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The Synthesis and Use of pp60^{src}-Related Peptides and Phosphopeptides as Substrates for Enzymatic Phosphorylation Studies†

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Abstract—A series of peptides and phosphopeptides corresponding to the auto-phosphorylation site of pp60^{src}, -Asn-Glu-Tyr^{416}-Thr-Ala-, were prepared by either Boc/solution or Fmoc/solid phase peptide synthesis and used as substrates to study their enzymatic phosphorylation by various casein kinases. The Tyr(*P*)-containing peptide, Asn-Glu-Tyr(*P*)-Thr-Ala, was prepared by the use of Fmoc-Tyr(PO_3Bzl_2)-OH in Fmoc/solid phase peptide synthesis followed by acidolytic treatment of the peptide–resin with 5% anisole/CF_3CO_2H. Both Asn-Glu-Tyr-Thr-Ala and Asn-Glu-Ser(*P*)-Thr-Ala were prepared by the Boc/solution phase peptide synthesis and employed hydrogenolytic deprotection of the protected peptides. Enzymatic phosphorylation studies established that (A) the Tyr residue acted as an unusual positive determinant for directing phosphorylation to the Thr-residue, (B) the rate of Thr-phosphorylation was markedly facilitated by a change from the Tyr-residue to the Tyr(*P*)-residue, and (C) a Ser(*P*)-residue was as effective as the Tyr(*P*)-residue in facilitating Thr-phosphorylation. A subsequent structure–function study using Asn-Glu-Phe-Thr-Ala, Asn-Glu-Tyr(Me)-Thr-Ala (prepared by Fmoc/solid phase peptide synthesis) and Asn-Glu-Cha-Thr-Ala (prepared by hydrogenation of Asn-Glu-Tyr-Thr-Ala) established that the rate of Thr-phosphorylation was influenced by the extent of hydrophobic–hydrophobic interactions by the aralkyl side-chain group (either aromatic or aliphatic) of the 416-residue with casein kinase-2; the rate of Thr-phosphorylation being decreased by the introduction of methyl or hydroxyl groups at the 4-position of the aromatic group {i.e. Tyr(Me) and Tyr respectively} but enhanced by the introduction of the hydrophilic phosphate group {i.e. as Tyr(*P*)}.

Introduction

The tyrosine protein kinases of the *src*-family possess a highly conserved peptide sequence in which Tyr-416 of the sequence -Asn-Glu-Tyr-Thr-Ala- is autophosphorylated *in vivo*[2] and correlates with activation of the enzyme.[3] In addition, a number of seryl- and threonyl-residues are also phosphorylated in this enzyme but the consequence of these phosphorylations to its overall activity or regulatory function remain unclear.[4,5] In order to obtain a better understanding of the factors that are involved in the phosphorylation of this particular sequence, we describe here the synthesis of Asn-Glu-Tyr-Thr-Ala, Asn-Glu-Tyr(*P*)-Thr-Ala and several other derivatives of the Asn-Glu-Xxx-Thr-Ala sequence and their use as substrates in ascertaining the affect of different residues at the 416 site on the phosphorylation of the Thr residue by casein kinase-2.

Results and Discussion

Synthesis of Asn-Glu-Tyr(P)*-Thr-Ala and Asn-Glu-Tyr-Thr-Ala*

In order to establish the relative effect of phosphorylation of the tyrosyl residue, the first stage of this study involved the preparation of Asn-Glu-Tyr-Thr-Ala and its phosphorylated derivative, Asn-Glu-Tyr(*P*)-Thr-Ala. The synthesis of the non-phosphorylated peptide was readily accomplished by the Boc mode of solution phase peptide synthesis using the mixed anhydride coupling procedure. The protected pentapeptide, *Z*-Asn-Glu(OBzl)-Tyr(Bzl)-Thr(Bzl)-Ala-OBzl (**7**) was obtained in good overall yield from Boc-Thr(Bzl)-Ala-OBzl[6] (**1**) by the use of 4 M HCl/dioxane for the removal of the Boc group and the isobutoxycarbonyl mixed anhydride coupling procedure for successive incorporation of Boc-Tyr(Bzl)-OH, Boc-Glu(OBzl)-OH and *Z*-Asn-OH (coupling yields of 89, 89 and 65% respectively). Pentapeptide (**7**) was readily deprotected by palladium-catalysed hydrogenolysis in 50% CF_3CO_2H/ CH_3CO_2H[7,8] and gave Asn-Glu-Tyr-Thr-Ala (**8**) in high yield and purity.

In early work, Valerio and coworkers[6] described the first synthesis of Asn-Glu-Tyr(*P*)-Thr-Ala by the use of Boc-Tyr(PO_3Me_2)-OH[9] in the Boc mode of solution phase peptide synthesis followed by bromotrimethylsilane cleavage of the methyl phosphonate groups. Although successful, a limitation of this work was that the methyl phosphonate groups were relatively acid stable and required harsh acidolytic or silylitic treatments for their complete cleavage. As Fmoc/solid phase peptide synthesis has recently been used for the synthesis of Tyr(*P*)-containing peptides, the synthesis of this phosphopeptide was

†Part of this work has appeared in preliminary form.[1]
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therefore re-undertaken using Fmoc-Tyr(PO_3Bzl_2)-OH[10] as the protected phosphoamino acid; the selection of this protected derivative being based on the marked sensitivity of the benzyl phosphonate group to acidolytic conditions.

The Fmoc/solid phase synthesis[11] of Asn-Thr-Tyr(*P*)-Thr-Ala (**11**) was readily accomplished on an Applied Biosystems 431A Peptide Synthesizer operating in the DCC/HOBt mode of peptide chemistry with 20% piperidine/DMF being used for repetitive cleavage of the Fmoc group. The synthesis commenced with Fmoc-Ala-Resin (**9**) as the polymer support with peptide assembly performed by the successive incorporation of Fmoc-Thr(^{t}Bu)-OH, Fmoc-Tyr(PO_3Bzl_2)-OH, Fmoc-Glu(O^{t}Bu)-OH and Fmoc-Asn(Trt)-OH as their preformed symmetrical anhydride (2 equiv.). On completion of peptide synthesis, the treatment of peptide–resin (**10**) with 5% anisole/CF_3CO_2H readily effected the cleavage of the peptide from the resin and removal of all side-chain protecting groups, the acid-labile benzyl phosphonate groups also being cleaved under these conditions. The crude Tyr(*P*)-pentapeptide was determined to be >95% pure by capillary electrophoresis and C_8 RP-HPLC, and was readily purified to homogeneity by a semi-preparative C_8 HPLC step. The purified peptide gave a satisfactory amino acid analysis and its structure was confirmed by ^{13}C NMR spectroscopy and FAB mass spectrometry. In contrast to the harsh cleavage of methyl phosphonate groups by bromotrimethylsilane/thioanisole/ CF_3CO_2H treatment (16 h),[6] the use of benzyl phosphonate protection permits the use of milder acidolytic conditions with the benzyl groups being rapidly cleaved (1 h) and with reduced by-product formation.

