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Solid-Phase Synthesis of c(RGDfK) Derivatives: On-Resin Cyclisation and Lysine Functionalisation

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Abstract—The cyclic pentapeptide c(RGDfK), a selective ligand for the $\alpha_v\beta_3$ integrin, was synthesised on solid phase. All synthetic operations including the cyclisation step and the appendage of the Bolton–Hunter reagent was conducted on-resin. © 2002 Published by Elsevier Science Ltd.

Angiogenesis and metastasis are cell adhesion processes mediated by heterodimeric transmembrane glycoprotein receptors of the integrin family.[1,2] Tumour-induced angiogenesis is a consequence of ligation by extracellular matrix proteins to the $\alpha_v\beta_3$ integrin[3] which is highly expressed on many tumour cells.[4] A common binding motif in these matrix proteins is the amino acid sequence arginine-glycine-aspartic acid (RGD).[1] Blocking tumour-induced angiogenesis by inhibition of the $\alpha_v\beta_3$ integrin is now a major target for cancer chemotherapy[2,5,6] and many RGD-containing peptides[7–13] and RGD peptidomimetics[14–21] have been evaluated as antagonists of integrins. For example, Kessler and co-workers showed that the small cyclic pentapeptide c(RGDfV) is a selective inhibitor of the $\alpha_v\beta_3$ integrin and angiogenesis in tumours implanted in chick embryos.[22] In 1998, Ruoslahti and co-workers[23] reported that RGD peptides could also be used to deliver drugs to tumours selectively. A covalent conjugate between doxorubicin and the nonapeptide CDCRGDCFC significantly enhanced the survival rates of mice bearing human MDA-MB-435 breast carcinomas.

We are currently investigating $\alpha_v\beta_3$ integrin antagonists as delivery vehicles for highly cytotoxic natural product warheads. The cyclic pentapeptide c(RGDfK), first designed and synthesised by Kessler and co-workers,[9] was selected as a prototypical delivery vehicle because the lysine side-chain provides a convenient handle for attaching the warhead or reporter groups and diverse relatives are amenable to parallel synthesis. The original synthesis of c(RGDfK) employed an *o*-chlorotrityl chloride resin and an Fmoc strategy. Fully protected, linear (DfKRG) was cleaved from the resin and cyclisation performed in solution, by activation of the carboxyl terminus, using diphenylphosphorylazide under high dilution conditions. A higher yielding synthesis of c(RGDfK) was recently reported[24] that adopts a strategy similar to Kessler's, involving solid-supported peptide chain elongation, solution-phase cyclisation and simultaneous amino acid side-chain deprotections. We now describe a practical and efficient solid-phase synthesis of Kessler's c(RGDfK) peptide and its conjugation with three reporter groups via the lysine amino function. A noteworthy feature of our approach is that the lysine functionalisation and cyclisation reactions are performed on-resin thereby circumventing intermediate purification steps and solubility problems. A similar strategy was described recently by Boturyn and Dumy.[25]

NovaSyn TGT alcohol resin **1** (4-carboxytrityl alcohol group linked to an amino PEG-polystyrene resin, 0.20–0.30 mmol/g loading) was converted to its active

Abbreviations: All, allyl; Dde, (4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl; DIPEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; Fmoc, 9-fluorenylmethyoxycarbonyl; HATU, *O*-(7-Azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; TFA, trifluoroacetic acid.

