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Synthesis of Protected Peptide Fragments and Release from a Solid Support under Neutral Conditions[1]

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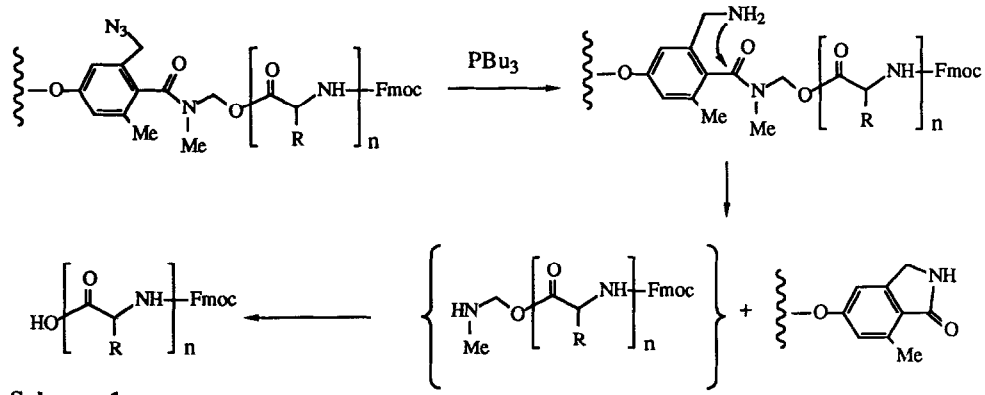
***Abstract** : A linker has been developed for use in solid-phase peptide synthesis which allows a protected peptide to be cleaved from the resin under neutral conditions (nBu_3P, DMF, imidazole buffer pH 7) whilst retaining tBuO-, Boc- and Fmoc-protecting groups. The linker comprises a 2-azidomethyl-4-hydroxy-6,N-dimethylbenzamide moiety, which is coupled to polystyrene resin as a phenol ether and to the peptide via a N-hydroxymethyl group. The protected peptide fragments so produced may be useful, for example, in subsequent fragment condensations using the Fmoc-chemistry.*

Stepwise SPPS[1], using either the Fmoc- or tBoc- groups for N-terminal protection[2,3], is a highly refined technology which *in favourable cases* can provide access to polypeptides in the 50-100 residue range. A related, convergent strategy involves fragment (or segment) coupling, in which protected peptide fragments are assembled into the target polypeptide on a solid support[4]. There is great interest in the fragment coupling strategy, especially as a means for deriving small synthetic proteins which are not easily accessible by stepwise SPPS due to low overall yields and difficulties in product purification. The potential advantages of this convergent approach would be enhanced further by making use of the Fmoc-chemistry during fragment coupling steps, rather than the tBoc-chemistry, since this would avoid the need to expose the final product to strong acids such as HF or CF_3SO_3H during cleavage from the resin. The required peptide fragments should then be Fmoc-protected at the N-terminus and have compatible tBoc- or related TFA-labile side chain protecting groups. In this paper we describe a novel linker unit which allows the assembly of such protected peptides, on a solid-phase using standard Fmoc-protocols, and their cleavage into free solution under neutral conditions mainly with retention of Fmoc- and side-chain protecting groups.

Over the past few years a large number of linkers have been developed which allow the release of protected peptide fragments from a solid-phase under (relatively) mild conditions, including some that are cleaved by nucleophiles[5], or mild acid[6] or base[7], or with fluoride[8], through photochemical activation[9], or with catalysis by Pd(0)[10] or by an enzyme[11]. However, many of these do not allow the

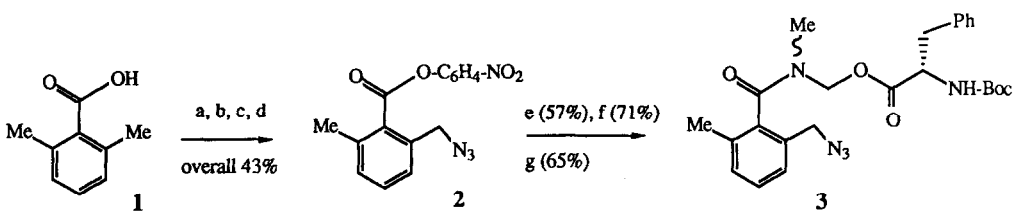
retention of both acid- (tBoc and -OtBu) and base-labile (Fmoc-) protecting groups in the peptide fragment, especially those requiring acid, base or fluoride in the cleavage reaction.

The linker developed here contains an aryl azidomethyl group, which, after reduction to an aminomethyl group with nBu_3P, undergoes an intramolecular reaction to release finally the protected peptide, as shown below (Scheme-1):



Scheme-1

The concept uses (a) an azide as a latent amino group which can be unmasked under conditions that are compatible with the functional groups typically found in protected peptides, (b) a sterically buttressed amide to which the peptide is attached through an N-hydroxymethyl link, and (c) a favourable intramolecular cyclization to initiate release of product. As a first step we used a model system to establish that the following could be achieved in free solution, i) formation of an appropriate N-methyl-N-hydroxymethylamide, ii) the coupling to this of an activated and protected amino acid, and iii) the final reduction-cleavage step with $(Bu)_3P$, with release of the required product. For these purposes the compound (**3**) was prepared (Scheme-2).



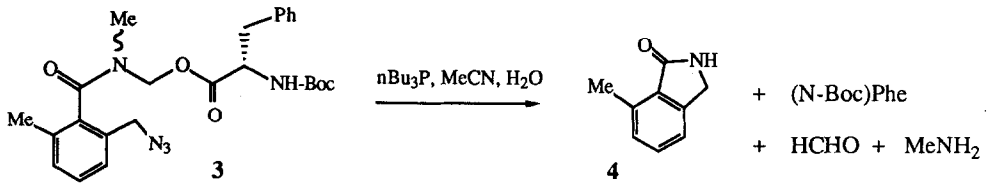
Scheme-2 **Reagents**: a, PCl_5 ; b, HO-C_6H_4-NO_2 ; c, NBS, dibenzoylperoxide, CCl_4 ; d, NaN_3 ; e, $MeNH_2$; f, HCHO, K_2CO_3 ; g, (Boc)Phe, DIPCDI, DMAP, DMF.