Enzymatic phosphorylation study of peptides (8) and (11)

The enzymatic phosphorylation of Asn-Glu-Tyr-Thr-Ala and Asn-Glu-Tyr(*P*)-Thr-Ala with CK-1, CK-2 and PK-C showed that both peptides were inert to CK-1 and PK-C but were phosphorylated by CK-2 with phosphorylation rates of 0.082 and 0.27 pmol/min respectively* (Figure 1, Panel A). Although the phosphorylation rates of both peptide substrates is significantly less than other known peptide substrates, the obtained data presents the first reported finding which shows that there is a significant increase in the phosphorylation rate of a particular target residue through the conversion of the Tyr-residue to its Tyr(*P*)-derivative. In addition, the phosphorylation of the Thr-residue in this sequence was somewhat unexpected since the -Glu-Tyr-Thr- sequence does not correspond with the Ser-Xxx-Xxx-Glu motif which has previously been well established for casein kinase-2.[14] The role of the anionic phosphate group of the Tyr(*P*)-residue for the increased phosphorylation rate of pentapeptide (**11**) was further substantiated by its dependence on the solution pH, the phosphorylation rate being at its maximum at pH 8 and decreasing significantly as the pH approached 6.5 (Figure 2). This phosphorylation behaviour is consistent with that observed for Ser-Xxx-Xxx-Ser(*P*)-peptides (unlike

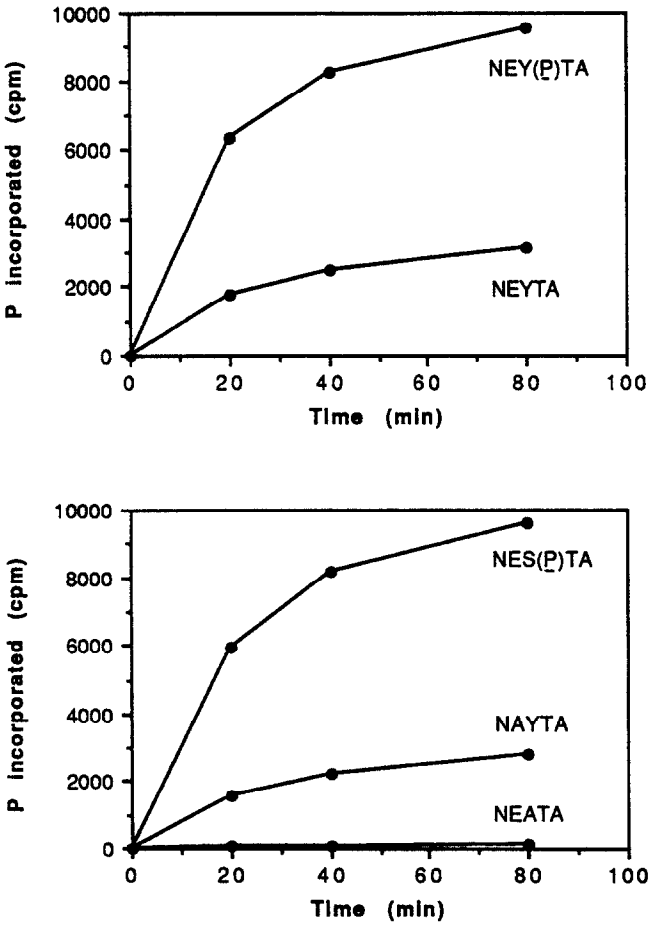

Figure 1. Panel A: Time course profiles for the phosphorylation of Asn-Glu-Tyr-Thr-Ala and Asn-Glu-Tyr(*P*)-Thr-Ala by CK-2. Panel B.: Time course profiles for the phosphorylation of Asn-Ala-Tyr-Thr-Ala, Asn-Glu-Ala-Thr-Ala and Asn-Glu-Ser(*P*)-Thr-Ala by CK-2

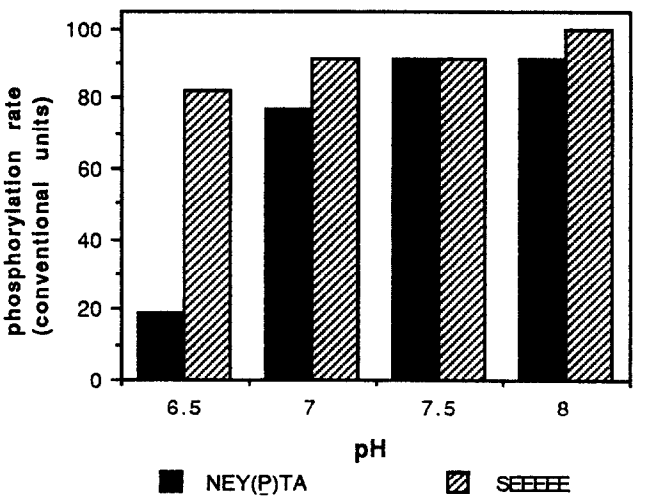

Figure 2. pH Dependence of the phosphorylation rate of Asn-Glu-Tyr(*P*)-Thr-Ala by CK-2 and its comparison with SEEEEE (which is set at 100% at pH 8)

*The residues adjacent to the Thr-residue of NEY(*P*)TA were found to be solely responsible for phosphorylation of the Thr-residue since, in a later study, an identical phosphorylation rate was obtained for the larger tridecapeptide sequence, RLIEDNEY(*P*)TARQG.[12,13]

Glu-containing peptides) which display increased phosphorylation by casein kinase-2 when the pH is increased from pH 7 to 7.5 and indicate that the phosphate group must be fully dissociated to act as a specificity determinant. The pH effect on phosphorylation was not an effect on the kinase *per se* as there was no significant variation in phosphorylation rate of SEEEEE at the pH values tested.

