Pergamon

Tetrahedron Letters 43 (2002) 5021–5024

TETRAHEDRON LETTERS

A general method for functionalising both the C- and N-terminals of Tyr^3-octreotate

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Accepted 29 May 2002

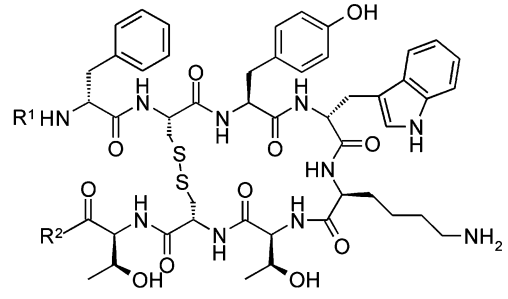
Abstract—A new method has been developed for solid-phase peptide synthesis (SPPS) via the Fmoc strategy that allows functionalisation of both the C- and N-terminals of Tyr^3-octreotate. *N*-α-Fluorenylmethyloxycarbonyl-L-threonine allyl ester, the starting amino acid, was loaded onto the 3,4-dihydro-2*H*-pyran-2-ylmethoxymethyl polystyrene (DHP) resin via its side chain alcohol group and the cyclic Tyr^3-octreotate was synthesised. The C- and N-terminals were functionalised with DOTA (1,4,7,10-tetraazacyclodecane-*N′*,*N″*,*N‴*,*N⁗*-tetraacetic acid) and HYNIC (6-hydrazinonicotinic acid).

The cyclic tetradecapeptide somatostatin is a potent inhibitor in the secretion of growth hormones.[1] In addition to this a large number of carcinoid tumours have been shown to express receptors for somatostatin.[2] However, the clinical use of this peptide is severely limited due to an in vivo half-life of 2–3 min.[3] Consequently, smaller cyclic peptides have been discovered which incorporate D-amino acids to prolong the in vivo half-life by inhibiting amino- and carboxypeptidases. One of these discoveries octreotide, a cyclic eight-membered peptide with a disulphide bridge, has been shown to have an in vivo half-life in humans of 60–90 min[4] and to be 2000 more times effective in inhibiting growth hormone secretions.[5]

Peptides containing chelating substituents for various radiolabels can be used for the diagnosis and therapy of somatostatin receptor positive tumours. 1,4,7,10-Tetraazacyclodecane-*N′*,*N″*,*N‴*,*N⁗*-tetraacetic acid (DOTA)[6] and 6-hydrazinonicotinic acid (HYNIC)[7] are appropriate chelators for this purpose (Fig. 1).

Recently, the potential of DOTATOC (1,4,7,10-tetraazacyclodecane-*N′*,*N″*,*N‴*,*N⁗*-tetraacetic acid-D-Phe^1-Tyr^3-octreotide) labelled with either ^{68}Ga for positron emission tomography (PET)[8] or ^{90}Y for radionuclide therapy[9] and HYNIC-D-Phe^1-Tyr^3-octreotide labelled with ^{99m}Tc for single photon emission tomography (SPET)[10] have proven to be effective tools for nuclear medicine.

For incorporation of these chelators into the SSTR–affine peptides, the suitably protected derivatives DOTA-tris *tert*-butyl ester[11] and 6-(2′-*tert*-butoxycarbonylhydrazino)nicotinic acid (Boc-HYNIC)[7] were used.



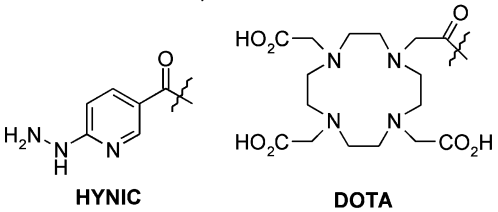


Figure 1. Chemical structures of the conjugates synthesised.

