Potential mechanism-based tyrosine kinase inhibitors. Part 2.[1] Design and synthesis of peptides containing heterocyclic tyrosine analogues

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The Fmoc derivatives of two homochiral tyrosine analogues, a pyridine *N*-oxide and a pyridone, have been prepared in high stereochemical purity. Solid-phase synthesis has been used to prepare a decapeptide substrate for the tyrosine kinase domain of epidermal growth factor. Two decapeptides, which incorporate the tyrosine analogues in place of tyrosine, and thereby have the potential to act as mechanism-based inhibitors of epidermal growth factor tyrosine kinase, have been synthesised and found to inhibit the aforementioned kinase.

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein which catalyses the phosphorylation of tyrosine residues of its carboxy terminal domain (autophosphorylation) and of protein(s) within the cell in response to extracellular signals.[2] This tyrosine kinase activity is one of the primary signals which culminates in cell division, and aberrant levels of activity can result in unregulated cell proliferation associated with cancer.[3] A range of reversible inhibitors of these enzymes have been described.[3] We report the design and synthesis of two potential mechanism-based irreversible inhibitors[4] which might provide a starting point for the rational design of anti-tumour agents. This work has involved the preparation of two homochiral tyrosine analogues; the preparation and testing of a synthetic substrate, **11**, for the enzyme; and synthesis of related peptides **12** and **13** which incorporate these tyrosine analogues. These peptides have been evaluated as tyrosine kinase inhibitors.[5]

Appropriately substituted heterocyclic isosteres of tyrosine, when incorporated into molecules which closely mimic the normal enzyme substrate, might be phosphorylated in an analogous fashion to tyrosine. If the resulting phosphorylated heterocycle is activated with respect to nucleophilic substitution, covalent attachment to an active-site nucleophile might lead to irreversible enzyme inhibition.[6] Phosphorylation is a known chemical[7] and biological[8] mechanism for the activation of pyridones and analogues with respect to nucleophilic substitution. On this basis a suitable peptide containing the amino acid **4** can be viewed as a potential inhibitor of tyrosine kinases.‡ We have also identified a pyridine *N*-oxide-containing amino acid **6** as a tyrosine analogue which might act in this fashion.[1] Fig. 1 illustrates a possible mode of mechanism-based enzyme inhibition for peptides derived from these compounds. Phosphorylation of either pyridine *N*-oxide or pyridone derivatives will generate an electrophilic aromatic ring; active site nucleophiles would be expected to intercept this species. In each case nucleophilic attack may be followed by loss of phosphate leading to irreversible enzyme inhibition.

Results and discussion

The fluorenylmethoxycarbonyl, Fmoc, derivative **5** of pyridone **4** was prepared from the fluoropyridyl malonate derivative **1**[10] (see Scheme 1). The synthesis of the (*S*)-amino acid was closely related to that reported for the racemic compound.[10] Base-catalysed hydrolysis and decarboxylation of **1** gave the racemic *N*-acetyl amino acid **2**. Resolution to generate the free (*S*)-amino acid **3** was accomplished by incubation with hog kidney acylase and ion-exchange chromatographic purification. Acid-catalysed hydrolysis of the fluoropyridine moiety gave the pyridone **4** which was protected as its Fmoc derivative **5** on treatment with 9-fluorenylmethylsuccinimido carbonate (Fmoc–O–Su).[11]

We examined several possible routes to (*S*)-β-(1-oxido-4-pyridyl)alanine, **6**. One approach, analogous to the one used in the successful preparation of the (*S*)-amino acid **4**, was unsuccessful as the corresponding *N*-acetylamino acid **7** was found to be a poor substrate for hog kidney acylase. In view of these difficulties we elected to follow a literature resolution of the racemic precursor, β-(4-pyridyl)alanine,[12] and effect oxidation to the *N*-oxide subsequently (Scheme 2). Resolution of β-(4-pyridyl)alanine, *via* the diastereoisomeric hydrogen tartrate salts,[13] gave (*S*)-amino acid **8** which was protected as its Fmoc derivative **9** in a similar fashion to the preparation of **5**. Oxidation to the corresponding *N*-oxide **10** was best accomplished with *m*-chloroperbenzoic acid.

In order to determine the stereochemical purity of the Fmoc protected amino acids **5** and **10** they were deprotected to the free amino acids **4** and **6** and then subjected to chiral HPLC. They were both judged to be greater than 96% stereochemically pure.