While Asn-Glu-Tyr(*P*)-Thr-Ala is a poor substrate for CK-2, its phosphorylation efficiency nevertheless correlates well with Ser-Ala-Ala-Glu-Ala-Ala (see Table 1) which fulfils the minimum structural requirement for CK-2 that an acidic residue is located in the +3 position from the Ser-residue but lacks other positive determinants that maximise phosphorylation. While the K_m of Asn-Glu-Tyr(*P*)-Thr-Ala is more favourable than that of Ser-Ala-Ala-Glu-Ala-Ala, its lower V_{max} is a reflection of the intrinsic properties of the threonyl residue, which generally displays 10-fold lower V_{max} values in identical peptide sequences which contain the seryl residue.

Table 1. Kinetic constants for the phosphorylation of NEY(*P*)TA and SAAEAA with CK-2.

Peptide	V_{max} (nmol/min/mg)	K_m (mM)	Efficiency (V_{max}/K_m)
NEY(*P*)TA	1.0	3.2	0.31
SAAEAA	7.2	18.9	0.38

Elucidation of the specificity determinants of NEYTA

While the enzymatic phosphorylation data showed that Thr-phosphorylation of NEY(*P*)TA was due to the influence of the Tyr(*P*)-residue, the unexpected phosphorylation of Asn-Glu-Tyr-Thr-Ala suggested that residues on the N-terminus of the Thr-residue were acting as specificity determinants for Thr-phosphorylation. In order to establish the influence of N-terminal residues for directing phosphorylation to the Thr-residue of Asn-Glu-Tyr-Thr-Ala, the next stage of this study required the synthesis of Asn-Glu-Ala-Thr-Ala (**12**) and Asn-Ala-Tyr-Thr-Ala (**13**) in which the Glu- and Tyr-residues were selectively replaced by the non-functional Ala-residue. In addition, the Ser(*P*)-peptide, Asn-Glu-Ser(*P*)-Thr-Ala, was also selected as a peptide substrate to test whether the acidic Ser(*P*)-residue, which is normally a favourable specificity determinant for casein kinase-2, could act as a surrogate for the Tyr(*P*)-residue in this particular peptide sequence.

The synthesis of both the non-phosphorylated peptides was readily accomplished by Fmoc/solid phase peptide synthesis (PyBOP® coupling method) followed by treatment of the peptide–resins with 5% anisole/CF_3CO_2H. Both peptides were obtained in high yield and purity, and were readily characterized by ^{13}C NMR spectroscopy and amino acid analysis. Taking account of the fact that the base sensitivity of protected Ser(PO_3R_2)-residues precludes the use of the Fmoc-based synthetic approach, the synthesis of this peptide was undertaken by the use of Boc-Ser(PO_3Ph_2)-OH (**14**) in the Boc mode of solution phase peptide synthesis.[15] The synthesis of Z-Asn(Trt)-Glu(OBzl)-Ser(PO_3Ph_2)-Thr(Bzl)Ala-OBzl (**19**) was readily accomplished in good overall yield from Boc-Thr(Bzl)-Ala-OBzl[6] (**1**) by the use of 50% CF_3CO_2H/CH_2Cl_2 for repetitive cleavage of the Boc group and the successive incorporation of Boc-Ser(PO_3Ph_2)-OH, Boc-Glu(OBzl)-OH and Z-Asn(Trt)-OH (coupling yields of 96, 91 and 84% respectively) as their isobutoxycarbonyl mixed anhydride. In this synthesis, the N-terminal Asn-residue was incorporated as Z-Asn(Trt)-OH since two prior attempts using Z-Asn-OH resulted in extensive dehydration and the formation of the β-cyanoalanyl residue. Pentapeptide (**19**) was deprotected by initial treatment with CF_3CO_2H for 2 h to cleave the trityl group followed by cleavage of the benzyl and phenyl phosphonate groups by platinum-mediated hydrogenolysis of pentapeptide (**19**) in 50% CF_3CO_2H/CH_3CO_2H[16] using 1.1 equiv. of platinum oxide per phenyl phosphonate group. The crude isolated peptide was found to be homogeneous by C_8 HPLC analysis and gave a satisfactory amino acid analysis.

The enzymatic phosphorylation study of Asn-Glu-Ala-Thr-Ala (**12**) and Asn-Ala-Tyr-Thr-Ala (**13**) with casein kinase-2 established that the former peptide was inert to phosphorylation while the later peptide was phosphorylated with a rate of 0.069 pmol/min (Figure 1, Panel B); the data indicating that the non-acidic Tyr-residue was acting as a specificity determinant for Thr-phosphorylation. Two significant conclusions from this data is that firstly, the Tyr-residue in the sequence -Glu-Tyr-Thr- is responsible for directing phosphorylation to the downstream Thr-residue and secondly, that conversion of the Tyr-residue to the Tyr(*P*)-residue facilitates enzymatic phosphorylation of the Thr-residue. These findings are of interest from a physiological point of view since they provide the first findings that CK-2 can recognize an atypical acidic residue on the N-terminal side of the target residue and that phosphorylation of the tyrosyl residue is a positive modification for facilitating further enzymatic Thr-phosphorylation. In addition, the phosphorylation rate of Asn-Glu-Ser(*P*)-Thr-Ala was similar to that of Asn-Glu-Tyr(*P*)-Thr-Ala and indicates that in the case of this particular peptide sequence, the Ser(*P*)-residue can effectively act as a surrogate for the Tyr(*P*)-residue (Figure 1, Panel B).

Effect of structural variations in the Tyr-residue

The improved phosphorylation of Asn-Glu-Tyr(*P*)-Thr-Ala over Asn-Glu-Tyr-Thr-Ala suggested that structural factors of the central residue of the -Glu-Xxx-Thr- sequence also played a minor role in 'fine-tuning' the efficacy of Thr-phosphorylation. In order to study the effect of structural variations in the central Tyr-residue, the three peptides, Asn-Glu-Tyr(Me)-Thr-Ala, Asn-Glu-Phe-Thr-Ala and Asn-Glu-Cha-Thr-Ala were selected as substrates to be studied in conjunction with Asn-Glu-Tyr-Thr-Ala. While the first peptide represents the introduction of a hydrophobic methyl

group to the tyrosyl phenoxy group, the second and third peptides constitute the loss of the tyrosyl hydroxy moiety and loss of the aromatic phenyl moiety respectively. The cyclohexylalanyl (Cha)-derivative was selected so as to observe the effect on phosphorylation caused by perturbation of the planar aromatic group of the Phe-residue to the chair-conformation of the β-(cyclohexyl)alanyl residue.

