mmol, 3 equivalent) in DMF–$CHCl_3$–AcOH–NMM (18.5 : 18.5 : 2 : 1, v/v/v/v; 10 cm^3) for 3 h. The resin-bound peptide **11** was washed successively with DMF, DMF containing DIEA (0.5% v/v) and sodium diethyldithiocarbamate (0.5% w/v), DMF, methanol and finally *tert*-butyl methyl ether. Peptide-resin assembly **11** was dried *in vacuo* (over KOH) overnight.

Peptide-resin **11** (25 mg) was cleaved using TFA–triethylsilane (95 : 5, v/v; 2 cm^3) for 2 h and the peptide isolated as described above. Analytical HPLC [gradient 10–90% B] showed the presence of a single major peak of retention time 10.2 min with ESMS at 1084.4 (theoretical mass = 1084.3).

Preparation of Ac-Tyr(Bu^t)-Glu-Lys(Boc)-Glu(OBu^t)-Ile-Lys-Ala-Tyr(Bu^t)-NovaSyn TGR (peptide-resin 12; Scheme 3)

Peptide-resin assembly **11** (100 mg, 0.02 mmol) was suspended in DMF containing HATU (0.1 mmol, 38 mg), HOAt (0.1 mmol, 15.2 mg) and NMM (0.19 mmol, 19.2 mg) for 6 h to effect cyclisation. On completion of the reaction, the resin was washed successively with DMF, methanol and *tert*-butyl methyl ether and dried *in vacuo* (over KOH pellets). A small quantity of dried resin (≈20 mg) was treated with TFA–triethylsilane (95 : 5, v/v; 1 cm^3) for 2 h and the peptide **13** isolated as described above. The residue was dried in vacuum over KOH (1 h) and then dissolved in MeCN–0.1% aq. TFA (1 : 2, v/v; 1 cm^3). Analytical HPLC [gradient 10–90%B] showed the presence of two species (1 : 4 ratio) with retention times (peak 1) 10.1 min and (peak 2) 12.0 min. ESMS analysis of the collected peaks gave: peak 1, 1084.4 and peak 2, 1066.3 Da (theoretical molecular mass = 1066.3 Da). Peak 2 was isolated by semi-preparative HPLC (Phenomenex C4 column, 7.0 × 250 mm). Amino acid analysis [Found (expected): Glu, 2.03 (2); Ala, 0.98 (1); Ile 0.95 (1); Tyr, 2.02 (2); Lys, 1.06 (2)].

Alternatively, peptide-resin assembly **11** (100 mg, 0.02 mmol) was suspended in DMF containing HATU (0.1 mmol, 38 mg), HOAt (0.1 mmol, 13.6 mg) and NMM (0.19 mmol, 19.2 mg) for 6 h. Small-scale cleavage of the peptide was effected as described above to yield peptide **13**. Analytical HPLC [gradient 10–90%B] showed the presence of a single species with a retention time of 10.2 min. ESMS analysis of the collected peak gave MH^+ of 1066.4 Da (theoretical molecular mass = 1066.3 Da).

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