We next sought an oligopeptide sequence which would bind effectively at the active-site of EGFR tyrosine kinase and which was amenable to efficient solid-phase synthesis. This peptide would provide a carrier for our tyrosine analogues. On the basis of literature precedent[14] we designed and synthesised the peptide **11** as a substrate for EGFR tyrosine kinase. For our enzyme studies we used a genetically truncated, soluble form of EGFR comprising the intracellular kinase domain. This protein retains the kinase activity of the original receptor but is more amenable to detailed kinetic study. Peptide **11** was found to be phosphorylated efficiently by this preparation. The Michaelis constant, K_m, was found to be 70 μmol dm^{-3}, similar to an earlier

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‡ L-3-Deoxymimosine, another heterocyclic amide, has recently been identified as a possible component of a tyrosine kinase inhibitor in an analogous fashion to that described here.[9]

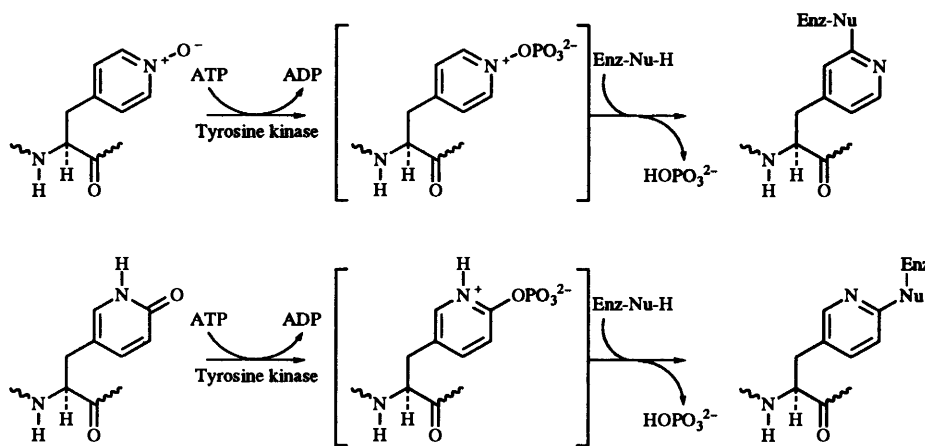
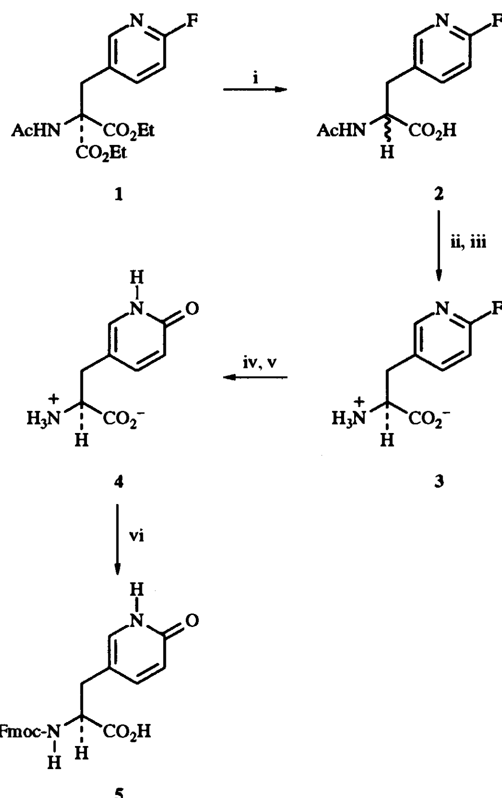
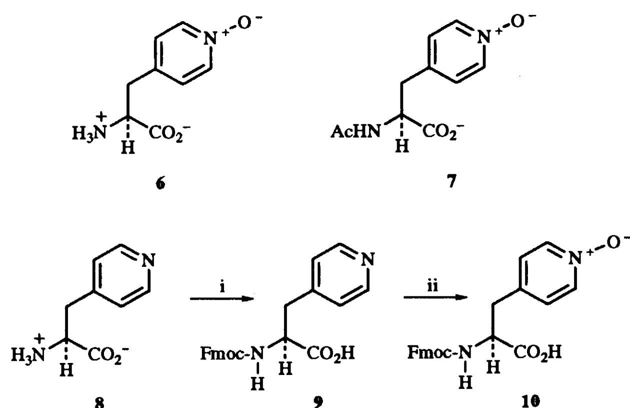


Fig. 1

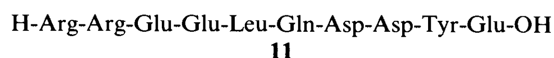


Scheme 1 *Reagents and conditions:* i, NaOH, heat; ii, hog kidney acylase; iii, Dowex; iv, HCl; v, Amberlite; vi, Fmoc–O–Su, Na_2Ca_3



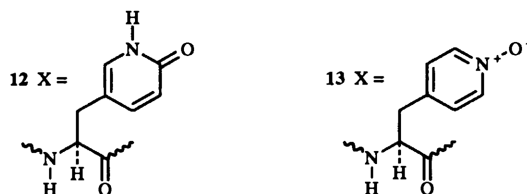
Scheme 2 *Reagents:* i, Fmoc–O–Su, Na_2CO_3; ii, 3-$ClC_6H_4CO_3H$

estimate for a dodecapeptide analogue[14] phosphorylated by EGFR, suggesting high active-site affinity for this peptide sequence. Hence **11** was judged to be a suitable carrier for the heterocyclic tyrosine analogues **4** and **6**.