In the case of NEY(Me)TA (**21**) and NEFTA (**22**), the synthesis of these two peptides were accomplished in high yield by manual Fmoc/solid phase synthesis with the incorporation of the Fmoc amino acids by PyBOP®-mediated couplings (3 equiv.) in DMF/CH_2Cl_2 solution (the N-terminal Asn-residue being incorporated as Boc-Asn-OH). Final peptide–resin cleavage and peptide deprotection was effected by treatment with 5% anisole/CF_3CO_2H and gave each of the peptides in excellent yield and purity. In the case of Asn-Glu-Cha-Thr-Ala (**23**), this peptide was readily obtained by the platinum-mediated hydrogenation of Asn-Glu-Tyr-Thr-Ala (**1**) in 50% CF_3CO_2H/CH_3CO_2H using 1.1 equiv. of platinum oxide, reduction of the side-chain aromatic group of the Tyr-residue proceeding smoothly and giving the Cha-containing peptide in near-quantitative yield. For all three peptides, their structure was readily established by ^{13}C NMR spectroscopy, FAB mass spectrometry and amino acid analysis.

Enzymatic phosphorylation studies

The data obtained from the treatment of phosphopeptide (**11**) and peptides (**21–23**) with CK-2 showed that the conversion of Tyr-residue to the more hydrophobic Tyr(Me)-residue caused a major reduction in phosphorylation efficacy and that the loss of the phenoxy hydroxyl group (Tyr→Phe) resulted in a minor increase in Thr-phosphorylation (Figure 3). Surprisingly, loss of the aromatic moiety (Phe→Ala) resulted in a marked reduction in Thr-phosphorylation and conversion of the aromatic group to the cyclohexyl group (Phe→Cha) resulted in improved phosphorylation; the level of phosphorylation being on par with Asn-Glu-Tyr(*P*)-Thr-Ala.

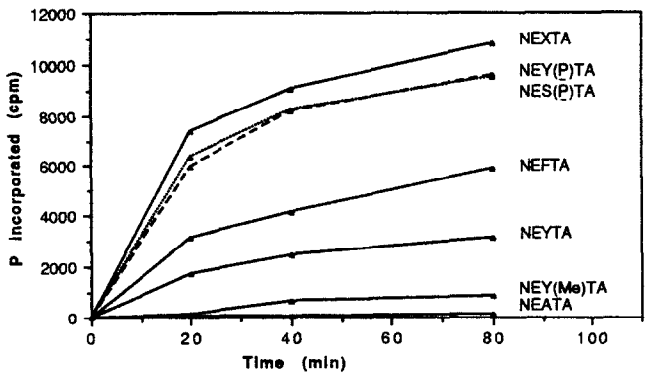

Figure 3. Time course profiles for the phosphorylation of peptides by CK-2 (X = Cha)

The analysis of the total kinetic data indicates that phosphorylation of the Thr-residue of the Asn-Glu-Xxx-Thr-Ala sequence is influenced by both hydrophobic and hydrophilic interactions of the central Xxx-residue with the active phosphorylation site of CK-2. As the order of Thr-phosphorylation was found to be NEXTA (X = Cha) > NEFTA > NEATA, the unexpected performance of the Cha- and Phe-peptides suggests that a hydrophobic moiety in the side-chain of the central Xxx residue is able to undergo hydrophobic–hydrophobic interactions with the kinase; the level of peptide/kinase interaction being increased through conversion of the planar aromatic ring of the Phe-residue to the chair conformation of the cyclohexyl group of the Cha-residue. Alternatively, as the order of Thr-phosphorylation was found to be NEY(*P*)TA > NEYTA > NEY(Me)TA, this indicates that the extent of peptide/kinase interaction is also a function of a hydrophilic group at the C4 position of the Tyr-residue. Since masking of the Tyr hydroxyl group produced an unfavourable interaction and conversion of the Tyr hydroxyl group to its phosphate derivative produced a favourable interaction, it is likely that the phenolic hydroxy group of the Tyr-residue is able to participate in weak hydrogen bonding with the active site of the kinase and that this interaction is markedly increased when the phenolic group is converted to a phosphate group.

Conclusion

In respect to chemical methodology, this study has demonstrated that Fmoc-Tyr(PO_3Bzl_2)-OH can be readily used for the efficient and rapid synthesis of Tyr(*P*)-containing peptides and is based on the marked sensitivity of the benzyl phosphonate group to mild acidolytic conditions. Furthermore, the above study demonstrates how synthetic phosphopeptides such as Asn-Glu-Tyr(*P*)-Thr-Ala and Asn-Glu-Ser(*P*)-Thr-Ala can be used as substrates to reveal how phosphorylated residues affect enzymatic phosphorylation processes and how the conversion of the Tyr-residue to its Tyr(*P*)-residue influences the rate of enzymatic threonyl phosphorylation. By the use of various phosphorylated and non-phosphorylated substrates in enzymatic phosphorylation studies with casein kinase-2, the obtained kinetic data showed for the first time that the -Glu-Tyr-Thr- sequence can act as an atypical recognition sequence and that Thr-phosphorylation is markedly facilitated by conversion of the Tyr-residue to the Tyr(*P*)-residue. While the physiological ramifications of CK-2 mediated phosphorylation of Thr^{417} in $pp60^{src}$ remains unclear, the principal finding from this study is the observation that a Tyr(*P*)-residue is able to act as a CK-2 specificity determinant in particular peptide sequences.

Experimental

^{13}C NMR spectra were obtained on a Jeol FX-90Q instrument operating at 22.5 MHz and were referenced to internal tetramethylsilane for $CDCl_3$ solutions and internal dioxane set to 66.5 ppm for D_2O solutions. ^{31}P NMR spectra were obtained on a Jeol FX-100 instrument operating at 40.26 MHz and referenced to external 85% H_3PO_4. FAB mass spectra were obtained on a Jeol AX-505H mass spectrometer equipped with a FAB source and

used argon as ionization gas. Analytical HPLC was performed on an Applied Biosystems instrument comprising a 140A solvent delivery system linked to a 1000S diode-array detector. The instrument was fitted with an Aquapore RP-300 C_8 reversed-phase column (4.6 x 22.0 cm) and elution was performed using either a linear gradient of 0.1% CF_3CO_2H/0–80% CH_3CN over 20 min. All solvents were of AnalaR grade and trifluoroacetic acid was obtained from Merck–Schuchardt and used without further purification. Peptide synthesis reagents, Fmoc-Ala-Wang Resin and protected amino acid derivatives were obtained from Novabiochem. Amino acid analyses of the peptides were performed by vapour hydrolysis in 5.7 M HCl for 24 h at 110 °C followed by analysis of the PTC-derivatised hydrolysate on a Waters HPLC instrument fitted with a C_{18} Picotag column. Melting points were determined on a Kofler hot stage and are uncorrected. Dipeptide (1) was prepared as described elsewhere.[6]