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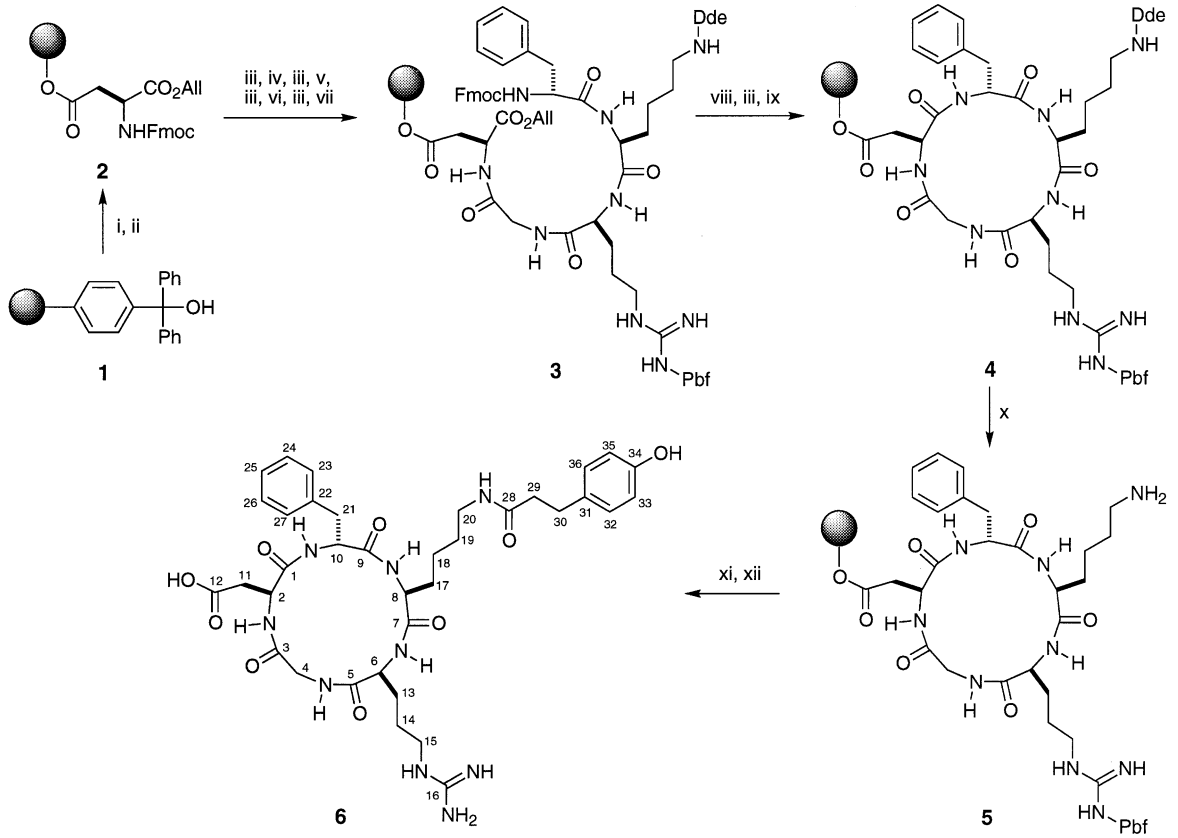

Scheme 1. Reagents and conditions: (i) AcCl (50 equiv), PhMe, 60 °C, 3 h; (ii) *N*-α-Fmoc-Asp-OAll (2.5 equiv), DIPEA (10 equiv), CH_2Cl_2, rt, 1.5 h (× 2); (iii) piperidine-DMF (1:4), rt, 4 min, (× 2); (iv) *N*-α-Fmoc-Gly-OH (2 equiv), HATU (2 equiv), DIPEA (4 equiv), rt, 1 × 30 min, then 1 × 1 h; (v) *N*-α-Fmoc-Arg(Pbf)-OH (2 equiv), HATU (2 equiv), DIPEA (4 equiv), rt, 1 × 30 min then 1 × 1 h; (vi) *N*-α-Fmoc-Lys(Dde)-OH (2 equiv), HATU (2 equiv), DIPEA (4 equiv), rt, 1 × 30 min then 1 × 1 h; (vii) *N*-α-Fmoc-Phe-OH (2 equiv), HATU (2 equiv), DIPEA (4 equiv), rt, 1 × 30 min then 1 × 1 h; (viii) $Pd(PPh_3)_4$ (3 equiv), $CHCl_3$–AcOH–*N*-methylmorpholine (37:2:1), rt, 2 h; (ix) HATU (2 equiv), DIPEA (4 equiv), DMF, rt, 16 h; (x) hydrazine monohydrate–DMF (2:98), rt, 3 min, (× 2); (xi) succinimidyl ester, DIPEA, DMF, rt, 2 h; (xii) TFA–CH_2Cl_2 (1:1), rt, 2 h.

chloride form by gently heating with a large excess of acetyl chloride in toluene for 3 h (Scheme 1). Aspartic acid was then easily attached, via its side-chain carboxyl group, by mixing the chlorinated resin with a solution of Fmoc-Asp-OAll (2.5 equiv) and DIPEA (10 equiv) in dry CH_2Cl_2. The resin was capped by washing with a solution of dry CH_2Cl_2–MeOH–DIPEA (17:2:1) to give **2**. The Fmoc protecting group was removed with a solution of piperidine–DMF (1:4) and the linear pentapeptide **3** was assembled using standard Fmoc procedures by consecutive addition of the protected amino acid (2 equiv), HATU (2 equiv) and DIPEA (4 equiv) in DMF. All Fmoc groups were removed using piperidine-DMF (1:4). After addition of the last amino acid, the C-terminal allyl ester was removed from **3** with $Pd(PPh_3)_4$ in $CHCl_3$–AcOH–*N*-methylmorpholine (37:2:1), under N_2, by occasional gentle agitation for 2 h, before washing with DIPEA-DMF (5:95) followed by diethyldithiocarbamic acid sodium salt. The N-terminal Fmoc group was then removed with piperidine–DMF (1:4) before addition of HATU (2 equiv) and DIPEA (2 equiv) to effect on-resin cyclisation. Removal of the (4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) protecting group[26] from the amino side-chain of lysine in **4** was easily achieved by hydrazine monohydrate–DMF (2:98) for 3 min.