H-Arg-Arg-Glu-Glu-Leu-Gln-Asp-Asp-Tyr-Glu-OH
11

Peptides **12** and **13**, containing heterocyclic tyrosine analogues **4** and **6**, respectively became our target inhibitors. These were synthesised in an analogous fashion to **11**, utilising the Fmoc-protected amino acids **5** and **10**. The *N*-oxide and pyridone functional groups were unaffected by the conditions of the synthesis.



Peptides **12** and **13**, obtained in pure form by the above procedure, were then tested as inhibitors of the aforementioned kinase. Peptide phosphorylation activity was measured using, as substrates, peptides **11**; H-Arg-Arg-Lys-Gly-Ser-Thr-Ala-Glu-Asn-Ala-Glu-Tyr-Leu-Arg-Val-OH (**14**, corresponding to the major autophosphorylation site of the receptor); and H-Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly-OH (**15**, corresponding to a pp60^{src} kinase phosphorylation site). We also tested **12** and **13** for substrate activity, and found that they were not phosphorylated significantly.

Peptide analogues **12** and **13** had similar effects on the phosphorylation of added peptide substrates (Table 1). At 1.4 mmol dm^{-3} (a concentration comparable to that of the peptide substrates) these effects were slight, but 40–50% inhibition was obtained at approximately 7 mmol dm^{-3} inhibitor. The inhibitory effect appeared to be more pronounced as substrate concentrations were increased, suggesting that the type of interaction between peptides and kinase might not be purely competitive. The effects of **12** and **13** on the rate of autophosphorylation were similar to those observed in the phosphorylation of synthetic peptide substrates, with inhibition increasing to approximately 50% at inhibitor concentrations of 5–10 mmol dm^{-3}.

We investigated the possibility that the observed inhibition of enzyme activity in the presence of **12** and **13** might be due to

Table 1 Inhibition of EGF receptor peptide tyrosine kinase activity[a]

	% Peptide phosphotransferase activity					
	Substrate **11**		Substrate **14**		Substrate **15**	
	0.15 mmol dm^{-3}	0.73 mmol dm^{-3}	0.14 mmol dm^{-3}	0.7 mmol dm^{-3}	0.28 mmol dm^{-3}	1.4 mmol dm^{-3}
12						
1.4 mmol dm^{-3}	112.5 ± 5.3	89.8 ± 1.3	100.3 ± 7.8	98.9 ± 5.3	100.7 ± 1.2	76.4 ± 4.5
7.0 mmol dm^{-3}	50.7 ± 6.0	44.2 ± 1.0	69.4 ± 4.8	68.2 ± 5.5	54.9 ± 2.3	50.4 ± 4.4
13						
1.5 mmol dm^{-3}	116.7 ± 23	81.3 ± 0.6	87.1 ± 0.5	97.2 ± 10	104.3 ± 8.3	104.9 ± 7.0
7.5 mmol dm^{-3}	59.3 ± 5.5	39.6 ± 5.5	66.9 ± 5.2	66.9 ± 7.1	54.0 ± 3.9	49.4 ± 1.6

[a] Peptide phosphorylation in the presence of peptide analogues is expressed as percentage of enzyme activity (in pmol phosphate transferred to peptide substrate min^{-1} $pmol^{-1}$ kinase in the presence of the analogue compared to its absence [taken to represent 100% activity]). Mean values averaged from two experiments are shown, together with the differences from the means of the two determinations.

chelation of Mn^{2+} (an activating metal ion, used at 10 mmol dm^{-3} in the assay system) by the multiple carboxylate groups in these peptides. Similar patterns of inhibition were observed at 2, 10 and 20 mmol dm^{-3} Mn^{2+}, indicating that the mechanism of inhibition did not involve depletion of Mn^{2+}.

In conclusion the decapeptides, **12** and **13**, containing the synthetic tyrosine analogues were found to inhibit both the peptide and autophosphorylation activities of EGFR tyrosine kinase. We have not investigated the type of inhibition. Relatively high concentrations of the peptide analogues were required, which suggests that the replacement of the tyrosine side chain by the pyridone and *N*-oxide moieties greatly reduced the affinity of the kinase for these peptides.

