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Tyrosine Analogues as Alternative Substrates for Protein Tyrosine Kinase Csk: Insights into Substrate Selectivity and Catalytic Mechanism

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Abstract—Protein tyrosine kinases are critical enzymes in cell signal transduction but relatively little is known about the molecular recognition of the tyrosine substrate by these enzymes. Details of tyrosine substrate specificity within the context of a short peptide were investigated for protein tyrosine kinase Csk. It was found that aryl ring functional group substitutions the size of methyl group or smaller were generally well tolerated by the protein tyrosine kinase Csk whereas larger groups caused a decline in substrate efficiency. Extension of the phenol from the peptide backbone by a single methylene was acceptable for phosphorylation whereas removal of a methylene nearly abolished reactivity. Only the L-tyrosine derivative was processed. A negative charge *ortho* to the phenol hydroxyl was incompatible with substrate reactivity, consistent with previous pH rate profiles which indicated the importance of the neutral phenol. Overall, these studies confirmed the interpretation of a previous linear free energy relationship analysis which suggested that the enzyme followed a dissociative transition state mechanism.

Introduction

Protein tyrosine kinases catalyze the transfer of the γ-phosphoryl group from ATP to the phenol side chain of protein tyrosine residues (Fig. 1). Interest in the biology of protein tyrosine kinases has intensified in recent years. These enzymes have emerged as key signalling switches that can affect cell growth and differentiation.[1] Specificity of protein kinases for their target substrates is intimately associated with the regulation and dysregulation of cell signal transduction pathways.[2] Defining the characteristics of natural and unnatural molecules that lead to protein tyrosine kinase active site recognition should be instructive in the generation of specific protein kinase inhibitors.[3] While it is now well-recognized that the nucleotide binding site of protein kinases can be exploited to generate highly specific protein kinase inhibitors,[4] the peptide/protein substrate binding site could in principle provide an even greater source of specificity for the design of protein kinase inhibitors targeted to an individual protein kinase. Relatively little is known in a general sense about features of the tyrosine phenol side-chain, or potential substitutions on the aromatic ring, that could enhance or detract from binding or catalysis.

The catalytic mechanism of protein tyrosine kinase Csk, an enzyme important in modulating immune system activation and neuro-development, has been suggested to involve a dissociative transition state with relatively minor nucleophile involvement.[5] Among the experiments used to support a dissociative transition state include the analysis of linear free energy relationships (measurements of β_{nuc} and $\beta_{leaving\ group}$) using a series of peptides containing fluorotyrosine derivatives as substrates.[5a,b] The observation was made that fluoro-substitution of the aromatic ring of the substrate, while significantly altering the phenol pK_a, had minimal impact on recognition and phosphorylation efficiency by Csk. Steric and electronic impacts of the fluorine atoms on enzyme active site binding were assumed to be minor. In addition, the paradoxical observation was made that the phenoxide anion species of the fluorotyrosine containing substrates was found to be less enzymatically active than the corresponding neutral phenol. Electrostatic repulsion of the anions within the enzyme active site in the transition state was assumed to be the dominant factor here.

A systematic investigation is lacking of the tolerance of tyrosine kinases toward unnatural tyrosine derivatives

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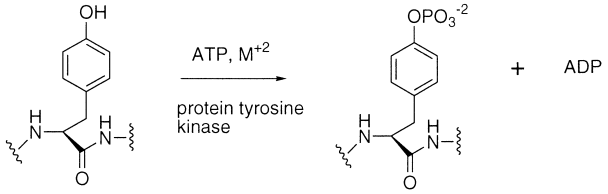

Figure 1. Protein tyrosine kinase reaction.

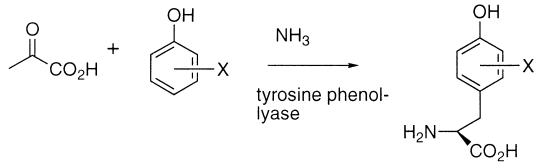

Figure 3. Tyrosine phenol-lyase (TPL) reaction used to make tyrosine analogues.

with varying features of size, polarity, charge and stereochemistry. To extend our understanding of substrate recognition by protein kinases, we undertook a more detailed investigation of the effects of ring substitution and phenol positioning on protein tyrosine kinase Csk catalytic processing, and report the results herein.