The reaction of 2-azidomethyl-6,N-dimethylbenzamide (Scheme-2) with formalin in basic solution at room temperature afforded the N-hydroxymethyl derivative in 71% yield as a mixture of **E** and **Z** isomers (**E**:**Z**, 65:35). Heating the reaction to 70°C for 1 hour did not increase the proportion of

product at equilibrium (^{1}H n.m.r.). Hydroxymethyl amides tend to revert to the amide in the absence of formaldehyde, especially under acidic or basic conditions or at elevated temperature. This instability of hydroxymethyl amides imparts an advantage on a solid support incorporating the azidomethyl linker. Any free hydroxyl groups present on the resin after the first coupling reaction need not be blocked, since the hydroxymethyl group should revert back to the amide and formaldehyde under basic (Fmoc-deprotection) conditions. The amide can then no longer participate in chain elongation.

L-N-Boc-Phenylalanine was activated with DIPCDI and coupled to the N-hydroxymethyl group to afford the desired product (**3**) in 65% yield (unoptimised). The ^{1}H n.m.r. spectrum of **3** was complicated by the presence of two diastereomers (axial chirality), each present as **E** and **Z** isomers due to slow rotation at the amide (CO)-N bond. The **E**:**Z** ratio was approximately 2:1 by integration, whereas the diasteromers were present in a 1:1 ratio.

The reaction of **3** with nBu_3P could be followed conveniently by UV-spectroscopy, as well as by t.l.c., since the product (**4**) has λ_{max} 283 nm, which is absent in **3**. In DMF with water (10 equiv.) and nBu_3P (3 equiv.) the formation of **4** and N-Boc-phenylalanine occurred almost quantitatively in 60 min. at room temperature. In addition, **3** was stable in 20% piperidine in DMF (typical conditions for Fmoc-deprotection) over several days.

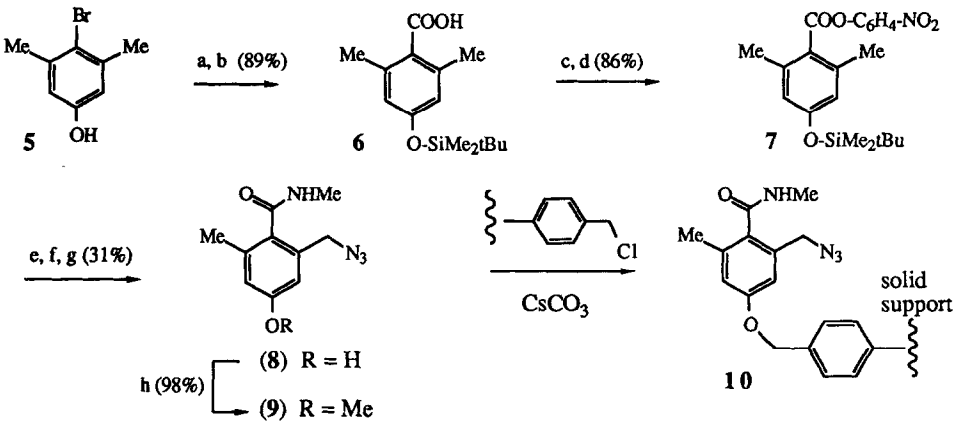


3 4

Scheme-3

The stage was therefore set to produce a suitable linker molecule which could be attached to a solid support. The target was compound **8**, which was synthesised according to Scheme-4, starting from commercially available 4-bromo-3,5-dimethylphenol. Although the synthesis of phenol **8** required seven steps, the overall yield is 23 %, and each reaction is relatively straightforward to carry out on a multi-gram scale. In step-b care must be taken to avoid using an excess of nBu-Li (Wurtz-type coupling). Steps e-to-g can be performed without purifying the intermediates, and only the purification of phenol **8** required a chromatographic step. The *p*-nitrophenol ester formed in step-d, was isolated as a stable colourless crystalline solid, and the phenol **8** was stable over a six month period under nitrogen at -20°C.

The methylamide **9** also reacted with formalin in basic solution at room temperature to afford the expected N-methyl-N-hydroxymethyl derivative in 60% yield as a mixture of **E** and **Z** isomers (**E**:**Z**, 2:1). Resonances for the **E** and **Z** isomers were assigned on the basis of NOE's between the N-methyl group and the aromatic methyl in the minor isomer (**Z**), and between the N-hydroxymethyl group and the aromatic methyl in the major isomer (**E**). To show that the *para*-methoxy substituent does not affect significantly the later steps, N-Boc-phenylalanine was then coupled via its symmetrical



Scheme-4 **Reagents**: a, t-BuMe$_2$SiCl, imidazole, DMF ; b, n-BuLi, then CO_2, with H_3O^+ work-up ; c, $(COCl)_2$; d, *p*-nitrophenol ; e, NBS, dibenzoylperoxide, CCl_4 ; f, NaN_3 ; g, $MeNH_2$; h, MeI, K_2CO_3.

anhydride to the free hydroxymethyl group, and the resulting conjugate upon treatment with nBu_3P again underwent reduction and cleavage to release N-Boc-phenylalanine.

The phenol **8** was attached in quantitative yield to chloromethylated Wang resin by reaction in DMF in the presence of $CsCO_3$ (Scheme-4). The extent of linker coupling could be determined by nitrogen elemental analysis after washing the resin sequentially with water, DMF and dichloromethane, and drying *in vacuo*. The coupling reactions have been performed on up to five gram batches of chloromethyl resin without problems, and the product **10** (referred to below as "azidomethyl resin") is stable upon storage at -20°C for at least a year.