Experimental

Mps were determined on a Gallenkamp hot-stage apparatus, and are uncorrected. IR spectra were recorded on a Perkin-Elmer 1750 Fourier transform instrument. ^{1}H NMR spectra were recorded on a Gemini 200 (at 200 MHz), or on a Brüker AM500 (at 500 MHz). ^{13}C NMR spectra were recorded on a Gemini 200 (at 50 MHz), on a Brüker AM250 (at 63 MHz), or on a Brüker AM500 (at 125 MHz). ^{19}F NMR spectra were recorded on a Brüker AM250 at 235 MHz. *J* Values in Hz. Mass spectra were generated by fast atom bombardment (FAB^+) on a Finnegan Mat 8400, or by chemical ionisation, CI^+, recorded on a VG Autospec or VG Micromass ZAB IF by Dr R. Aplin and R. Procter. Amino acid analyses were determined using a Biotronik LC5001 or LKB-4400 amino acid analyser. Optical rotations were recorded at 20 °C and are quoted in units of 10^{-1} deg cm^{-2} g^{-1}. All solvents were purified by conventional methods and moisture sensitive reactions were carried out under an anhydrous inert atmosphere. Capilliary zone electrophoresis (CZE) was carried out at 30 °C on an ABI 270A eluting through a 50 µm × 72 cm capilliary assembly at 20 kV with detection at 210 nm.

(*R,S*)-*N*-Acetyl-β-(2-fluoro-5-pyridyl)alanine 2

A solution of ethyl *N*-acetyl-2-(2-fluoro-5-pyridylmethyl)-malonate **1**[9] (3.038 g, 9.319 mmol) and sodium hydroxide (764 mg, 19.1 mmol) in distilled water (65 cm^3) and absolute ethanol (65 cm^3) was heated under reflux for 5 h. The mixture was then cooled and washed with ethyl acetate (2 × 100 cm^3). After acidification to pH 2 with HCl (1 mol dm^{-3}), the aq. layer was saturated with sodium chloride and extracted with ethyl acetate (4 × 200 cm^3). Evaporation of the combined extracts afforded the title compound **2** as a viscous oil (2.00 g, 95%); δ_H(D_2O, 200 MHz) 1.68 [3 H, s, C(O)Me], 2.73 (1 H, dd, J_{ABX} 9, 14, CH_2), 2.96 (1 H, dd, J_{ABX} 6, 14, CH_2), 4.38 (1 H, dd, J_{ABX} 6, 9, CH), 6.75 (1 H, dd, *J* 2, 9, Py–H), 7.56 (1 H, dt, *J* 2, 8, Py–H) and 7.72 (1 H, d, *J* 2, Py–H); δ_C(D_2O, 63 MHz) 22.4, 33.8, 54.3, 110.5 (d, J_F 36), 131.4, 144.2 (d, J_F 8), 147.6 (d, J_F 12), 165.2, 174.5 and 175.0; δ_F(D_2O) −75.8; *m/z* [CI^+(NH_3)] 227 (MH^+, 100%) and 208 (MH^+ − F, 20%).

(*S*)-β-(2-Fluoro-5-pyridyl)alanine 3

(*R,S*)-*N*-Acetyl-β-(2-fluoro-5-pyridyl)alanine **2** (2.00 g, 8.85 mmol) was dissolved in water (25 cm^3) and the solution brought to pH 7.5 by the addition of aq. ammonia (1 mol dm^{-3}). Hog kidney acylase (5 mg) was added and the flask incubated in a shaker at 80 rpm and 35 °C. The reaction was monitored using ^{1}H NMR (after removing the solvent). After 24 h, the aq. solution was acidified to pH 2, washed with ethyl acetate (15 cm^3) and loaded onto a freshly prepared Dowex [50*8(H)] column. After eluting the column with water (500 cm^3), the column was washed with ammonia (1 mol dm^{-3}). The basic eluent was reduced in volume on a rotary evaporator, taken up in water (50 cm^3) and then freeze dried to give a white solid. This was dissolved in the smallest volume of water to which acetone was added until crystallisation commenced. The title compound **3** was afforded as white crystals (200 mg, 89%), mp 240–242 °C (decomp.); $[\alpha]_D^{20}$ −9.45 (*c* 0.22, H_2O); δ_H(D_2O, 200 MHz) 3.20 (1 H, dd, J_{ABX} 6, 15, CH_2), 3.26 (1 H, dd, J_{ABX} 6, 15, CH_2), 3.97 (1 H, t, *J* 6, CH), 7.10 (1 H, dd, *J* 2, 8, Py–H), 7.88 (1 H, dt, *J* 2, 8, Py–H), 8.06 (1 H, d, *J* 2, Py–H); δ_C(D_2O, 63 MHz) 33.9, 56.3, 110.9 (d, J_F 36), 144.2 (d, J_F 8), 147.9 (d, J_F 11), 155.3, 165.5 and 173.9; δ_F(D_2O) −74.7; *m/z* [CI^+(NH_3)] 185 (MH^+, 100%) (Found: M, 185.0725. Calc. for $C_8H_{10}FN_2O_2$: *M*, 185.0726).