CK-2 was obtained as described elsewhere.[17] The enzymatic phosphorylation of peptides was essentially performed and quantitated as described elsewhere[18] (procedure *a*) and involved incubating the peptides (1 mM) at 37 °C in a solution containing 50 mM Tris–HCl buffer (pH 7.5), 12 mM $MgCl_2$, 100 mM NaCl, 10 μM [γ-^{32}P]ATP (spec. act. 1000–1500 cpm/pmol) and the protein kinase. The phosphorylation was terminated by the addition of HCl (6N final concentration), the phosphopeptides hydrolysed at 110 °C for 4 h and the liberated phosphoserine and phosphothreonine (which comigrates with phosphotyrosine) isolated by high-voltage paper electrophoresis at pH 1.9 and quantitated. Discrimination of phosphothreonine and phosphotyrosine was effected by elution of the radioactive spot and subsequent 2D electrophoresis on cellulose plates.[19]

Solution phase peptide synthesis — general procedure

The Boc group was cleaved from the Boc-protected peptide by treatment with either 4 M HCl/dioxane or 50% CF_3CO_2H/CH_2Cl_2 (4 mL). After 30 min, the solvent was evaporated under reduced pressure and the peptide precipitated by the addition of diethyl ether (30 mL). The solvent was removed, the solid triturated with diethyl ether (30 mL) and then dried under high vacuum to constant weight.

N-Methylmorpholine (1.40 mmeq) in THF (1 mL) and isobutyl chloroformate (1.30 mmequiv.) in THF (1 mL) were successively added to a solution of the Boc amino acid (1.40 mmequiv.) in THF (3 mL) at -20 °C. After an activation period of 3 min, a solution of the peptide trifluoroacetate (1.00 mmequiv.) and *N*-methylmorpholine (1.00 mmequiv.) in CH_2Cl_2 (3 mL) was added at -20 °C and the reaction mixture stirred at -20 °C for 2 h. A solution of 5% $NaHCO_3$ (5 mL) was then added, the solution stirred at 20 °C for a further 30 min and then transferred to a separating funnel using diethyl ether or ethyl acetate (50 mL). The organic phase was washed with 5% $NaHCO_3$ (2 x 25 mL) and 1M HCl (2 x 25 mL), dried (Na_2SO_4) and filtered.

Solution phase synthesis of Asn-Glu-Tyr-Thr-Ala

Boc-Tyr(Bzl)-Thr(Bzl)-Ala-OBzl (3)

The mixed anhydride coupling was performed using dipeptide trifluoroacetate (**2**) (1.68 mmol) according to the general procedure with diethyl ether used as the extraction solvent. The crude solid (1.126 g) was recrystallised from ethyl acetate/diethyl ether to give tripeptide (**3**) as a white solid (1.086 g, 89%), m.p. 152–153 °C. NMR: δ (^{13}C) ($CDCl_3$) 15.2, Ala Cβ; 17.7, Thr Cγ; 28.1, Boc Me; 36.9, Tyr Cβ; 48.2, Ala Cα; 56.0 and 56.2, Tyr and Thr Cα; 66.8, Ala Bzl CH_2; 69.8, Tyr Bzl CH_2; 71.3, Thr Bzl CH_2; 73.9, Thr Cβ; 80.3, Boc CMe_3; 115.0, Tyr Ar C3; 127.3, 127.6, 127.8, 128.0, 128.3 and 128.4, Bzl C2, C3, C4 and Tyr Ar C1; 130.2, Tyr Ar C2; 135.4, Ala Bzl C1; 136.8, Tyr Bzl C1; 137.9, Thr Bzl C1; 155.4, Boc CO; 157.8, Tyr Ar C4; 168.9, Ala CO; 171.2, Tyr CO; 171.9, Thr CO.

Boc-Glu(OBzl)-Tyr(Bzl)-Thr(Bzl)-Ala-OBzl (5)

The mixed anhydride coupling was performed using tripeptide trifluoroacetate (**4**) (0.50 mmol) according to the general procedure with ethyl acetate used as the extraction solvent. The crude solid was recrystallised from ethyl acetate/diethyl ether to give tetrapeptide (**5**) as a white solid (0.417 g, 89%), m.p. 158–160 °C. NMR: δ (^{13}C) ($CDCl_3$) 15.1, Ala Cβ; 17.8, Thr Cγ, 27.3, Glu Cβ; 28.2, Boc Me; 30.2, Glu Cγ; 37.4, Tyr Cβ; 48.1, Ala Cα; 53.7 and 54.5, Glu and Tyr Cα; 56.3, Thr Cα; 66.3 and 66.8, Glu and Ala Bzl CH_2; 69.7, Tyr Bzl CH_2; 71.2, Thr Bzl CH_2; 74.2, Thr Cβ; 79.9, Boc CMe_3; 114.8, Tyr Ar C3; 127.3, 127.6, 128.1, 128.2 and 128.3, Bzl C2, C3, C4 and Tyr Ar C1; 130.2, Tyr Ar C2; 135.3 and 135.6, Ala and Glu Bzl C1; 136.9 and 137.9, Tyr and Thr Bzl C1; 155.4, Boc CO; 157.7, Tyr Ar C4; 168.8, Ala CO; 170.7, Glu CO; 171.6, Tyr CO; 172.0, Thr CO; 172.8, Glu δ-CO.

Z-Asn-Glu(OBzl)-Tyr(Bzl)-Thr(Bzl)-Ala-OBzl (7)

The mixed anhydride coupling was performed using tetrapeptide trifluoroacetate (**6**) (0.40 mmol) according to the general procedure followed by aqueous precipitation of pentapeptide (**7**) (0.283 g, 65%), m.p. 220–230 °C (dec.). NMR: δ (^{13}C) {$(CD_3)_2SO$} 16.3, Ala Cβ; 17.0, Thr Cγ, 27.2, Glu Cβ; 29.8, Glu Cγ, obs, Tyr Cβ; 47.8, Ala Cα; 51.8, Glu and Asn Cα; 54.3, Tyr Cα; 56.7, Thr Cα; 65.4, 65.5 and 65.9, Z, Glu and Ala Bzl CH_2; 69.1, Tyr Bzl CH_2; 70.5, Thr Bzl CH_2; 75.0, Thr Cβ; 114.3, Tyr Ar C3; 127.6, 127.7, 128.0 and 128.3, Bzl C2,3,4; 129.9, Tyr Ar C1; 130.2, Tyr Ar C2; 135.9 and 136.1, Ala and Glu Bzl C1; 136.8, Z C1; 137.2, Tyr Bzl C1; 138.6, Thr Bzl C1; 155.7, Z, CO; 156.9, Tyr Ar C4; 169.4, Ala CO; 170.7, Tyr CO; 171.1 and 171.6, Asn CO and β-CO; 171.2, Glu CO; 172.1, Thr CO; 172.2, Glu δ-CO.