The azidomethyl resin was next hydroxymethylated by rotating the resin (using a rotary evaporator) in either DMF or NMP with formaldehyde and $CsCO_3$ as a base. The formation of product was accompanied by a shift in the amide carbonyl IR stretching vibration from 1660 to 1640 cm^{-1}, although accurate quantification of the reaction yield was not possible. The resin was washed with water, DMF and dichloromethane to remove spent reagents, and dried *in vacuo* (< 40°C). Due to the aforementioned instability of hydroxymethyl amides, the product was used immediately for peptide synthesis.

As a first test of the new linker the relatively uncomplicated synthesis of the protected hexapeptide Fmoc-Gly-Pro-Gly-Arg(Pmc)-Ala-Phe-OH (**11**) was carried out. This carries Fmoc-N-terminal, and arginine-Pmc-side chain[12] protecting groups, and represents a sequence in the so-called V3 loop region[13] of the gp120 coat protein of the human immunodeficiency virus-1. The synthesis of **11** was performed on a 0.25 mmol scale using NMP as solvent, HBTU as the activating agent[14] during each chain elongation step (except for the first residue where DCC was used) and a four fold excess of the activated amino acid in each coupling cycle. After N-Fmoc-Phe had been coupled to the hydroxymethylated azidomethyl resin using DCC, the extent of substitution was found to be 0.65 mmol per gram, which corresponded to a 69% yield over the four steps required to couple the linker to Wang resin and N-Fmoc-Phe to the linker. Thereafter Kaiser[15] or isatin[16] tests, as appropriate, indicated that each coupling during the synthesis had proceeded >99% to completion. The resin was

then washed with dichloromethane and dried *in vacuo* ; the resin weight and its amino acid composition were in agreement with expectation.

When the resin was treated with nBu_3P (3 equiv) in DMF and water (10 equiv) to release the desired protected peptide (**11**), a complete loss of the Fmoc group occurred over the course of the reaction (10 h), to afford the peptide H_2N-Gly-Pro-Gly-Arg(Pmc)-Ala-Phe-OH (**12**). This loss of the Fmoc-group presumably occurs due to the generation of a basic moiety, possibly the iminophosphorane which is an intermediate in the Staudinger reaction[17] between azides and phosphines. However, with nBu_3P (3 equiv.) in DMF containing imidazole buffer (1M, pH 7, 20% v/v) this deprotection was largely suppressed and **11** and **12** were formed in a 84 : 16 ratio. The peptide products were precipitated with ether, redissolved in methanol and chromatographed on LH20 Sephadex in methanol to afford **11** in 71% yield and of 96% purity by reverse phase h.p.l.c. It was not possible to further suppress the undesired loss of the Fmoc-protecting group. However, in principle, this deprotected material could be reprotected with Fmoc-Cl to maximise the yield of desired peptide.

To test whether similar protocols could be used for the synthesis of peptides containing a more diverse array of acid-sensitive side-chain protecting groups, the peptide Fmoc-Thr(OtBu)-Ser(OtBu)-Asp(OtBu)-Tyr(OtBu)-Trp-Ser(OtBu)-OH (**13**) was chosen, derived from complementarity determining region-1 of the heavy chain of the anti-lysozyme antibody HYHEL-10[18]. The first residue, Fmoc-Ser(OtBu)-OH, was activated with DCC and coupled to the N-hydroxymethylated azidomethyl resin to achieve a substitution level of 0.43 mmol/gm. The same conditions were used for chain elongation as described in the previous synthesis, and upon completion the product was cleaved from the solid phase (nBu_3P (3 equiv.) in DMF containing imidazole buffer (1M, pH 7, 20% v/v)). After chromatography on LH20 Sephadex in methanol, **13** was obtained in 60% yield and > 90% purity by h.p.l.c., along with the corresponding N-deprotected material in ≈ 10% yield.

The results described above demonstrate that the linker, 2-azidomethyl-4-hydroxy-6,N-dimethylbenzamide, can be used in solid-phase peptide synthesis with the standard Fmoc-chemistry to produce peptide fragments retaining Fmoc-N-terminal and Boc/OtBu-side chain protecting groups. Such protected peptides should be useful, for example, in the assembly of larger synthetic oligopeptides using a fragment condensation approach.

EXPERIMENTAL

2,6-Dimethylbenzoic acid 4-nitrophenyl ester

2,6-Dimethylbenzoic acid (5.8 g, 38.6 mmol) and PCl_5 (8.0 g, 38.4 mmol) were heated to 80°C for 30 min. *p*-Nitrophenol (5.4 g, 38.8 mmol) was added, and stirred for a further 30 min. After cooling to room temp. and dissolving in dichloromethane (50 ml) the product was washed with sat. NaCl (2 x 50 ml), the organic layer dried over $MgSO_4$ and the solvent was removed in vacuo to give **product** as a colourless crystalline solid (9.95 g, 95%), m.p: 106-107°C (from petroleum ether (60-90)) (Found: C, 66.3; H, 4.55; N; 5.3. Calc. for $C_{15}H_{13}NO_4$ C, 66.4; H, 4.8; N, 5.2%); $\upsilon_{max.}$ (KBr) 3120w, 2970w, 1740s, 1522s, and 1350s cm^{-1}; $\lambda_{max.}$ ($CHCl_3$) 270.0 nm (ε 9,850 $dm^3mol^{-1}cm^{-1}$); δ_H (80 MHz; $CDCl_3$) 2.47 (6H, s, CH_3-Ar), 7.15-7.22 (3H, m, Ar-H), 7.28-7-7.48 (2H, m, Ar-H), and 8.27-8.38 (2H, m, Ar-H); *m/z* (EI) 133 (M^+-$C_6H_4NO_3$, 100%) and 105.

2-Bromomethyl-6-methylbenzoic acid 4-nitrophenyl ester

To the forgoing ester (5.0 g, 18.45 mmol) in CCl_4 (100 ml) was added NBS (3.0 g, 16.9 mmol) and dibenzoyl peroxide (50 mg, 0.22 mmol). The reaction was refluxed for 7 h, filtered, and solvent removed *in vacuo*. The product was used in this form in the next step. A small sample was purified by silica column chromatography (10% EtOAc, 90% n-hexane) for characterisation to give a colourless solid, m.p. 81-83°C (Found: C, 51.5; H, 3.7; N, 4.0; Br, 22.85. $C_{15}H_{12}BrNO_4$ requires C, 51.45; H, 3.45; N, 4.3; Br, 22.85%); δ_H (300 MHz; $CDCl_3$) 2.55 (3H, s, CH_3-Ph), 4.70 (2H, s, CH_2Br), 7.14-7.70 (5H, m, H-Ph) and 8.2-8.5 (2H, m, Ar-H).