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0040-4039/02/$ - see front matter
PII: S0040-4039(02)01021-3

The notable similarity in the traditional SPPS of derivatives of octreotide is that they are all functionalised at the N-terminal. The main reason for this is that the C-terminal amino acid, threonine, is usually loaded onto the resin via its carboxylic acid moiety, thus, blocking this terminal for manipulation whilst still being attached to the resin. In this approach the first amino acid, threonine, was loaded onto a resin using it's secondary alcohol side chain moiety. Then the Tyr^3-octreotate was synthesised using standard Fmoc peptide chemistry. For future functionalisation the carboxylic acid group of threonine needed to be suitably protected. The protecting group chosen was the allyl group, as this would not be cleaved during the octreotate synthesis and could be removed easily without affecting the cyclic peptide.

The *N*-α-fluorenylmethyloxycarbonyl-L-threonine allyl ester (Fmoc-Thr-OAllyl) **6** was synthesised from Fmoc-Thr-OH **5** with caesium carbonate and allyl bromide according to literature procedures.[12] The Fmoc-Thr-OAllyl **6** was loaded onto the 3,4-dihydro-2*H*-pyran-2-ylmethoxymethyl polystyrene resin (Ellman's dihydropyran resin) in the presence of pyridinium *p*-toluenesulfonate (PPTS) in 1,2-dichloroethane (DCE) at 60°C for 16–20 h to give **7** (Scheme 1).[13]

Fmoc quantification of the amino acid bound to the solid support was found to be consistently around 0.60–0.66 mmol g^{-1}. The linear octapeptides R^1-D-Phe-Cys(Acm)-Tyr(*t*Bu)-D-Trp(Boc)-Lys(Boc)-Thr(tBu)-Cys-(Acm)-Thr(THP resin)-*O*-Allyl **8** (R^1 = Boc) and **9** (R^1 = Fmoc) were synthesised stepwise using an Fmoc protocol, i.e. 4 molar equiv. of the amino acid, 3.8 equiv. of 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 10 equiv. of diisopropylethylamine (DIPEA) in DMF at 25°C. The formation of the disulphide bridge between the Cys^2 and Cys^7 amino acids giving the cyclic peptides **8** and **9** was achieved with 4 equiv. of thallium(III) trifluoroacetate in DMF at 25°C (Scheme 2).

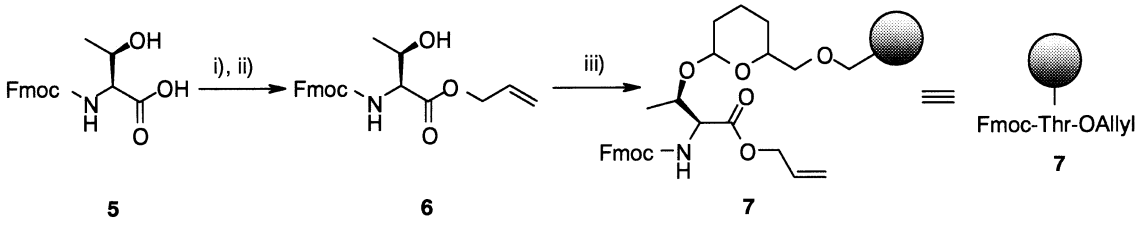
The final amino acid, D-phenylalanine, used in the synthesis was either Boc protected (Boc-D-Phe-OH) or Fmoc protected (Fmoc-D-Phe-OH). The Boc protection was considered to be more suitable than the Fmoc group for investigating the conditions to functionalise the C-terminal carboxylic acid. The octreotate derivatives **8** and **9** could now be selectively functionalised at the C- or N-terminal.

The first step in functionalising the C-terminal of the octreotate was the removal of the allyl ester protecting group. A number of methods were studied and the best method was found to be with Pd(PPh$_3$)$_4$ (0.1 equiv.) and phenylsilane (24 equiv.) in dichloromethane.[14]