Results and Discussion

A series of tyrosine analogues with varied steric, electronic and stereochemical features was incorporated in place of tyrosine into a short, well-characterized Csk substrate peptide sequence Glu-Asp-Asn-Glu-Tyr-Thr-Ala ($K_m \approx 5$ mM, $k_{cat} \approx 20\ min^{-1}$). The tyrosine derivatives used in this study were prepared either using the enzyme, tyrosine phenol-lyase (**2**–**5**, **9**), or obtained from commercially available sources (**6**–**8**) (Fig. 2). Tyrosine phenol-lyase has been found to be generally useful in preparing L-tyrosine derivatives using the corresponding phenols, ketoacid and ammonia (Fig. 3).[6] These synthetic tyrosine analogues were converted to the corresponding *N*-Fmoc derivatives without side-chain protection using standard conditions. The corresponding Fmoc derivatives were used in automated solid phase peptide synthesis to generate the desired peptides, purified by reversed phase HPLC and characterized by electrospray mass spectrometry. To produce the 3-hydroxymethyltyrosine (**3**) containing peptide, it proved necessary to alter the standard resin-cleavage/deprotection conditions (omitting the anisole from trifluoroacetic acid/water/anisole). Agents such as anisole were shown to undergo acid-catalyzed attack at the benzylic position of **3** in the peptide. To prepare the salicylic acid tyrosine (**10**) containing peptide, prior to cleavage from the resin and deblocking the peptide containing **3** was oxidized with tetrapropyl ammonium perruthenate and mCPBA (Fig. 4).[7] A combination of the MS and NMR data of the purified peptide was used to confirm product identity in this case.

Each peptide was tested as a protein tyrosine kinase Csk alternative substrate using an established HPLC assay. To verify the structural identity of the product of the phosphorylation reaction, the reaction products were separated from the unphosphorylated peptides and the phosphorylation state was confirmed by mass spectrometric analysis. We propose that, given the increased availability and efficiency of modern mass spectrometry (MALDI, ESI) in

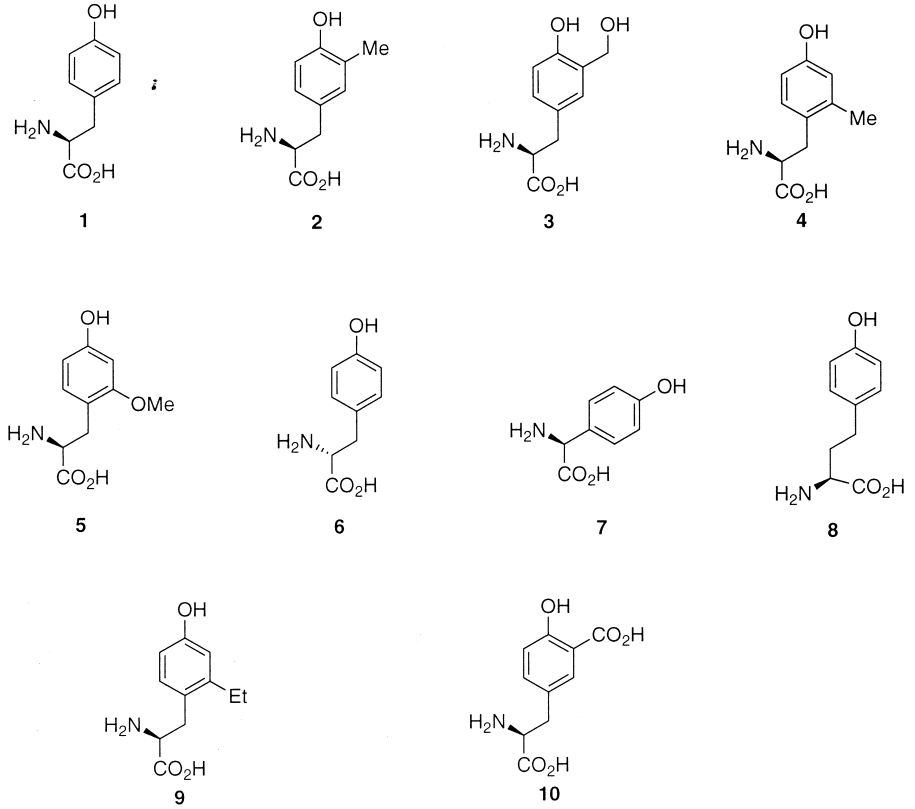

Figure 2. Tyrosine analogues investigated in this work.