2-Azidomethyl-6-methylbenzoic acid 4-nitrophenyl ester (2)

To the forgoing product in ethanol (85 ml) was added sodium azide (1.08 g, 16.6 mmol). The solution was stirred at room temp. for 40 h then sat. NaCl (100 ml) was added, the mixture was extracted with EtOAc (2 x 100 ml), the organic layer dried over $MgSO_4$, and solvent removed *in vacuo*. The product was purified by silica column chromatography (90% n-hexane, 10% EtOAc) to give a white solid (2.59 g, 45% over previous two synthetic steps), m.p. 82-84°C (Found: C, 57.6; H, 3.9; N, 17.7. $C_{15}H_{12}N_4O_4$ requires C, 57.7; H, 3.85; N, 17.95%); $\upsilon_{max.}$ (KBr) 3120w, 2930w, 2105s, 1750s, 1525s, and 1345s cm^{-1}; $\lambda_{max.}$ ($CHCl_3$) 270 nm (ε 15100 $dm^3mol^{-1}cm^{-1}$); δ_H (300 MHz; $CDCl_3$) 2.55 (3H, s, CH_3-Ph), 4.54 (2H, s, CH_2-Ph), 7.26-7.51 (5H, m, Ar-H), and 8.33-8.38 (2H, m, Ar-H); *m/z* (Cl, C_4H_{10}) 270.1 (M^+-N_3, 19%), 174.1 (49) and 146.1 (100).

2-Azidomethyl-6,N-dimethylbenzamide

To the forgoing product (0.685 g, 2.20 mmol) in MeOH (28.5 ml) was added $MeNH_2$ (40% in MeOH, 28.5 ml, 368 mmol), and the solution was stirred at room temp. for 6 h then diluted with EtOAc (30 ml), and washed with aq. $NaHCO_3$ (3 x 10 ml), the organic layer was dried over $MgSO_4$, and solvent removed *in vacuo*. The resulting oil was purified by silica column chromatography (60% n-hexane, 40% EtOAc) to give a colourless oil (0.257 g, 57%) (Found: C, 59.1; H, 5.95; N, 27.7. $C_{10}H_{12}N_4O$ requires C, 58.8; H, 5.9; N, 27.45%); $\upsilon_{max.}$ (KBr); 3450m, 3000m, 2100s, 1660s, 1540s, 1460m, and 1415m cm^{-1}; $\lambda_{max.}$ (MeOH) 267.5 (ε 560 $dm^3\ mol^{-1}\ cm^{-1}$), 273.5 (530); δ_H (300 MHz; $CDCl_3$) 2.35 (3H, s, CH_3-Ar), 3.04 (3H, d *J* 4.9Hz, CH_3-N), 4.34 (2H, s, CH_2-Ar), 5.80 (1H, bs, N-H), and 7.1-7.45 (3H, m, ArH); *m/z* (EI) 175 (M^+-CH_3N+H, 19%), 161 (40), 146 (49), 133 (48) and 83(100).

2-Azidomethyl-N-hydroxymethyl-6,N-dimethylbenzamide

To the forgoing amide (450 mg, 2.20 mmol) in MeCN (17 ml) was added K_2CO_3 (607 mg, 4.40 mmol) and formaldehyde (0.89 ml, 11 mmol) and stirred at room temp. for 5 h. The reaction was poured into sat. NaCl (25 ml), extracted into EtOAc (3 x 20 ml), the organic layers pooled and dried over $MgSO_4$ and solvent removed *in vacuo*. The resulting oil was purified on silica chromatography (50% EtOAc, 50% n-hexane) to give product 0.37 g (71%) as a colourless oil (Found: C, 56.65; H, 6.15; N, 23.65. $C_{11}H_{14}N_4O_2$ requires C, 56.4; H, 6.0; N, 23.95%); $\upsilon_{max.}$ (Film) 3350bm, 2920w, 2100s, 1640s, and 1400m cm^{-1}; λmax. (MeOH) 274.5 (ε 470 $dm^3mol^{-1}cm^{-1}$) and 266.9 (540) nm; δ_H (300 MHz; $CDCl_3$) 2.24 and 2.30(3H, 2 x s, CH_3-Ar), 2.90 and 3.22 (3H, 2 x s, CH_3-N), 3.52 (dd, *J* 7.95 and 7.95Hz) and 3.58 (1H, dd, *J* 4.09 and 11.13Hz, OH), 4.31-4.42 (2H, m, CH_2N_3), 4.46 (dd, *J*=11.03 and 11.04Hz) and 4.69 (dd, *J* 4.09 and 11.13Hz) and 5.02 (dd, *J*=7.91 and 10.12 Hz) and 5.07 (2H, dd, *J*=7.93 and 10.24 Hz, CH_2O), 7.18, (3H, m, Ar-H); *m/z* (EI) 235 (M^++H, 4%), 205 (25), 189 (29), 174 (52) and 146 (100).