(*S*)-β-(2-Oxo-1,2-dihydro-5-pyridyl)alanine 4

A solution of (*S*)-β-(2-fluoro-5-pyridyl)alanine **3** (350 mg, 1.902 mmol) in hydrochloric acid (3 mol dm^{-3}; 30 cm^3) was heated under reflux for 8 h and then reduced in volume to 20 cm^3. The residue was taken up in water (200 cm^3) and the solution reduced in volume to 15 cm^3. The hot solution (80 °C) was passed through a hot (80 °C) column of Amberlite IRA-93. The filtrate was evaporated under reduced pressure to give a white solid which was dissolved in the smallest volume of water to which acetone was added until crystallisation commenced. The title compound **4** was afforded as white crystals (280 mg, 81%); mp 219 °C (decomp.); $[\alpha]_D^{20}$ −17.0 (*c* 0.20, H_2O); δ_H(D_2O, 500 MHz) 2.97 (1 H, dd, J_{ABX} 7, 15, CH_2), 3.03 (1 H, dd, J_{ABX} 6, 15, CH_2), 3.88 (1 H, dd, J_{ABX} 6, 7, CH), 6.61 (1 H, d, *J* 9, Py–H), 7.41 (1 H, d, *J* 3, Py–H) and 7.62 (1 H, dd, *J* 3, 9, Py–H); δ_C(D_2O, 50 MHz) 33.0, 55.8, 116.8, 120.0, 135.2, 145.7, 164.8 and 174.2; ν_{max}(KBr)/cm^{-1} 3436 and 1652; *m/z* [CI^+(NH_3)] 183 (MH^+, 45%) and 139 (MH^+ − CO_2, 100%) (Found: M, 183.0773. Calc. for $C_8H_{10}N_2O_3$: *M*, 183.0770).

(*S*)-*N*-(Fluoren-9-ylmethoxycarbonyl)-β-(2-oxo-1,2-dihydro-5-pyridyl)alanine 5
(*S*)-β-(2-Oxo-1,2-dihydro-5-pyridyl)alanine **4** (240 mg, 1.20 mmol) was dissolved in water (14 cm^3) containing sodium carbonate (254 mg, 2.40 mmol) and the solution cooled to 0 °C. A solution of *N*-(fluoren-9-ylmethoxycarbonyl)-*N*-hydroxy succinimide (337 mg, 1.00 mmol) in DMF (9 cm^3) at 5 °C was added to it in one portion with vigorous stirring and mixing continued for 10 min at room temp. The mixture was diluted with water (220 cm^3), and extracted with diethyl ether (80 cm^3) and ethyl acetate (2 × 100 cm^3). The aqueous phase was cooled and acidified to pH 2 with hydrochloric acid (1 mol dm^{-3}) and the precipitated product and aqueous phase were extracted with ethyl acetate (3 × 200 cm^3). Evaporation of the combined extracts after washing with ice-cold water, afforded the title compound **5** as a white solid (318 mg, 79%); mp 187–190 °C (decomp.); $[\alpha]_D^{20}$ −19.5 (*c* 0.2, DMF); δ_H([$^2H_6$]DMSO, 500 MHz), 2.60 (1 H, dd, J_{ABX} 11, 14, $PyCH_2$), 2.82 (1 H, dd, J_{ABX} 5, 14, $PyCH_2$), 4.01 (1 H, m, OCH_2CH), 4.24 (3 H, m, OCH_2 and NCHCO), 6.25 (1 H, d, *J* 9, Py–H), 7.23 (1 H, d, *J* 2, Py–H), 7.45 (5 H, m, 4 Fmoc–H and Py–H), 7.66 [3 H, m, 2 Fmoc–H and NH (D_2O ex.)] and 7.88 (2 H, d, *J* 8, Fmoc–H); δ_C([$^2H_6$]DMSO, 125 MHz) 32.4, 46.8, 55.1, 65.9, 114.3, 119.7, 120.3, 125.4, 127.3, 127.9, 134.5, 140.9, 142.9, 143.9, 156.2, 162.0 and 173.4; ν_{max}(KBr)/cm^{-1} 3436, 3315, 1694 and 1659; *m*/*z* [$CI^+(NH_3)$] 405 (MH^+, 100%) (Found: M, 405.1448. Calc. for $C_{23}H_{21}N_2O_5$: *M*, 405.1450).