H-Asn-Glu-Tyr-Thr-Ala-OH·CF_3CO_2H (8)

A solution of pentapeptide (**7**) (0.109 g, 0.1 mmol) in 50% CF_3CO_2H/CH_3CO_2H (4 mL) containing 10%

palladium/charcoal (0.20 g) was charged with hydrogen and vigorously stirred until cessation of hydrogen uptake (< 1 h). The catalyst was removed by gravity filtration and the solvent evaporated under reduced pressure. The residue was triturated with diethyl ether (3 x 30 mL) and then dried under high vacuum to give pentapeptide (**8**) as a white solid (0.070 g, 99%). NMR: δ (^{13}C) (D_2O) 16.1, Ala Cβ; 18.6, Thr Cγ; 26.1, Glu Cβ; 29.6, Glu Cγ; 34.8, Asn Cβ; 36.1, Tyr Cβ; 48.8 and 49.7, Asn and Ala Cα; 53.2, Glu Cα; 55.1, Tyr Cα; 58.7, Thr Cα; 67.2, Thr Cβ; 115.4, Tyr Ar C3; 127.8, Tyr Ar C1; 130.4, Tyr Ar C2; 154.4, Tyr Ar C4; 168.7, 170.7, 172.3, 172.6 and 172.8, Asn, Glu, Tyr and Thr CO, Asn β-CO; 176.3 and 176.7, Ala CO and Glu δ-CO. FAB mass spectrum (argon, positive mode)* m/z (rel. ratio) 663 (M - 2H + 3Na, 4%), 641 (M - H + 2Na, 8), 619 (M + Na, 24), 597 (M + H, 7), 508 (2), 413 (16), 375 (6), 317 (11), 279 (7), 244 (22), 237 (20), 221 (14), 216 (14), 191 (11), 180 (15), 159 (22), 149 (32), 136 (55), 131 (100), 102 (42), 91 (67), 73 (51). Amino acid analysis: Ala 1.14 (1), Asx 0.87 (1), Glu 1.03 (1), Thr 1.00 (1), Tyr 1.03 (1).

H-Asn-Glu-Cha-Thr-Ala-OH·CF_3CO_2H (23)

A solution of pentapeptide (**8**) (0.053 g, 0.075 mmol) in 50% CF_3CO_2H/CH_3CO_2H (4 mL) containing 83% platinum oxide (0.070 g) was charged with hydrogen and vigorously stirred until cessation of hydrogen uptake (< 1 h). The platinum was removed by gravity filtration and the solvent evaporated under reduced pressure. The residue was triturated with diethyl ether (3 x 15 mL) and dried under high vacuum to give pentapeptide (**23**) as a white foam (0.051 g, 98%). NMR: δ (^{13}C) (D_2O) 16.4, Ala Cβ; 18.7, Thr Cγ, 25.6, 25.7 and 25.8, Cha C3,3',4; 26.2, Glu Cβ; 30.0, Glu Cγ; 31.7 and 33.0, Cha C2,2'; 33.7, Cha C1; 34.8, Asn Cβ; 38.1, Cha Cβ; 49.2 and 49.7, Asn and Ala Cα; 52.0, Glu Cα; 53.2, Tyr Cα; 58.8, Thr Cα; 67.1, Thr Cβ; 168.8, 170.9, 172.6, 172.6 and 174.6, Asn, Glu, Cha and Thr CO, Asn β-CO; 176.8 and 177.0, Ala CO and Glu δ-CO. FAB mass spectrum (argon, positive mode)* m/z (rel. ratio) 653 (M - 2H + 3Na, 4%), 631 (M - H + 2Na, 10), 609 (M + Na, 18), 587 (M + H, 8), 550 (4), 522 (4), 455 (5), 366 (7), 309 (10), 238 (21), 180 (22), 159 (77), 131 (55), 126 (72), 109 (29), 102 (37), 87 (65), 65 (100). Amino acid analysis: Ala 1.17 (1), Asx 0.83 (1), Glu 1.07 (1), Thr 0.99 (1).

Solution phase synthesis of Asn-Glu-Ser(P)*-Thr-Ala*

Boc-Ser(PO_3Ph_2)-Thr(Bzl)-Ala-OBzl (15)

The mixed anhydride coupling was performed using dipeptide trifluoroacetate (1.00 mmol) (**2**) according to the general procedure with diethyl ether used as the extraction solvent. Tripeptide (**15**) was obtained as a light yellow oil (0.759 g, 96%). NMR: δ (^{13}C) ($CDCl_3$) 15.2, Ala Cβ; 17.7, Thr Cγ; 28.1, Boc Me; 48.2, Ala Cα; 55.3, $J_{P,C}$ 6.6 Hz, Ser Cα; 56.6, Thr Cα; 66.8, Ala Bzl CH_2; 67.9, $J_{P,C}$ 6.6 Hz, Ser Cβ; 71.4, Thr Bzl CH_2; 73.9, Thr Cβ; 80.9, Boc CMe_3; 119.9, $J_{P,C}$ 5.5 Hz, Ser Ph C2; 125.5, 127.6, 128.0, 128.1, 128.2, 128.4 and 129.8, Ph and Bzl C2,3,4; 135.4, Ala Bzl Cl; 137.9, Thr Bzl Cl; 150.1, $J_{P,C}$ 6.6 Hz, Ser Ph Cl; 155.5, Boc CO; 168.4 and 168.8, Ser and Ala CO; 171.9, Thr CO. δ (^{31}P) ($CDCl_3$) -11.5.