[(2-Azidomethyl-6-methylbenzoyl)(methyl)amino]methyl-2-[[(tert-butoxy)-carbonyl]amino]-3-phenylpropanoate (3)

To the forgoing product (250 mg, 1.06 mmol) in DMF was added (Boc)Phe (1.13 g, 4.26 mmol), DIPCDI (8.5 ml, 0.5 M in DCM, 4.25 mmol) and 4-DMAP (104 mg, 0.85 mmol). The reaction was stirred at room temp. for 17 h, then dichloromethane (10 ml) was added, washed with $NaHCO_3$ (2 x 10 ml), the organic layer dried over Na_2SO_4 and solvent removed *in vacuo*. The resulting oil was purified by silica column (30% EtOAc, 70% n-hexane), giving (3) as a colourless oil (280mg, 65%) (Found: C, 62.5; H, 6.65; N, 14.35. $C_{25}H_{31}N_5O_5$ requires C, 62.35; H, 6.45; N, 14.55%); $\upsilon_{max.}$ (film) 3340w, 2980m, 2920m, 2100s, 1740s, 1710s, and 1655s cm^{-1}; $\lambda_{max.}$ ($CHCl_3$) 241 (ε 1230 $dm^3mol^{-1}cm^{-1}$), 259 (620), 265 (600), and 274 nm (505); δ_H (300 MHz; $CDCl_3$) 1.41 (9H, bs, tBu), 2.23 and 2.24 and 2.25 and 2.27 (3H, 4 x s, CH_3-Ph), 2.78 and 2.82 and 3.10 and 3.11 (3H, 4 x s, CH_3-N,), 3.02 (dd) and 3.11 (2H, dd, CH_2-Ph), 4.18-4.32 (2H, m, CH_2-N_3), 4.52 and 4.66 (1H, 2 x m, C_aH), 4.91 and 5.01 (1H, 2 x br.m, N-H), 5.1 and 5.17 and 5.61 and 5.71 (2H, 4 x m, NCH_2O), and 7.03-7.4 (8H, m, Ar-H); *m/z* (CI, NH_3) 499.4 (M^++NH_4, 77%), 482 3 (M^++H, 64), 454.3 (32), 426.2 (18) and 379.3 (100).

7-Methyl-2,3-dihydroisoindole-1-one (4)

To the forgoing ester (38 mg, 0.079 mmol) in DMF (1 ml) was added water (14.2 ml, 0.79 mmol), tributylphosphine (48 mg, 0.238 mmol). At intervals 10 μl was removed and diluted with methanol (3 ml). The u.v. absorbance at 283 nm was measured against 100% methanol as blank. The extent of azide reduction was followed by t.l.c. (silica, 50% EtOAc, 49% n-hexane, 1% AcOH). After five h the reaction mixture was diluted with dichloromethane (0.7 ml) and the product was purified on a silica column (20% n-hexane, 80% EtOAc) and dried at 60°C under high vacuum to give 4 as a colourless solid, m.p. 126-128°C (Found: C, 73.7; H, 6.35; N, 9.3. Calc. for C_9H_9NO C, 73.5; H, 6.1; N, 9.5%); $\upsilon_{max.}$ (KBr) 3450bm, 3200m, 3080m, 1680s, 1610m cm^{-1}; $\lambda_{max.}$ (MeOH) 275 (ε 1140 $dm^3mol^{-1}cm^{-1}$), and 283 nm (1940); δ_H (300 MHz; $CDCl_3$) 2.74 (3H, s, CH_3), 4.40 (2H, s, CH_2), 7.15 (1H, bs, N-H), and 7.18-7.45 (3H, m, Ar-H); *m/z* (EI) 147 1 (M^++H, 100), 133.1 (76), 132.1 (67) 119.1 (27) and 118.1 (33).

2-Bromo-5-tert-butyldimethylsilyloxy-1,3-dimethylbenzene

To a solution of 4-bromo-3,5-dimethylphenol (35.9 g, 179 mmol) in DMF (400 ml) was added *tert*-butyldimethylsilyl chloride (53.88 g, 357 mmol) and imidazole (24.39 g, 357 mmol). After stirring at room temp. for 3.5 h, n-hexane was added (400 ml), the organic layer washed with water (2 x 400 ml), and the solvent removed *in vacuo*. *tert*-Butyldimethylsilyl hydroxide was removed under high vacuum to give the product as a yellow oil (55.2 g, 98%) (Found: C, 53.35; H, 7.45; Br, 25.2. $C_{14}H_{23}BrOSi$ requires: C, 53.55; H, 7.3; Br, 25 5%); $\upsilon_{max.}$ (CH_2Cl_2) 2960m, 2940m, 2860m, 1565m, 1480s, 1325s, and 1170s cm^{-1}; λ_{max} ($CHCl_3$) 241 (ε 3240 $dm^3mol^{-1}cm^{-1}$), 276 (870), and 283 nm (850); δ_H (300 MHz; $CDCl_3$) 0.20 (6H, s, Si-$(CH_3)_2$), 0.99 (6H, s, Si-$C(CH_3)_3$), 2.36 (6H, s, CH_3-Ph), and 6.59 (2H, s, H-Ph); *m/z* (EI) 316 (M^++2, 25), 314 (M^+, 20%), 259 (79), 257 (4), 178 (97) and 177 (100) .

4-tert-Butyldimethylsilyloxy-2,6-dimethylbenzoic acid (6)

To the forgoing product (15.5 g, 49.2 mmol) in THF (100 ml) at -78°C was added nBuLi (29.8 ml, 1 65 M, 49.2 mmol) dropwise over 15 min After a further 10 min. dry carbon dioxide was blown into the solution for 10 min.

The reaction was rapidly warmed to room temp, sat. NaCl (100 ml) was added, and the pH adjusted to 4.7 by addition of neat AcOH. The organic layer was removed, the aqueous layer extracted with EtOAc (2 x 100 ml), the organic fractions combined and dried over $MgSO_4$ and solvent was removed in vacuo. The product gave white crystals from MeOH / H_2O (12.5 g, 91%), m.p. 132-133°C (Found: C, 64.3; H, 8.5. $C_{15}H_{24}SiO_3$ requires C, 64.3; H, 8.6%); $\upsilon_{max.}$ (KBr) 3420w, 2960m,1690s and 1600s cm^{-1}; $\lambda_{max.}$ ($CHCl_3$) 248.1 nm (ε 9920 $dm^3mol^{-1}cm^{-1}$); δ_H (300 MHz; $CDCl_3$) 0.22 (6H, s, $(CH_3)_2$-Si), 0.99 (9H, s, $(CH_3)_3$-Si), 2.42 (6H, s, CH_3-Ar), and 6.55 (2H, s, Ar-H) ; *m/z* (EI) 280 (M^+, 21%), 223 (100) and 179 (34).