(*S*)-*N*-(9-Fluorenylmethoxycarbonyl)-β-(4-pyridyl)alanine 9
(*S*)-β-(4-Pyridyl)alanine **8**[12] (280 mg, 1.69 mmol) was suspended in water (14 cm^3) containing sodium carbonate (358 mg, 3.37 mmol) and the suspension cooled to 0 °C. A solution of *N*-(9-fluorenylmethoxycarbonyl)*N*-hydroxy-succinimide (475 mg, 1.41 mmol) in DMF (8 cm^3) at 5 °C was added to it in one portion with vigorous stirring and mixing continued for 15 min at room temp. The mixture was then diluted with water (280 cm^3) and extracted with diethyl ether (80 cm^3) and ethyl acetate (2 × 100 cm^3). The aq. phase was cooled and acidified to pH 2 with HCl (10 mol dm^{-3}). The precipitated product and aqueous phase were extracted with ethyl acetate (7 × 25 cm^3). Evaporation of the combined extracts on a rotary evaporator afforded the title compound **9** as a white solid (429 mg, 65%), mp 197–198 °C (decomp.); $[\alpha]_D^{20}$ −49.5 (*c* 0.2, DMF); δ_H([$^2H_6$]DMSO, 200 MHz) 2.81–3.20 (2 H, m, Py–CH_2), 4.12–4.28 (4 H, m, OCH_2CH, OCH_2, NCHCO), 7.21–7.42 (8 H, m, Fmoc–H), 7.75 (1 H, br s, D_2O ex., NH), 7.85 (2 H, d, *J* 7, Py–H) and 8.42 (2 H, d, *J* 7, PyH); δ_C([$^2H_6$]DMSO, 50 MHz), 35.7, 46.6, 54.4, 65.6, 120.1, 124.6, 125.2, 127.1, 127.7, 140.7, 143.8, 147.1, 149.4, 155.9 and 173.0; ν_{max}(KBr)/cm^{-1} 3290 and 1716; *m*/*z* (FAB^+) 389 (MH^+, 25%) (Found: *M*, 389.1509. Calc. for $C_{23}H_{21}N_2O_4$: *M*, 389.1501).

(*S*)-*N*-(9-Fluorenylmethoxycarbonyl)-β-(1-oxido-4-pyridyl)-alanine 10
A suspension of dichloromethane (30 cm^3) containing (*S*)-*N*-(fluoren-9-ylmethoxycarbonyl)-β-(4-pyridyl)alanine **9** (370 mg, 0.954 mmol) and *m*-chloroperbenzoic acid (50%; 329 mg, 0.954 mmol) was stirred at room temp. for 16 h. The pale yellow solution was then concentrated on a rotary evaporator to give a yellow paste. This was taken up in methanol–acetonitrile (1:3, 50 cm^3) and the solution then reduced in volume to 3 cm^3 on a rotary evaporator to afford a yellow oil. Methanol–acetonitrile (1:3, 80 cm^3) were added to this to precipitate white crystals which were washed with ice-cold water to give the title compound **10** (230 mg, 57%); mp 135–136 °C (decomp.); $[\alpha]_D^{20}$ −20.0 (*c* 0.2, DMF); δ_H([$^2H_6$]DMSO, 500 MHz) 3.00 (1 H, dd, J_{ABX} 10, 14, Py–CH_2), 3.26 (1 H, dd, J_{ABX} 5, 14, $PyCH_2$), 4.18 (1 H, t, *J* 6, OCH_2CH), 4.35 (2 H, m, OCH_2), 4.46 (1 H, dd, J_{ABX} 5, 10, NCHCO), 7.28–7.40 (6 H, m, Fmoc–H), 7.58 (2 H, t, *J* 8, Fmoc–H), 7.75 (1 H, br s, NH, D_2O ex.), 7.87 (2 H, d, *J* 7, PyH) and 8.09 (2 H, d, *J* 7, PyH); δ_C([$^2H_6$]DMSO, 125 MHz) 37.1 (under solvent), 55.5, 67.7, 120.9, 126.1, 128.1, 128.9, 139.8, 142.6, 142.8, 145.1, 145.3, 158.3 and 174.0; ν_{max}(KBr)/cm^{-1} 3310, 1700 and 1253; *m*/*z* (FAB^+) 405 (MH^+, 100%) and 389 (MH^+ − O, 25%) (Found: M, 405.1454. Calc. for $C_{23}H_{21}N_2O_5$: *M*, 405.1451).

Determination of the stereochemical purity of Fmoc amino acids
Fmoc amino acids **5** and **10** were stirred with 20% piperidine in dichloromethane for 10 min at room temp. and the deprotected amino acids then extracted with water. The extract was evaporated under reduced pressure and the resulting amino acids were examined by chiral HPLC on a TECHOCEL OA-5000 column eluting with 15% methanol in aq. copper sulfate (2 mmol dm^{-3}). Comparison was made with racemic standards.

Peptide synthesis: general method
Peptide syntheses were carried out, using Fmoc-protected amino acids and TBTU-mediated coupling, on a Biosearch 9500 peptide synthesiser. 20% Piperidine in DMF was used for Fmoc deprotection. On completion of the synthesis the resin was washed with diethyl ether (3 × 20 cm^3) and dried overnight *in vacuo* to give the crude, resin-bound peptide, of which a portion was cleaved by stirring with CF_3CO_2H, PhOH and Et_3SiH (18:1:1, 20 cm^3) at room temp. for 2.5 h. The mixture was filtered and the filter washed with CF_3CO_2H (2 × 5 cm^3). The combined filtrate and washings were evaporated to *ca.* 1 cm^3 on a rotary evaporator and the resulting viscous yellow oil was transferred to a pre-weighed centrifuge vessel. The flask was washed with CF_3CO_2H (2 × 0.5 cm^3) and the washings added to the centrifuge vessel. The combined filtrate and washings were successively triturated and centrifuged with diethyl ether (25 cm^3), ethyl acetate–diethyl ether (3:2, 25 cm^3) and diethyl ether (25 cm^3) and then dried under a stream of nitrogen.