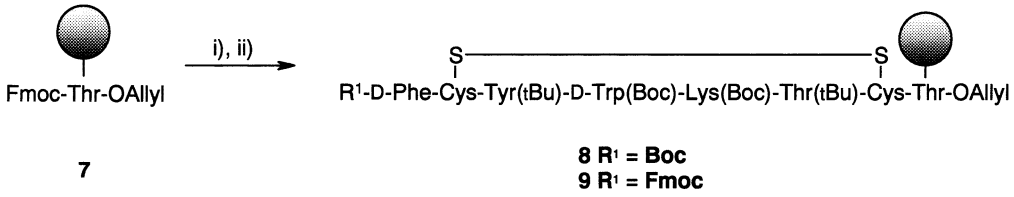
The conditions for coupling at the C-terminal carboxylic acid were investigated with numerous coupling reagents (PyBOP, HATU, HBTU, DIC, etc.) in various solvents (DMF, NMP, DMSO, etc.) and at varying temperatures (25–60°C). The best conditions, using 3-phenylpropylamine as the test amine, were to activate the carboxylic acid with 10 equiv. of 7-hydroxyazabenzotriazole (HOAt) and diisopropylcarboimide (DIC) in DMSO/DMF (1:3) at 60°C, then to add 10 equiv. of the amine.

The linker chosen to link the Tyr^3-octreotate to DOTA and HYNIC was 1,2-diaminoethane as this would give a free amino group for standard solid-phase couplings. The *N*^{2}-Fmoc-1,2-diaminoethane[15] linker was coupled to the C-terminal carboxylic acid using the conditions described above and the Fmoc group was removed with piperidine in DMF to give **10**. The coupling reactions were carried out with Boc-HYNIC and DOTA-tris *tert*-butyl ester and gave the desired octreotate derivatives. Treatment of these resin bound octreotate derivatives with TFA:water:triisopropylsilane (95:2.5:2.5) afforded the unprotected octreotate derivatives **1** and **2** (Scheme 3, Table 1).[16,17]

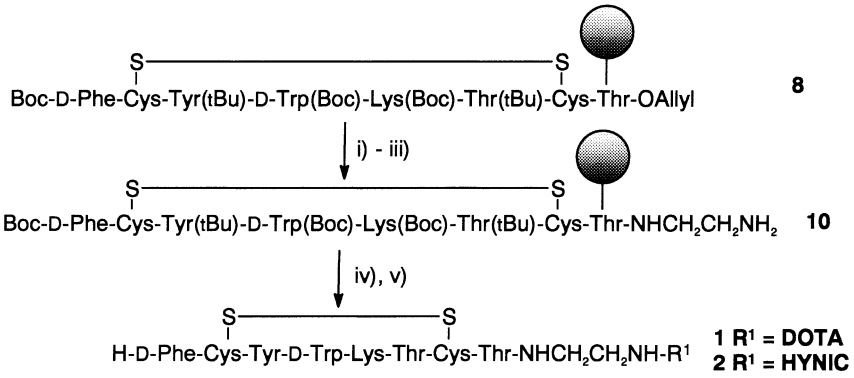
To show that this method was versatile, the functionalisation of the N-terminal was carried out with DOTA



Scheme 1. *Reagents and conditions*: (i) Cs_2CO_3, MeOH, H_2O, 25°C; (ii) allyl bromide, DMF, 25°C; (iii) DHP resin, PPTS, DCE, 60°C.



Scheme 2. *Reagents and conditions*: (i) conditions for the standard peptide synthesis: amino acid, HBTU, DIPEA, DMF; DMF washes; then piperidine, DMF; DMF washes; (ii) Tl(TFA)$_3$ in DMF, 25°C.



Scheme 3. *Reagents and conditions*: (i) $Pd(PPh_3)_4$, $PhSiH_3$, DCM, 25°C; (ii) Fmoc-$NHCH_2CH_2NH_2$·HCl, DIC, HOAt, NMM or DIPEA, DMSO, DMF, 60°C; (iii) piperidine, DMF, 25°C; (iv) DOTA-tris *tert*-butyl ester or Boc-HYNIC, HBTU, DIPEA, DMF, 25°C; (v) TFA:triisopropylsilane:H_2O, 95:2.5:2.5, 25°C.

Table 1.