4-tert-Butyldimethylsilyloxy-2,6-dimethylbenzoic acid 4-nitrophenyl ester (7)

To the forgoing acid (29.7 g, 107 mmol) in benzene (130 ml) was added oxaloyl chloride (20.21 g, 159 mmol) The mixture was stirred at room temp. for 1 h, the benzene and excess oxaloyl chloride were removed *in vacuo*, and the oil dried under high vacuum. *p*-Nitrophenol (14.7 g, 106 mmol) was then added, and stirred for 3 h adding enough benzene during this time to ensure efficient stirring. The benzene was removed *in vacuo*, the product was filtered through a silica pad (15% EtOAc, 85% n-hexane) and the resulting solid was recystallised from n-hexane to give a colourless crystalline **product** (36.9 g, 86%), m.p. 92-93°C (Found: C, 62.8; H, 6.7; N, 3.4. $C_{21}H_{27}NO_5Si$ requires C, 62.8; H, 6.7; N, 3.50%); $\upsilon_{max.}$ (KBr) 3450w, 2960m, 2935m, 2860m, 1750s, 1590s, 1530s cm^{-1}; $\lambda_{max.}$ ($CHCl_3$) 273 nm (ε 19600 $dm^3mol^{-1}cm^{-1}$); δ_H (300 MHz; $CDCl_3$) 0.23 (6H, s, $(CH_3)_2$-Si), 1.00 (9H, s, $(CH_3)_3$-Si), 2.44 (6H, s, CH_3-Ar), 6.59 (2H, s, Ar-H), 7.41 (2H, m, Ar-H), 8.33 (2H, m, Ar-H); *m/z* (CI, C_4H_{10}) 402.4 (M^++H, 50%), 372.4 (10) and 263.3 (100).

2-Azidomethyl-4-tert-butyldimethylsilyloxy-6-methylbenzoic acid 4-nitrophenyl ester

The ester forgoing ester (11.30 g, 28.2 mmol), NBS (4.52 g, 25 4 mmol), and dibenzoyl peroxide (75 mg, 0.33 mmol) were refluxed in CCl_4 (150 ml) for 12 h. This mixture was then filtered and the solvent evaporated *in vacuo*. Ethanol (150 ml) was then added, followed by sodium azide (3.66 g, 56 mmol) with stirring at room temp. for 24 h. The solvent was reduced to about 50 ml *in vacuo* , sat. NaCl (50 ml) was added and organic material was extracted into ethyl acetate (3 x 50 ml) and dried over $MgSO_4$. The solvent was removed and the resulting oil filtered through a pad of silica (20% EtOAc, 80% n-hexane) to give a yellow oil which solidified on standing. This material was used directly in the next reaction without further purification. A small portion was purified on silica gel (4% EtOAc, 96% n-hexane) for analysis (Found: C, 57 3; H, 5.9; N, 12.4. $C_{21}H_{26}N_4O_5Si$ requires C, 57.0; H, 5.9; N, 12.7%); $\upsilon_{max.}$ (Film) 2960s, 2930s, 2860s, 2100s, 1745s, 1590s, 1525s, 1490s, 1350s, and 1320s cm^{-1}; $\lambda_{max.}$ (MeOH) 270 (ε 13940 $dm^3mol^{-1}cm^{-1}$); δ_H (300 MHz; $CDCl_3$) 0.23 (6H, s, $(CH_3)_2$-Si), 1.01 (9H, s, $(CH_3)_3$-Si), 2.51 (6H, s, CH_3-Ar), 4.51 (2H, s, CH_2N_3), 6.75 (2H, s, Ar-H), 7.47 (2H, m, Ar-H), and 8.36 (2H, m, Ar-H); *m/z* (CI, NH_3) 460.3 (M^++NH_4^+, 8%), 293.2 (100) and 276.2 (75).

2-Azidomethyl-4-hydroxy-6,N-dimethylbenzamide (8)

To the forgoing product was added EtOH (125 ml) and $MeNH_2$ (33% in EtOH, 216 ml) with stirring at room temp. for 20 h. The $MeNH_2$ and EtOH were removed *in vacuo* (40°C) to give a yellow oil, which was purified by column chromatography (60% EtOAc, 39% n-hexane, 1% AcOH) to give (*8*) as a colourless oil, which solidified on standing (1.94 g, 31% over last three synthetic steps), m.p. 131-132°C (Found: C, 54.7, H, 5.2; N, 25.25. $C_{10}H_{12}N_4O_2$ requires C, 54.5; H, 5.45; N, 25.45%); $\upsilon_{max.}$ (KBr) 3260bs, 2920m, 2100s, 1610m cm^{-1}; $\lambda_{max.}$ (MeOH) 280.1 nm (1170 $dm^3mol^{-1}cm^{-1}$); δ_H (300 MHz; d_6 acetone) 2.21 (3H, s, CH_3-Ph), 2.90

(3H, d, NMe), 4.31 (3H, s, CH_2N_3), 6.65 (2H, m, Ar-H), 7.41 (1H, bs, N-H), and 8.88 (1H, s, Ar-OH); *m/z* (CI, NH_3) 221.4 (M^++H, 100), 193.3 (7), 179.3 (47) and 162.2 (31).