All peptides were purified by preparative reversed-phase HPLC, using a Dynamax C_{18} 41 × 300 mm column, eluting with a gradient of water–CF_3CO_2H (99.9:0.1; buffer A) and acetonitrile–water–CF_3CO_2H (90:10:0.5; buffer B), at a flow rate of 45 cm^3 min^{-1}.

Analytical HPLC was performed on a Brownlee-Aquapore RP300 C_8 reversed-phase column with a Varian 9000 liquid chromatograph, eluting with a gradient of water–CF_3CO_2H (99.9:0.1; buffer A) and acetonitrile–water–CF_3CO_2H (90:10:0.5; buffer B), at a flow rate of 1 cm^3 min^{-1}.

Arginyl-arginyl-glutamyl-glutamyl-leucyl-glutaminyl-aspartyl-aspartyl-tyrosyl-glutamate 11. The following Fmoc-protected amino acids (0.75 mmol) were loaded into the peptide synthesiser in the order shown: Tyr (Bu^t) OH (340 mg), Asp (OBu^t) OH (300 mg), Asp (OBu^t) OH (300 mg), Gln (Trt) OH (450 mg), Leu OH (260 mg), Glu (OBu^t) OH (310 mg), Glu (OBu^t) OH (310 mg), Arg (Pmc) OH (490 mg) and Arg (Pmc) OH (490 mg). Each reservoir was also loaded with TBTU (240 mg, 0.75 mmol) and coupling was accomplished using *N*-methylmorpholine (NMM, 99 mm^3) and 1-hydroxybenzotriazole (HOBt, 95 mg) in DMF (3 cm^3) per cycle.

N,*N*-Dimethylaminopyridine, DMAP (16 mg, 0.125 mmol) in DMF (250 cm^3) was added to a solution containing Fmoc-Glu (OBu^t) OH (640 mg, 1.5 mmol) and diisopropylcarbodiimide, DIPCI, (117 mm^3, 0.75 mmol) in DMF (3.5 cm^3). This mixture was immediately added to freshly washed ALH polystyrene resin[15] (0.78 mmol g^{-1}; 320 mg, 0.25 mmol) in the reaction vessel and the synthesis sequence using the amino acids above was commenced. Upon completion of the synthesis, the resin gave a positive TNBSA (2,4,6-trinitrobenzenesulfonic acid) test.

A portion (367 mg) of the crude, resin-bound peptide (837 mg) was cleaved to afford crude peptide (153 mg, quantitative). Preparative HPLC purification of 55 mg, of this product followed by removal of the solvent under reduced pressure gave the title compound **11** (30 mg, 55%); *m/z* (FAB$^+$) 1353 (MH$^+$, 5%); CZE (sodium citrate, pH 2.5), 98%; amino acid analysis (18 h hydrolysis), 2 Asx, 4 Glx, 1 Leu, 1 Tyr and 2 Arg.

General method for the synthesis of peptides incorporating tyrosine analogues. The following Fmoc-protected amino acids (0.60 mmol) were loaded into the peptide synthesiser in the order shown: Asp (OBu^t) OH (250 mg), Asp (OBu^t) OH (250 mg), Gln (Trt) OH (370 mg), Leu OH (210 mg), Glu (OBu^t) OH (260 mg), Glu (OBu^t) OH (260 mg), Arg (Pmc) OH (400 mg) and Arg (Pmc) OH (400 mg). Each reservoir was also loaded with TBTU 192 mg, 0.60 mmol) and coupling was accomplished using *N*-methylmorpholine (NMM, 80 mm^3) and 1-hydroxybenzotriazole (HOBt, 76 mg) in DMF (3 cm^3) per cycle.

DMAP (12.8 mg, 0.100 mmol) in DMF (200 cm^3) was added to a solution containing Fmoc-Glu (OBu^t) OH (511 mg, 1.2 mmol) and DIPCI (94 mm^3, 0.60 mmol) in DMF (2.8 cm^3). This mixture was immediately added to freshly washed ALH polystyrene resin (0.78 mmol g^{-1}; 256 mg, 0.20 mmol) in the reaction vessel and the synthesis sequence was commenced. After this first coupling cycle an aliquot of a solution of HOBt (1.58 g) and NMM (1.65 cm^3) in DMF (50 cm^3) was added to a mixture of the Fmoc-protected tyrosine analogue **5** or **10** and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate, TBTU, and this mixture was applied to the peptide resin. The synthetic sequence was then continued until complete. A portion of the crude resin-bound peptide was cleaved and purified by preparative HPLC. After purification by preparative HPLC the isolated peptides were homogeneous by analytical HPLC and CZE.