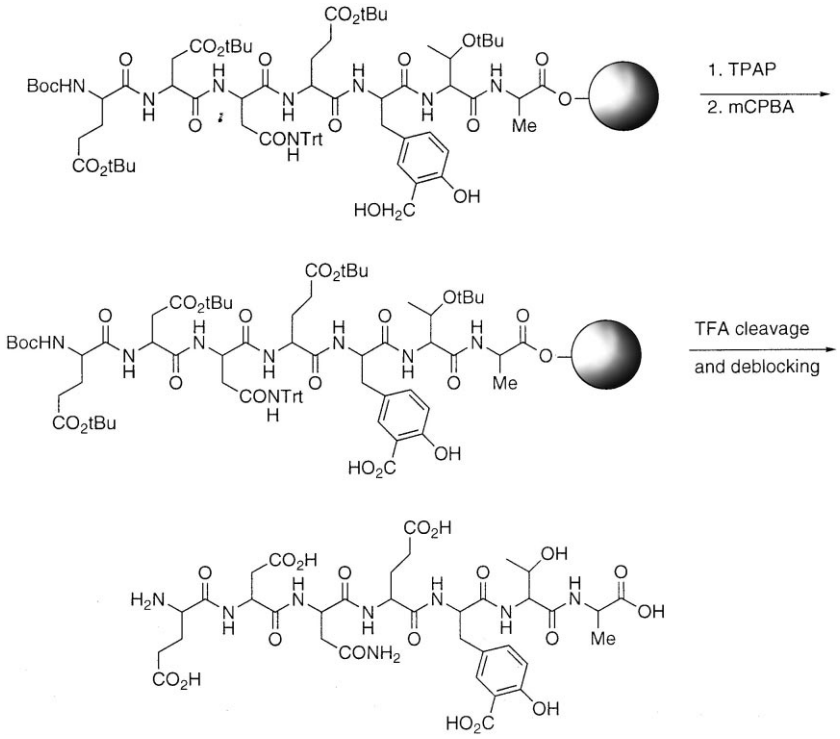

Figure 4. Conversion of salicylic alcohol containing peptide to the salicylic acid derivative.

analyzing biopolymers, this should become a routine procedure for investigators carrying out kinase assays for the first time with unnatural tyrosine derivatives, to avoid artifacts associated with less direct approaches. All peptides which were Csk substrates showed linear kinase activity versus time and enzyme concentration, allowing determination of the catalytic efficiency, k_{cat}/K_m, and the results are shown in Table 1.

The range of substituents allowed us to address several questions: (i) How would increased steric bulk *ortho* or *meta* to the phenol hydroxyl influence catalysis? (ii) How would hydrogen bond donating/accepting groups *ortho* to the phenol affect recognition or catalysis? (iii) Can a benzylic hydroxyl change phosphorylation site selectivity? (iv) How is a peptide containing D-tyrosine handled by the enzyme? (v) How important is the spacing between the phenol and the peptide backbone for kinase processing? (vi) What are the effects of introducing a negative charge *ortho* to the phenol hydroxy?

Table 1. Catalytic efficiency of peptides containing tyrosine analogues as Csk tyrosine kinase substrates[a]

Tyrosine analogue	Relative k_{cat}/K_m
L-Tyrosine (**1**)	100%
3-Methyltyrosine (**2**)	38%
3-Hydroxymethyltyrosine (**3**)	23%
2-Methyltyrosine (**4**)	90%
2-Methoxytyrosine (**5**)	12%
D-Tyrosine (**6**)	< 0.5%
L-Hydroxyphenylglycine (**7**)	0.75%
Homotyrosine (**8**)	26%
2-Ethyltyrosine (**9**)	21%
3-Carboxytyrosine (**10**)	< 0.5%

[a]The relative efficiencies (k_{cat}/K_m) are referenced to substrate peptide with L-tyrosine and measured as described in Experimental. Estimated standard errors for the values (given as percent of maximal activity) are ±20%.