Boc-Glu(OBzl)-Ser(PO_3Ph_2)-Thr(Bzl)-Ala-OBzl (17)

The mixed anhydride coupling was performed using tripeptide trifluoroacetate (0.835 mmol) (**16**) according to the general procedure with diethyl ether used as the extraction solvent. Tetrapeptide (**17**) was obtained as a crunchy off-white solid (0.768 g, 91%), m.p. 106–108 °C. NMR: δ (^{13}C) ($CDCl_3$) 15.1, Ala Cβ; 17.7, Thr Cγ; 27.6, Glu Cβ; 28.1, Boc Me; 30.2, Glu Cγ, 48.1, Ala Cα; 53.7, $J_{P,C}$ 6.6 Hz, Ser Cα; 53.8, Glu Cα; 56.6, Thr Cα; 66.3 and 66.8, Glu and Ala Bzl CH_2; 67.6, $J_{P,C}$ 5.5 Hz, Ser Cβ; 71.3, Thr Bzl CH_2; 73.9, Thr Cβ; 80.0, Boc CMe_3; 119.9, $J_{P,C}$ 4.4 Hz, Ser Ph C2; 125.5, 127.5, 128.0, 128.1, 128.3 and 129.7, Ph and Bzl C2,3,4; 135.3 and 135.6, Glu and Ala Bzl C1; 137.9, Thr Bzl C1; 150.1, $J_{P,C}$ 6.6 Hz, Ser Ph Cl; 155.4, Boc CO; 167.7 and 168.6 Ser and Ala CO; 172.0, 172.3 and 172.8, Glu and Thr CO, Glu δ-CO. δ (^{31} P) ($CDCl_3$) - 11.4.

Z-Asn(Trt)-Glu(OBzl)-Ser(PO_3Ph_2)-Thr(Bzl)-Ala-OBzl (19)

The mixed anhydride coupling was performed using tetrapeptide trifluoroacetate (0.10 mmol) (**18**) according to the general procedure with diethyl ether used as the extraction solvent. Evaporation of the solvent under reduced pressure gave a crude yellow solid which was recrystallised from dichloromethane/diethyl ether to give pentapeptide (**19**) as a white solid (0.118 g, 84%), m.p. 113–115 °C (dec.). NMR: δ (^{13}C) ($CDCl_3$) 15.4, Ala Cβ; 17.7, Thr Cγ; 25.6, Glu Cβ; 30.5, Glu Cγ; 38.3, Asn Cβ; 48.3, Ala Cα; 51.8, Asn Cα; 53.8, Glu Cα; 54.2, $J_{P,C}$ 6.6 Hz, Ser Cα; 56.7, Thr Cα; 66.5, 66.9, 67.1, Z, Glu and Ala Bzl CH_2; 67.6, $J_{P,C}$ 4.4 Hz, Ser Cβ; 70.8, Thr Bzl CH_2; 71.4, Trt CPh_3; 74.3, Thr Cβ; 120.0, $J_{P,C}$ 5.5 Hz, Ser Ph C2; 125.5, Ser Ph C4; 127.0, 127.6, 127.9, 128.1, 128.4, 128.5, Ph, Bzl and Trt Ph C2,3,4; 129.8, Ser Ph C3; 135.5, 135.7, 136.0, Z, Glu and Ala Bzl C1; 138.1, Thr Bzl C1; 144.1, Trt C1; 150.2, $J_{P,C}$ 6.6 Hz, Ser Ph C1; 156.2, Z, CO; 168.0, 168.8, 170.1, 171.7 (x2), 172.1, 173.1, Asn, Glu, Ser, Thr, Ala CO, Asn β-CO, Glu δ-CO. δ (^{31}P) ($CDCl_3$) -12.4.

H-Asn-Glu-Ser(P)*-Thr-Ala-OH·CF_3CO_2H (20)*

Pentapeptide (**19**) (0.113 g, 0.081 mmol) was dissolved in CF_3CO_2H (2 mL) and stood for 1 h. The solution was evaporated, the residue dried under vacuum and then triturated with diethyl ether (10 mL). A solution of the white solid in 50% CF_3CO_2H/CH_3CO_2H (4 mL) containing 83% platinum oxide (0.050 g) was charged with hydrogen and stirred vigorously until hydrogen uptake ceased (<1 h). The platinum was removed by gravity filtration and the solvent evaporated under reduced pressure. The residue was triturated with diethyl ether (3 x 30 mL),

*For all of the reported FAB mass spectral data, the molecular ion M is defined as the non-protonated peptide.

dried under high vacuum and lyophilized from water to give pentapeptide (**20**) as a white foam (0.047 g, 81%). NMR: δ (^{13}C) (D_2O) 16.1, Ala Cβ; 18.7, Thr Cγ; 25.7, Glu Cβ; 29.9, Glu Cγ, 34.7, Asn Cβ; 49.0, Asn Cα; 49.7, Ala Cα; 53.5, Glu Cα; 54.7, $J_{P,C}$ 7.3 Hz, Ser Cα; 59.0, Thr Cα; 63.9, $J_{P,C}$ 2.9 Hz, Ser Cβ; 67.0, Thr Cβ; 169.0, 171.1 (x2), 172.7, 173.1, 176.9, Asn, Glu, Ser, Thr, Ala CO, Asn β-CO, Glu δ-CO. δ (^{31}P) (D_2O) +0.7. FAB mass spectrum (argon, positive mode) m/z (rel. ratio) 639 (M + K, 6%), 623 (M + Na, 9), 601 (M + H, 58), 521 (62), 505 (10), 477 (5), 428 (9), 337 (14), 262 (31), 244 (53), 216 (57), 191 (78). Amino acid analysis: Ala 1.02 (1), Asx 0.96 (1), Glu 0.98 (1), Ser/Ser(*P*) 0.91 (1), Thr 0.93 (1).

FmoclSolid phase synthesis—general procedure

Fmoc/solid phase synthesis was performed on an ABI 430A instrument operating in the DCC/HOBt mode of peptide chemistry and used Fmoc-Ala-Wang Resin (0.20 mmol) as the polymer support {Wang Resin = 4-(hydroxymethyl)phenoxymethyl-copoly(styrene–1% divinyl benzene)resin}. On completion of peptide assembly, the peptide–resin was treated with 5% anisole/CF_3CO_2H (5 mL) for 1 h. The resin was then removed by gravity filtration, the solvent evaporated under reduced pressure and the peptide precipitated by the addition of diethyl ether.