Arginyl-arginyl-glutamyl-glutamyl-leucyl-glutaminyl-aspartyl-aspartyl-β-(2-oxo-1,2-dihydro-5-pyridyl)alanyl-glutamate 12. Using (*S*)-*N*-(fluoren-9-ylmethoxycarbonyl)-β-(2-oxo-1,2-dihydro-5-pyridyl)alanine **5** (242 mg, 0.60 mmol), TBTU (192 mg, 0.60 mmol) and a 3.0 cm^3 aliquot of HOBt–NMM–DMF solution for the second coupling cycle, crude, resin-bound peptide (520 mg) was obtained, of which 250 mg, was cleaved to afford the title compound **12** (104 mg). Purification of a portion (60 mg) by preparative HPLC followed by removal of solvent under reduced pressure gave a homogeneous sample (25 mg, 33%); *m/z* (FAB$^+$) 1354 (MH$^+$, 25%); amino acid analysis (18 h hydrolysis), 2 Asx, 4 Glx, 1 Leu, 1 pyridone **4** and 2 Arg.

Arginyl-arginyl-glutamyl-glutamyl-leucyl-glutaminyl-aspartyl-aspartyl-β-(1-oxido-4-pyridyl)alanyl-glutamate 13. Using (*S*)-*N*-(fluoren-9-ylmethoxycarbonyl)-β-(1-oxido-4-pyridyl)-alanine **10** (226 mg, 0.56 mmol), TBTU (184 mg, 0.56 mmol) and a 2.8 cm^3 aliquot of HOBt–NMM–DMF solution for the second coupling cycle gave crude, resin-bound peptide (520 mg) a sample (238 mg) of which was cleaved to afford the title compound **13** (110 mg). Purification of a portion (60 mg) of this material gave a homogeneous sample (20 mg, 30%); *m/z* (FAB$^+$) 1354 (MH$^+$, 25%), 1337 (MH$^+$ − O, 20%); amino acid analysis (18 h hydrolysis) 2 Asx, 4 Glx, 1 Leu, 1 pyridine *N*-oxide **6** and 2 Arg.

Assay of the peptide phosphotransferase activity of the EGF receptor kinase in the presence and absence of putative inhibitors
A recombinant, soluble, cytoplasmic domain of the human EGF receptor[16] was used to measure tyrosine kinase activity. The protein was purified from insect cells and its activity was characterised.[17]

Peptide phosphotransferase activity was measured using the phosphocellulose paper binding assay.[18] Reactions were carried out at 22 °C in 50 mmol dm^{-3} Hepes [2-(2-hydroxyethyl)-piperazin-1-ylethane-2-sulfonic acid] buffer, pH 7.5 containing 0.1 mmol dm^{-3} sodium EDTA, 2 mmol dm^{-3} dithiothreitol, 5% glycerol, and 10 mmol dm^{-3} $MnCl_2$. The reaction was initiated by the addition of kinase (2.7 pmol) to a mixture of peptide substrate (at the indicated concentration) and [γ-^{32}P]-ATP (10 μmol dm^{-3}, approximately 5000 CPM pmol^{-1}) in the presence or in the absence of putative inhibitor peptides. The total reaction volume was 20 mm^3. After 12 min aq. trichloroacetic acid [10 mm^3, 25% (w/v)] was added to stop the reaction and precipitate the kinase which was removed by centrifugation. The supernatant (containing the peptides) was spotted on phosphocellulose paper (P81, Whatman International Ltd) to which [^{32}P]-labelled phosphopeptides bound. Excess of [γ-^{32}P]-ATP was washed away using 5% (v/v) phosphoric acid. The amount of [^{32}P]-labelled phosphopeptides was quantified by scintillation counting.

Autophosphorylation activity was measured under similar conditions, except that no peptide substrates were included in the assay mixture, and [^{32}P]-kinase was isolated by analytical SDS-polyacrylamide gel electrophoresis.[19] Reactions were carried out in the presence of 2–20 mmol dm^{-3} $MnCl_2$ (10 mmol dm^{-3} was used in the standard conditions). Kinase was pre-incubated with peptide inhibitor for 4 min at 22 °C before reactions were started by the addition of [γ-^{32}P]-ATP. Reactions were stopped by the addition of Laemmli sample buffer (20 mm^3, 2 × concentrated solution) and heated for 5 min at 100 °C.

Inhibition of kinase activity in peptide phosphorylations was assessed by comparing reaction rates obtained in the presence or absence of putative inhibitor. Representative results are shown in Table 1.

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