Steric bulk

Placement of a methyl group *ortho* to the phenol hydroxyl as present in **2** had a modest 2.5-fold effect on decreasing catalytic processing efficiency compared to tyrosine and the even bulkier substituent hydroxymethyl found in **3** decreased activity as a kinase substrate by 4-fold compared to tyrosine (see Table 1). *Meta* substitution of the tyrosine aromatic ring with a methyl group represented in **4** led to minimal impact on processing, whereas OMe substitution (peptide containing **5**) did cause an 8-fold drop in processing compared to tyrosine. Since it was uncertain whether rate reduction with OMe resulted from the electronic effect of oxygen or the increased steric effect due to a larger group, the ethyl substituted analogue **9** was evaluated. The ethyl derivative was similar to the OMe analogue as a Csk substrate, suggesting that electronic effects of the oxygen atom at the *meta* position were non-disruptive.[8]

Taken together, these results suggest that the steric contributions of fluorine atoms should have minimal effect on the β_{nuc} or $\beta_{leaving\ group}$ measured in previous studies.[5,8] Thus, a dissociative mechanism is still the favored interpretation of the small β_{nuc} reported previously. However, kinase processing is somewhat sensitive to (and disrupted by) substrate steric effects for larger substituents at the *ortho* position and to a lesser extent at the *meta* position of tyrosine in peptide substrates. It is also noteworthy that no enhancement above the rate of tyrosine processing was observed with any of the

derivatives evaluated in this study (which contrasts *meta*-fluorotyrosine behavior, which was a 2–3-fold more efficient substrate compared to tyrosine[5]). Thus, it is unlikely that increased stabilization of the transition state complex can be achieved by enhancing additional active site interactions compared to a standard tyrosine.

ortho Hydrogen bond donor

The presence of the hydroxymethyl *ortho* to the phenol hydroxy in **3** has the capacity to disrupt hydrogen bonding or augment hydrogen bonding to active site groups. In particular, **3** is expected to maintain an internal hydrogen bond between the phenol oxygen and the CH_2OH oxygen. The practical effect of this group was minimal (less than 2-fold compared to the corresponding methyl analogue **2**), suggesting that potential H-bonding between the hydroxymethyl and the phenol oxygen or active site groups does not interfere. Alternatively, the benzylic hydroxy group could fill space normally occupied by a water molecule in this position and the net effects on catalysis would be minor. It is worth noting that the benzylic hydroxy group could be converted into a leaving group if it were protonated by an active site acidic group. In principle a peptide containing **3** could therefore cause irreversible inactivation of Csk. However, no time-dependent loss of Csk kinase activity was detected with this peptide, arguing against the presence of such acid activation and/or a reactive enzyme nucleophile nearby. In future work, it will be worth investigating whether a better leaving group such as fluoride can enhance enzyme-induced alkylation.[9]

Does 3 get phosphorylated only on the phenol?

The capacity for the benzylic hydroxy function to receive a phosphate from ATP in the presence of the standard phenol was unknown. Modelling suggested that the *ortho* hydroxymethyl could extend far enough from the peptide backbone to simulate typical tyrosine phosphorylation without severe strain. The major product of phosphorylation was revealed to be the phenolic phosphate (UV, NMR). HPLC analysis of the reaction mixture revealed no evidence (less than 10% of the rate of phenol phosphorylation) of benzylic site phosphorylation. This contrasts with the findings of promiscuity observed in a system reported by Lawrence and colleagues,[10] although the C-terminal location of the benzyl hydroxy group in that instance could be responsible for enhanced tolerance of unnatural substrates (see below). The higher pK_a of the alkyl hydroxy group versus the phenol would be unlikely to explain the decreased reactivity since the β_{nuc} for the reaction is near zero.[5]