The *N*-acylation of the nascent amino function in **5**, deprotection of the pentamethyl-dihydrobenzofuran-5-sulfonyl (Pfb) group protecting the guanidine function[27] and cleavage of the peptide from the resin is illustrated by the synthesis of **6**: DMF (8 mL) was added to peptide resin **5** (76 μmol, 1 equiv) followed by the succinimidyl ester of 3-(*p*-hydroxyphenyl)propanoic acid (the Bolton–Hunter reagent, 263 mg, 1 mmol, 13 equiv) and DIPEA (0.20 mL, 1.14 mmol, 15 equiv). The resulting mixture was agitated for 2 h before exhaustive washing with DMF followed by CH_2Cl_2. TFA–CH_2Cl_2 (1:1, 8 mL) was then added and the resulting mixture agitated for 2 h before washing with TFA–CH_2Cl_2 (1:9). The acid washings were concentrated to approximately 1 mL and Et_2O was added until a white precipitate separated. The precipitate, which was isolated and dried in a stream of N_2, was purified by RP-HPLC [Hycrom RPB C18RP, 100 × 21.2 mm id, 20% MeCN-H_2O to 60% MeCN-H_2O (containing 0.1% TFA), 20 mL/min, 20 min run time] and lyophilised to give peptide **6** as a white, fluffy solid (26 mg, 35 μmol, 46% based on estimated loading of peptide-resin **2**: δ_H (500 MHz, D_2O) 7.33–7.27 (2H, m, C24H and C26H), 7.24–7.21 (1H, m, C25H), 7.18 (2H, d, $J = 7.0$ Hz, C23H and C27H), 7.07 (2H, d, $J = 8.5$ Hz, C32H and C36H), 6.79 (2H, d, $J = 8.5$ Hz, C33H and C35H), 4.57 (1H, dd, $J = 9.6$, 6.7 Hz, C10H),

4.56–4.52 (1H, m, C2H), 4.38 (1H, dd, $J=9.4$, 5.5 Hz, C6H), 4.22 (1H, d, $J=16.4$ Hz, C4H_AH_B), 3.72–3.70 (1H, m, C8H), 3.57 (1H, d, $J=16.4$ Hz, C4H_AH_B), 3.11 (2H, dt, $J=6.8$, 3.4 Hz, C15H_2), 3.04 (1H, dd, $J=13.2$, 6.7 Hz, C21H_AH_B), 2.96–2.86 (3H, m, C21H_AH_B and C20H_2), 2.80 (2H, t, $J=7.1$ Hz, C30H_2), 2.77–2.75 (2H, m, C11H_2), 2.46 (2H, t, $J=7.1$ Hz, C28H_2), 1.97–1.90 (1H, m, C13H_AH_B), 1.69–1.62 (1H, m, C13H_AH_B), 1.51–1.41 (3H, m, C14H_2 and C17H_AH_B), 1.36–1.29 (1H, m, C17H_AH_B), 1.11 (2H, quin, $J=7.3$ Hz, C19H_2), 0.70–0.60 (2H, m, C18H_2); δ_C (125 MHz, D_2O) 177.1 (C28), 176.6 (C7), 176.1 (C9), 175.1 (C12), 174.6 (C5), 173.2 (C1), 172.8 (C3), 158.3 (C16), 155.5 (C34), 137.4 (C22), 133.9 (C31), 131.4 (C32H and C36H), 130.7 (C23H and C27H), 130.5 (C24H and C26H), 129.0 (C25H), 116.9 (C33H and C35H), 57.1 (C3H), 56.8 (C10H), 54.3 (C6H), 51.9 (C2H), 44.5 (C4H_2), 42.0 (C15H_2), 40.3 (C20H_2), 39.2 (C21H_2), 38.9 (C29H_2), 37.6 (C11H_2), 32.2 (C30H_2), 31.7 (C17H_2), 29.1 (C19H_2), 28.8 (C13H_2), 26.1 (C14H_2), 23.7 (C18H_2); HRMS (ES^+): m/z calcd for $C_{36}H_{49}N_9O_9Na$ $(M+Na)^+$: 774.3551. Found 774.3524. Analytical HPLC revealed a purity of >98% @ 230 nm, $t_R=3.81$ min, Hycrom RPB C18RP, 100 × 4.6 mm id, 5% MeCN–H_2O to 95% MeCN–H_2O (containing 0.1% TFA), 2 ml/min, 10 min run time.

In conclusion we have devised a simple, practical and efficient solid-phase synthesis of the Kessler c(RGDfK) $\alpha_v\beta_3$ integrin antagonist that allows cyclisation and lysine functionalisation on resin. The procedure should be amenable to the synthesis of a wide range of analogous peptides including drug conjugates.

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