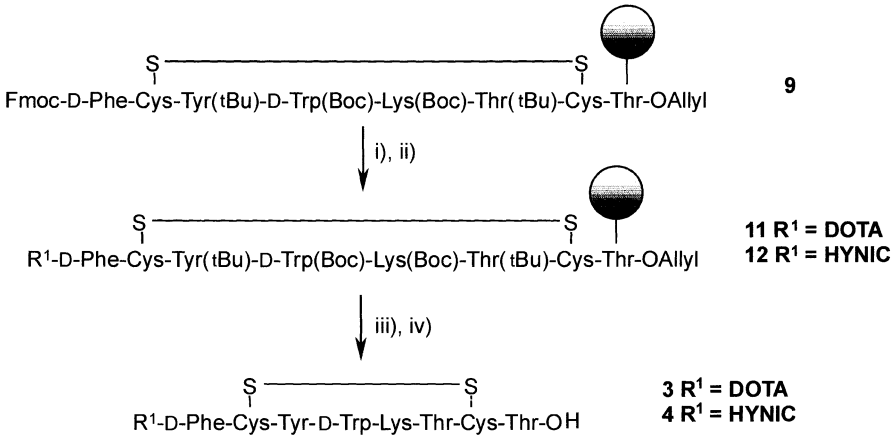
Compound	Retention time (min)[a]	Calculated $[M+H]^+$	Observed[b] $[M+H]^+$
1	13.3	1478.7	1478.3
2	13.1	1227.4	1226.7
3	14.2	1436.6	1436.2
4	14.6	1185.3	1184.8

[a] The HPLC column and conditions are described in Ref. 16.
[b] Electrospray ionisation mass spectrometry on a Finnigan MAT TSQ 7000 (Thermo Finnigan, San Jose, USA).

and HYNIC. The Fmoc protecting group was removed from **9** and the free amine was coupled to DOTA-tris *tert*-butyl ester to give **11** and to Boc-HYNIC to give **12**. The allyl group was removed under the same conditions described above with $Pd(PPh_3)_4$. Treatment of the resin bound octreotate derivatives with TFA:water:triisopropylsilane (95:2.5:2.5) afforded the unprotected octreotate derivatives **1** and **2** (Scheme 4).[16,17]

The same octreotate synthesis was carried out with the 4-(*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino)benzyl (Dmab) protecting group for carboxylic acids. The cyclic octreotate was formed and the Dmab group was successfully removed with 2% hydrazine hydrate in DMF to give the free carboxylic acid at the C-terminal. The coupling of amines to this carboxylic acid with numerous reagents and under varying conditions, had limited success with low conversions. This may be due to the retention of hydrazine with the resin-bound octreotate. Treatment of this resin with either 10% solutions of acetic acid or imidazole in DMF to eliminate the hydrazine gave slightly improved conversions.

In conclusion, the loading of threonine to the DHP resin via its side chain alcohol group allows the Tyr^3-octreotate to be functionalised at both the C- and N-terminal, thus, introducing a new scope to the range of octreotate derivatives that can be synthesised using



Scheme 4. *Reagents and conditions*: (i) piperidine, DMF, 25°C; (ii) DOTA-tris *tert*-butyl ester or Boc-HYNIC, HBTU, DIPEA, DMF, 25°C; (iii) $Pd(PPh_3)_4$, $PhSiH_3$, DCM, 25°C; (iv) TFA:triisopropylsilane:H_2O, 95:2.5:2.5, 25°C.

SPPS. The new C-terminal functionalised derivatives of octreotate with DOTA and HYNIC will be tested for their affinity to somatostatin receptors and the results will be reported in due course.

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16. Preparative reversed-phase HPLC (RP-HPLC) experiments were performed on a Gyncotech P580 instrument equipped with a Shimadzu SPD 6-A multi-wavelength detector. Retention times are given for gradient elution using the following conditions: column LiChrosorb RP-select B, 250×10 mm (10 μm); eluent A, 0.1% (v/v) TFA in H_2O; eluent B, 0.1% (v/v) TFA in acetonitrile; gradient, 5% B to 95% B over 30 min, flow rate 4.0 mL min^{-1}; absorbance, 254 nm. Molecular weight determinations were carried out by electrospray mass spectrometry on a Finnigan MAT TSQ 7000.
17. The octreotate derivatives **1**, **2**, **3** and **4** were white solids after lyophilisation. The overall yields of the purified octreotate derivatives for **1**, **2**, **3** and **4** were 18, 27, 38 and 35%, respectively.