2-Azidomethyl-4-methoxy-6,N-dimethylbenzamide (9)

The forgoing phenol (800 mg, 3.64 mmol) in acetonitrile (33 ml) with K_2CO_3 (1500 mg, 15.15 mmol) and MeI (1.033 g, 7.17 mmol) was stirred overnight at room temp. The mixture was poured into sat. NaCl (50 ml), extracted into ethyl acetate (3 x 50 ml), the organic portions were combined, dried over $MgSO_4$ and the solvent evaporated *in vacuo* to give (9) 0.83 g (98%) as a colourless oil. A portion was purified on silica (52% n-hexane, 48% ethyl acetate) for analysis. (Found: C, 56.4; H, 6.3; N, 23.7. $C_{11}H_{14}N_4O_2$ requires C, 56.4; H, 6.0; N, 23.9%); $\upsilon_{max.}$ (KBr) 3450m, 3000m, 2100s, 1660s, 1610s cm^{-1}; $\lambda_{max.}$ (MeOH) 278.1 nm (ε 1515 $dm^3mol^{-1}cm^{-1}$); δ_H (300 MHz; $CDCl_3$) 2.32 (3H, s, CH_3-Ph), 3.00 (3H, d, CH_3-N), 3.81 (1H, s, MeO), 4.31 (1H, s, CH_2N_3), 5.86 (1H, bs, N-H), and 6.70 (2H, s, Ar-H); *m/z* (CI, C_4H_{10}) 235.2 (M^++H, 100%), 207.2 (12) and 192.2 (5.5).

2-Azidomethyl-N-hydroxymethyl-4-methoxy-6,N-dimethylbenzamide

The forgoing product (0.75 g, 3.21 mmol) in acetonitrile (24 ml) with K_2CO_3 (953 mg, 9.63 mmol) and formaldehyde (37% in water, 1.27 ml) was stirred at room temp. for 19 h. The mixture was then poured into sat. NaCl (40 ml), extracted into ethyl acetate (3 x 40 ml), dried over $MgSO_4$ and the solvent was removed *in vacuo* to give **product** 0.87 g as a yellow oil. A portion was purified on silica gel (60% ethyl acetate, 40% n-hexane) for analysis (Found: C, 54.4; H, 6.3; N, 20.9. $C_{12}H_{16}N_4O_3$ requires C, 54.55; H, 6.1; N, 21.2%); $\upsilon_{max.}$ (CCl_4) 3450m, 2950m, 2100s, 1645s, and 1610m cm^{-1}; $\lambda_{max.}$ (MeOH) 278 nm (ε 1560 $dm^3mol^{-1}cm^{-1}$); δ_H (400 MHz; $CDCl_3$) 2.19 (s, CH_3-Ar, E), 2.25 (s, CH_3-Ar, Z), 2.89 (s, CH_3-N, Z), 3.17 (s, CH_3-N, E), 3.63 (dd, J=10.74 and 4.34Hz, OH, Z), 3.73 (dd, J=7.92 and 7.94Hz, OH, E), 3.81 (3H, s, O-Me, Z+E), 4.29-4.31 (2H, m, CH_2N_3, Z+E), 4.44 (dd, J=10.73Hz and 10.80Hz, NCH'O, E), 4.68 (dd, J=10.82 and 5.45Hz, NCH"O, E), 5.00 (dd, J=8.02 and 10.31Hz, NCH'O, Z), 5.03 (dd, J=7.86 and 10.30Hz, NCH"O, Z), and 6.70-6 75 (3H, m, Ar-H, Z+E); *m/z* (EI) 265 (M^++H, 7%), 235.4 (67), 207.3 (29), 204.3 (52), 190.3 (20) and 176.2 (100)

[(2-Azidomethyl-4-methoxy-6-methylbenzoyl)(methyl)amino]methyl-2-{[(tert-butoxy)-carbonyl]amino}-3-phenylpropanoate

To BocPhe (4.37 g, 16.5 mmol) in dichloromethane (50 ml) was added DIPCDI (0.5 M in dichloromethane, 16.5 ml, 8.25 mmol). After 25 min. at room temp. the symmetric anhydride was filtered into a flask containing the forgoing product (crude) and 4-DMAP (237 mg, 1.94 mmol) and stirred for 3 h. After washing with $NaHCO_3$ (50ml) the organic layer was dried over $MgSO_4$ and the product purified on silica gel (30% EtOAc, 70% n-hexane) to give **product** as a colourless oil (801 mg, 28% over last three synthetic steps), m.p. 118-121°C (Found: C, 61.0; H, 6.7; N, 13 5. $C_{26}H_{33}N_5O_6$ requires C, 61.1; H, 6.5; N, 13.7%); $\upsilon_{max.}$ (CCl_4) 3450w, 2990m, 2100s, 1745s, 1715s, and 1655s cm^{-1}; $\lambda_{max.}$ ($CHCl_3$) 278 nm (ε 1640 $dm^3mol^{-1}cm^{-1}$); δ_H (300 MHz; $CDCl_3$) 1.41 (9H, s, t-Bu), 2 20 and 2.21 (2 x s, CH_3-Ar, E), 2.22 and 2.24 (s, CH_3-Ar, Z), 2.78 and 2.81 (2 x s, CH_3-N, Z), 3.05 (m, CH_2-Ph, E), 3.1 (s, Me-N, E), 3.15 (m, CH_2Ph, Z), 3.81 (s, 3H, MeO), 4.20-4.30 (2H, m, CH_2-N_3), 4.51 (m, C_αH, E), 4.60 (m, C_αH, Z), 4.93 (bd, NH, E), 5.02 (bd, NH, Z), 5.1 - 5.2 (m, NCH_2O, E), 5.55 - 5.75 (m, NCH_2O, Z), 6.7 (m, 2H, Ar-H), and 7.15-7.35 (m, 5H, Ph-H); *m/z* (CI, NH_3) 512 (M^++H, 10%), 484.6 (9), 351 4 (55) and 204 3 (100); *m/z* (electrospray) 534.4 (M + Na) and 512 1 (M + H)

Chlorination of p-alkoxybenzyl alcohol resin[19]

p-Alkoxybenzylalcohol (Wang) resin (2.65 g, 0.97 mmol/g, 2.57 mmol) was added to triphenyldichlorophosphine (4.37 g, 13.1 mmol) and dichloromethane (54 ml) and the mixture was shaken at room temp. for 21 h. The resin was then washed sequentially with DMF / water (1:1, 4 x 45 ml), DMF (45 ml, 4 x 45 ml), EtOH (45 ml, 4 x 45 ml) and dichloromethane (4 x 45 ml).