D-Tyrosine as substrate

The ability of protein tyrosine kinases to discriminate between L- and D-tyrosine residues within peptide substrates has not been well studied. The insulin receptor tyrosine kinase has been reported to be unable to process a peptide containing D-tyrosine (**6**),[11] consistent with expectations about the standard stereospecificity of enzyme active sites. In contrast, Lawrence and colleagues found that both Abl and Src were capable of phosphorylating D-tyrosine residues at reasonable enzymatic rates.[10] This work employed an unnatural C-terminal tyrosine linkage making its insight into standard substrate recognition unclear. Investigation of the EGF receptor kinase revealed that a peptide containing D-tyrosine (**6**) was reported to be a fairly efficient substrate, although the phosphorylated peptide product was not rigorously characterized.[12] In the case of Csk and the standard substrate heptapeptide containing D-tyrosine (**6**), no detectable (<200-fold L-tyrosine phosphorylation rate) phosphorylation occurred. It is possible that protein tyrosine kinases are divergent in their ability to discriminate between D- and L-tyrosine residues in the context of peptides. This would be somewhat surprising given the high degree of similarity in the catalytic core of protein tyrosine kinases and further work is needed with a variety of systems to clarify this issue.

Homotyrosine (8) and hydroxyphenylglycine (7) studies

In a dissociative mechanism, the transition state bond formation between the nucleophilic hydroxyl and the ATP γ-phosphorus is minimal. It is therefore expected that the nucleophilic hydroxyl may be positioned quite far away from the γ-phosphorus in the transition state.[13] It might be expected that significant latitude would be tolerated in the distance between peptide backbone and attacking phenol. Peptides containing L-homotyrosine (**8**) and L-hydroxyphenylglycine (**7**) were prepared to examine this issue. Both residues were acceptable as substrates. The homotyrosine (**8**) containing peptide was only 4-fold down compared to tyrosine as a substrate, but hydroxyphenylglycine (**7**) was over 100-fold worse. Thus, positioning of the hydroxy group in the transition state is important in the enzyme catalyzed reaction. It has been suggested that the basis for protein tyrosine kinase selectivity versus serine kinases is primarily related to the distance between the hydroxyl and the peptide backbone.[14] The findings with peptide containing hydroxyphenylglycine (**7**) shown to be a poor Csk substrate would tend to support such a proposal, although orienation could also be important.

How do we rationalize these findings with a dissociative transition state of enzymatic phosphoryl transfer? Two broad possibilities include: (i) the binding of the peptide containing hydroxyphenylglycine (**7**) to Csk indirectly causes a distortion in Csk interaction with ATP in the ternary complex and thus slows leaving group (ADP) departure; (ii) there is significant entropy loss with improper nucleophile alignment even in a dissociative transition state. A detailed discussion concerning the second possibility has recently been reported in the nucleoside diphosphate kinase system.[15] Further experimental work will be needed in the Csk system to discriminate between these mechanistic models and is the subject of ongoing work in the lab.

Electrostatic repulsion

The inability of the salicylic acid (**10**) containing peptide to serve as a Csk substrate ($<0.5\%$ kinase rate with the

tyrosine containing peptide) provides strong evidence for the role of electrostatic repulsion in preventing the processing of the phenoxide anion species.[5] The pK_a of the benzoic acid function is estimated to be approximately 3 in free solution. While enzyme active sites can perturb substrate pK_a values, it is unlikely that Csk would raise the pK_a more than 1–2 units at this position[5] and thus this group should be fully deprotonated under the assay conditions (pH 7.4). While the large loss in substrate efficiency of the carboxylic acid substituted derivative could in principle be due to steric repulsion, the relative tolerance of the enzyme toward the peptide with the hydroxymethyl derivative **3** (4.5-fold rate reduction compared to peptide with tyrosine) suggests otherwise. The molecular volumes of CH_2OH versus CO_2H are similar.[16] It can be estimated that the electrostatic repulsion in the transition state by the carboxylate ion leads to at least a 40-fold effect, or a $\Delta G \geqslant 2.3$ kcal/mol. Assuming a similar electrostatic repulsion energy for the phenoxide anion in the Csk transition state, this free energy increase would be nearly enough to offset the modest gain in rate (50-fold assuming $\beta_{nuc} = 0.1$) expected in a dissociative mechanism with the increased nucleophilicity of the phenoxide anion. The loss of a hydrogen bond between the substrate tyrosine phenol and Asp-314 ($\Delta G \approx 3$ kcal/mol)[5] associated with the phenoxide anion species would also be expected to contribute to this species being a poor protein tyrosine kinase substrate.