H-*Asn-Glu-Tyr*(P)*-Thr-Ala-OH·CF_3CO_2H (11)*

Pentapeptide (**11**) was obtained as a white fluffy powder (0.142 g, 90%). NMR: δ (^{13}C) (D_2O) 15.5, Ala Cα; 16.0, Thr Cγ; 26.2, Glu Cβ; 29.6, Glu Cγ, 34.8, Asn Cβ; 35.7, Tyr Cβ; 48.8, Asn Cα; 49.5, Ala Cα; 52.7, Glu Cα; 53.8, Tyr Cα; 55.9, Thr Cα; 70.2, Thr Cβ; 120.5, d, $J_{P,C}$ 4.4 Hz, Tyr Ar C3; 130.4, Tyr Ar C1; 131.7, Tyr Ar C2; 150.9 d, $J_{P,C}$ 4.4 Hz, Tyr Ar C4; 166.1, 168.5, 170.7, 172.3 and 172.6, Asn, Glu, Tyr and Thr CO, Asn β-CO; 175.4 and 176.8, Ala CO and Glu δ-CO. δ (^{31}P) (D_2O) -4.4. FAB mass spectrum (argon, positive mode) m/z (rel. ratio) 677 (M + H, 24), 505 (10), 429 (6), 367 (28), 278 (82), 275 (100), 258 (50), 245 (70), 215 (98). Amino acid analysis: Ala 1.05 (1), Asx 0.93 (1), Glu 1.02 (1), Thr 1.02 (1), Tyr 0.99 (1).

H-*Asn-Glu-Ala-Thr-Ala-OH·CF_3CO_2H (12)*

Pentapeptide (**12**) was obtained as a white fluffy powder (0.113 g, 91%). NMR: (^{13}C) (D_2O) 16.2 and 16.4, $Ala^{3,5}$ Cβ; 18.7, Thr Cγ, 26.1, Glu Cβ; 29.8, Glu Cγ, 34.8, Asn Cβ; 48.8, Asn Cα; 49.7 and 49.8, $Ala^{3,5}$ Cα; 53.0, Glu Cα; 58.8, Thr Cα; 67.0, Thr Cβ; 168.8, 171.2, 172.4, 172.6, 174.8, 176.2 and 176.8, Asn, Glu, $Ala^{3,5}$ and Thr CO, Asn β-CO, Glu δ-CO. FAB mass spectrum (argon, positive mode) m/z (rel. ratio) 527 (M + Na, 5%), 505 (M + H, 92), 487 (10), 461 (6), 460 (6), 416 (10), 371 (12), 357 (2), 337 (6), 315 (34), 300 (25), 272 (15), 262 (22), 244 (82), 227 (10), 216 (47), 191 (82), 185 (100), 173 (55), 157 (32), 143 (100+), 115 (92), 102 (100+). Amino acid analysis: Ala 2.09 (2), Asx 1.01 (1), Glu 1.03 (1), Thr 0.92 (1).

H-*Asn-Ala-Tyr-Thr-Ala-OH·CF_3CO_2H (13)*

Pentapeptide (**13**) was obtained as a white fluffy powder (0.121 g, 93%). NMR: δ (^{13}C) (D_2O) 16.0, Ala Cβ; 16.4, Thr Cγ; 18.5, Ala Cβ; 34.8, Asn Cβ; 36.1, Tyr Cβ; 48.8, Asn Cα; 49.6 and 49.7, $Ala^{2,5}$ Cα; 55.1, Tyr Cα; 58.6, Thr Cα; 67.2, Thr Cβ; 115.3, Tyr Ar C3; 127.8, Tyr Ar C1; 130.4, Tyr Ar C2; 154.4, Tyr Ar C4; 168.6, 170.6, 172.6, 173.1, 174.1 and 176.3, Asn, Tyr, Thr, $Ala^{2,5}$ CO, Asn, β-CO. FAB mass spectrum (argon, positive mode) m/z (rel. ratio) 539 (M + H, 55 %), 525 (20), 506 (12), 450 (7), 425 (23), 407 (7), 349 (22), 337 (45), 321 (20), 259 (10), 245 (23), 191 (100+). Amino acid analysis: Ala 2.12 (2), Asx 0.88 (1), Thr 0.98 (1), Tyr 1.03 (1).

H-*Asn-Glu-Tyr(Me)-Thr-Ala-OH·CF_3CO_2H (21)*

Pentapeptide (**21**) was obtained as a white fluffy powder (0.139 g, 96%). NMR: δ (^{13}C) (D_2O) 16.1, Ala Cβ; 18.6, Thr Cγ 26.0, Glu Cβ; 29.5, Glu Cγ; 34.8, Asn Cβ; 36.0, Tyr Cβ; 48.8, Asn Cα; 49.7, Ala Cα; 53.4, Glu Cα; 55.0, Tyr Cα; 55.2, Tyr Me; 58.7, Thr Cα; 67.2, Thr Cβ; 114.0, Tyr Ar C3; 128.6, Tyr Ar Cl; 130.3, Tyr Ar C2; 157.7, Tyr Ar C4; 168.8, 170.6, 172.2, 172.5, 172.7, 176.2 and 176.6, Asn, Glu, Tyr, Thr and Ala CO, Asn β-CO, Glu δ-CO. FAB mass spectrum (argon, positive mode) m/z (rel. ratio) 633 (M + Na, 2%), 611 (M + H, 42), 595 (3), 567 (2), 522 (7), 477 (5), 421 (21), 404 (2), 368 (18), 348 (5), 300 (16), 279 (26), 244 (100), 216 (57), 191 (85), 161 (100+). Amino acid analysis: Ala 1.10 (1), Asx 1.10 (1), Glu 1.09 (1), Thr 0.88 (1), Tyr 0.95 (1).

H-*Asn-Glu-Phe-Thr-Ala-OH·CF_3CO_2H (22)*

Pentapeptide (**22**) was obtained as a white fluffy powder (0.129 g, 93%). NMR: δ (^{13}C) (D_2O) 16.3, Ala Cβ; 18.6, Thr Cγ; 26.1, Glu Cβ; 29.7, Glu Cγ; 34.8, Asn Cβ; 36.9, Phe Cβ; 48.9, Asn Cα; 49.7, Ala Cα; 53.3, Glu Cα; 54.9, Phe Cα; 58.7, Thr Cα; 67.2, Thr Cβ; 127.2, 128.7 and 129.1, Phe Ar C2,3,4; 136.2, Phe Ar C1; 168.8, 170.6, 172.3, 172.5, 172.7, 176.4 and 176.8, Asn, Glu, Phe, Thr and Ala CO, Asn β-CO, Glu δ-CO. FAB mass spectrum (argon, positive mode) m/z (rel. ratio) 603 (M + Na, 1%), 581 (M + H, 38), 563 (4), 537 (3), 492 (6), 447 (7), 391 (19), 374 (3), 338 (18), 300 (21), 272 (10), 244 (92), 216 (48), 191 (88), 171 (19), 143 (100), 102 (100+). Amino acid analysis: Ala 1.12 (1), Asx 0.96 (1), Glu 0.98 (1), Phe 0.97 (1), Thr 0.91 (1).

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