Coupling 2-azidomethyl-4-hydroxy-6,N-dimethylbenzamide to the resin

To the chlorinated resin (2.57 g, 2.5 mmol) was added the phenol **8** (0.71 g, 3.23 mmol), $CsCO_3$, (1.59 g, 8.24 mmol), DMF (34 ml) and the mixture was rotated for 37 h at room temp. The resin was then washed sequentially with water (4 x 40 ml), H_2O:DMF (1:1, 4 x 40 ml), DMF (4 x 40 ml), EtOH (4 x 40 ml), dichloromethane (4 x 40 ml) and dried *in vacuo* to give **10** (2.90 g). (Found: N, 4.37. 100% reaction over last two steps requires N, 4.41%); $\upsilon_{max.}$ (KBr) 3405m, 3305bm, 3010s, 2920s, 2100s, 1660s, 1600s, 1510m and 1490m.

Hydroxymethylation of azidomethyl resin

To the azidomethyl resin **10** (500 mg) in DMF (10 ml) was added $CsCO_3$ (770 mg, 3.98 mmol) and formaldehyde (386 µl, 37% in water), which were rotated for 24 h at room temp. The resin was then washed sequentially with water (5 x 10 ml), DMF:water (1:1, 5 x 7 ml), EtOH (5 x 7 ml), dichloromethane (5 x 7 ml) and dried at room temp. *in vacuo* to give the hydroxymethylated resin (485mg). $\upsilon_{max.}$ (KBr) 3420bm, 3010s, 2920m, 2100m, 1640s, 1600s, 1510m and 1490m.

Synthesis of Fmoc-GPGR(Pmc)AF-OH on azidomethyl Resin

(Fmoc)Phe (0.39 g, 4eq.) was coupled to the hydroxymethylated resin (0.35g) using DCC (0.21g) and DMAP (0.2 equiv.) in 1:1 DMF:dichloromethane (6.5ml). The substitution of (Fmoc)Phe on the resin was 0.65 mmol/g (69% over previous four steps). All subsequent coupling reactions were performed in a standard process which involved the following: each coupling step began with removal of the N-terminal Fmoc group using 20% piperidine in DMF. After washing the resin with NMP, the addition of each Fmoc-amino acid was carried out by activating 1 mmol (4 equiv) with HBTU (1 mmol) in the presence of HOBt (1 mmol) and diisopropylethylamine (2 mmol). The activated amino acid was then reacted with the resin with vigorous shaking for 30 min. The resin was then washed with NMP and the coupling efficiency was checked on a small aliquot (≈ 1 mg) of the resin using the Kaiser or isatin test, as appropriate. After completion of the synthesis the resin was washed with dichloromethane (5 x 5ml), dried *in vacuo* and weighed (0.58g). This resin with DMF (7.5ml), buffer (1M imidazole, pH 7, 2ml) and tributylphosphine (273 µl) were stirred vigorously at room temp. After 10 h the resin was filtered, washed with DMF (10 x 1ml), methanol (5 x 1ml), the organic washings were pooled, the solvent evaporated, the product (**11**) was precipitated with diethyl ether and then purified by chromatography on LH20 Sephadex (methanol). A small portion was further purified by h.p.l.c. (water:MeCN (30:70, +0.1% TFA) to 100% MeCN (+0.1% TFA), Vydac C_{18}) for analysis by 600MHz ^{1}H NMR in DMSO. Amino acid composition found (Fmoc-GPGR(Pmc)AF-OH) Phe:1.0, Pro: 0.93, Gly: 2.10, Ala: 1.09, Arg: 0 95. *m/z* (electrospray) 1092.9 (M^++H). The Fmoc group of the peptide was removed in 20% piperidine in methanol (5 minutes), the solvent was evaporated and the product (**12**) redissolved in methanol and purified by reverse phase h.p.l.c. (Waters C_{18} µbondapak, 40:60-water:MeCN to 20:80-water:MeCN (+0.1% v/v TFA) over 15 minutes). Amino

acid analysis (H_2N-GPGR(Pmc)AF-OH): Phe:1.0, Pro: 0.89, Gly: 2.14, Ala: 1.0, Arg: 1.08; *m/z* (electrospray) 870.9 (M^++H). The ^{1}H NMR spectra of both peptides at 600MHz in DMSO were entirely consistent with the proposed structure.

Synthesis of Fmoc-T(OtBu)S(OtBu)D(OtBu)Y(OtBu)WS(OtBu)OH on azidomethyl resin

This peptide was synthesized following the same protocols as used above, using 0.38g of hydroxymethylated azidomethyl resin. After coupling the first amino acid, the loading of Fmoc-Ser(OtBu) was 0.43 mmol/g. After completion of the synthesis the resin was washed with dichloromethane (5 x 5 ml), dried in vacuo and weighed 0.56g (87%). The product was released from the resin as before, and purified by chromatography on LH20 Sephadex (methanol). Relevant fractions were pooled and evaporated to afford **13** in 60% yield, and > 90% homogeneity by reverse phase h.p.l.c. (conditions as above). A small sample was purified (>98%) by reverse phase h.p.l.c. for characterization. Amino acid analysis: Asp:1.0, Ser:1.82, Thr:0.91, Tyr:0.53, *m/z* (electrospray): 1266.7 ($M+Li^+$) and 1282.5 ($M+Na^+$). The ^{1}H NMR spectrum at 600MHz in DMSO was entirely consistent with the proposed structure.

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REFERENCES AND NOTES

1. This work forms part of the Ph.D. dissertation of N. J. Osborn, Southampton University, 1992. **Abbreviations : SPPS**, solid-phase peptide synthesis; **Fmoc-**, fluoren-9-yl methoxycarbonyl- ; **tBoc-**, *tert*-butoxycarbonyl-; **TFA**, trifluoroacetic acid; **Pmc**, N_Y-2,2,5,7,8-pentamethylchroman-6-sulphonyl; **NMP**, N-methylpyrrolidone; **HBTU**, *o*-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate; **DCC**, dicyclohexylcarbodiimide; **DIPCDI**, diisopropylcarbodiimide; **DMAP**, 4-N,N-dimethylaminopyridine; **NBS**, N-bromosuccinimide.
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