Conclusion

This work provides new insights into the accessibility of a protein tyrosine kinase active site to unnatural tyrosine derivatives. Some of the key findings include: (i) the tyrosine kinase Csk can tolerate substantial steric bulkiness in the substrate aromatic ring, enhancing the confidence of the interpretations of the linear free energy relationship measurements with the fluorotyrosine derivatives reported previously; (ii) proper fit and alignment for the substrate tyrosine group in the tyrosine kinase active site are important factors in catalysis even in a dissociative transition state; (iii) electrostatic repulsion is an important consideration in the enhanced enzyme reactivity of the neutral phenol species of tyrosine substrates compared to the corresponding phenoxide anions. These studies allow a richer understanding of the substrate selectivity and mechanism of protein tyrosine kinases and should enhance the design of peptidomimetic tyrosine kinase inhibitors. They might also provide opportunities in 'bumps and holes' complementarity studies[17] that could ultimately allow specific control of cellular signal transduction pathways.

Experimental

General

Fmoc-D-tyrosine, Fmoc-L-homotyrosine and Fmoc-L-hydroxyphenylglycine were purchased from Novobiochem and used in peptide synthesis without further protection or purification. All other reagents not described below were obtained from commercial sources in reagent grade quality and used without further purification.

3-Methyltyrosine (**2**), 3-hydroxymethyltyrosine (**3**), 2-methyltyrosine (**4**), 2-methoxytyrosine (**5**), and 2-ethyltyrosine (**9**) were prepared using the enzyme tyrosine phenol-lyase (TPL).[6] Reactions were carried out as previously described using the recombinant enzyme according to the adapted procedures necessary for larger scale work.[5] In particular, reactions were carried out on a 500 mL scale and used 50–100 units of TPL. The typical yields were 0.5–1.5 g of derivative per reaction. Work up and purification by cation exchange chromatography were carried out as described previously. Each analogue was characterized using ^{1}H NMR, ^{13}C NMR, and MS and the spectroscopic data for these compounds are shown below.

3-Hydroxymethyltyrosine (3).[6,18] ^{1}H NMR (D_2O) δ 6.77 (s, 1H), 6.71 (d, $J = 8.4$ Hz, 1H), 6.47 (d, $J = 8.4$ Hz), 4.23 (s, 2H), 3.48 (dd, $J = 6.8$, 5.6 Hz, 1H), 2.69 (m, 2H); ^{13}C NMR (D_2O) δ 174.4, 153.0, 131.3, 129.7, 127.1, 117.1, 115.5, 59.8, 56.3, 35.8; HRMS calcd for $C_{10}H_{13}NO_4$ (MH+), 212.0923; found, 212.0926.

2-Methyltyrosine (4).[6] ^{1}H NMR (D_2O) δ 7.11 (d, $J = 8.0$ Hz, 1H), 6.80 (d, $J = 2.8$ Hz, 1H), 6.72 (dd, $J = 8.0$, 2.8 Hz, 1H), 3.82 (dd, 9.0, 5.8 Hz, 1H), 3.25 (dd, $J = 14.6$, 5.8 Hz, 1H), 2.93 (dd, $J = 14.6$, 9.0 Hz, 1H), 2.31 (s, 3H); ^{13}C NMR (D_2O) δ 180.4, 158.0, 139.0, 131.9, 125.8, 118.6, 114.3, 56.70, 36.5, 19.0; MS (MH^+) m/z 196.

3-Methyltyrosine (2).[6] ^{1}H NMR (D_2O) δ 7.01 (s, 1H), 6.93 (d, $J = 8.0$ Hz, 1H), 6.74 (d, $J = 8.0$ Hz, 1H), 3.58 (dd, $J = 7.4$, 5.4 Hz, 1H), 2.96 (dd, $J = 13.8$, 5.4 Hz, 1H), 2.77 (dd, $J = 13.8$, 7.4 Hz, 1H), 2.14 (s, 3H); ^{13}C NMR (D_2O) δ 179.9, 155.2, 132.2, 128.2, 126.9, 117.0, 115.4, 57.3, 38.5, 15.9; MS (MH^+) m/z 196.

2-Methoxytyrosine (5).[6,19] ^{1}H NMR (D_2O) δ 7.03 (d, $J = 8.0$ Hz, 1H), 6.51 (s, 1H), 6.43 (d, $J = 8.0$ Hz, 1H), 3.91 (dd, $J = 8.0$, 4.4 Hz, 1H), 3.80 (s, 3H), 3.22 (dd, $J = 14.6$, 4.4 Hz, 1H), 2.91 (dd, $J = 14.6$, 8.0, 1H); ^{13}C NMR (D_2O) δ 174.7, 158.9, 157.4, 132.4, 115.1, 107.8, 99.7, 55.6, 55.5, 31.2; HRMS calcd for $C_{10}H_{13}NO_4$ (MH^+), 212.0923; found, 212.0926.

2-Ethyltyrosine (9).[6] ^{1}H NMR (D_2O) δ 7.09 (d, $J = 9$ Hz, 1H), 6.80 (d, $J = 2$ Hz, 1H), 6.70 (dd, $J = 9$, 2 Hz, 1H), 3.81 (dd, $J = 9$, 6 Hz, 1H), 3.27 (dd, $J = 14$, 6 Hz, 1H), 2.91 (dd, $J = 14$, 9 Hz, 1H), 2.61 (q, $J = 7.5$ Hz, 2H), 1.16 (t, $J = 7.5$ Hz, 3H); ^{13}C NMR (D_2O) δ 175.3, 155.7, 145.8, 132.2, 125.5, 116.3, 113.7, 56.5, 33.7, 25.4, 15.1; HRMS calcd. for $C_{11}H_{15}NO_3$ (MH+), 210.1130; found 210.1120.

Conversion to the Fmoc derivatives

The Fmoc derivatives of all tyrosine analogues were prepared using sodium carbonate and 9-fluorenylmethyl *N*-succinimidyl carbonate in dioxane according to methods described previously, worked up as previously stated by extraction and used in peptide synthesis without further purification.[5]

Peptide synthesis

(a) The peptides incorporating the unnatural tyrosine analogues were synthesized and purified according to previously described methods.[5] Cleavage and deprotection of the peptide containing salicyl alcohol function were carried out in the absence of anisole to enhance the yield of the desired material. The molecular weight of each heptapeptide was confirmed by electrospray mass spectrometry and all were shown to be >95% pure by reverse-phase HPLC (C-18 column, H_2O, CH_3CN, trifluoroacetic acid).

(b) The salicylic acid containing peptide was prepared by oxidizing the salicylic alcohol containing peptide in the solid phase which had been prepared with an N-terminal Boc group. The resin bound peptide (795 mg; 0.1 mmol scale) was suspended in DMF (10 mL), treated with 141 mg (0.4 mmol) of tetrapropyl ammonium perruthenate and 4-methylmorpholine *N*-oxide (1.4 g, 12 mmol) with stirring for 5 h. The reaction mixture was filtered and washed with DMF, CH_2Cl_2, MeOH, water and dried under vacuum overnight. The resultant resin was treated with mCPBA (77%, 859 mg) in DMF (12 mL) and dimethoxyethane (5 mL) and after stirring for 28 h at 4 °C was washed (DMF, MeOH, CH_2Cl_2) and filtered. The resultant resin was treated with standard deprotection/cleavage conditions (5 mL trifluoroacetic acid/0.25 mL water/0.25 mL anisole mixture) and the peptide isolated by ether precipitation and purified by reverse-phase HPLC (typical yield of purified peptide was ~1–2 mg). The molecular weight was confirmed by electrospray MS and ^1H NMR of the purified peptide revealed the loss of the methylene signal attributed to the hydroxymethyl protons and appropriate aromatic shifts characteristic of the salicylic acid derivative.

Enzyme assays

Assays were carried out using previously described methods with quantification of the substrate and phosphopeptide product by HPLC integration at 214 nm.[5] Briefly, the conditions included saturating ATP (2 mM), 1 mM DTT, 5 mM $MnCl_2$, 60 mM Tris–HCl, pH 7.4, 30 °C. Electrospray mass spectrometry was used to confirm the identity of the phosphopeptide product in each case. All rates were measured in a linear range with respect to time and enzyme concentration with <10% turnover of the limiting substrate. Peptide substrate concentration ranges were varied from 0 to 4 mM and the data were fit to the Michaelis Menten equation which in most cases showed that the K_m was >4 mM so that only the k_{cat}/K_m could be reliably compared for each. All experiments were performed at least twice.

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