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Derivatization of phosphopeptides with mercapto- and amino-functionalized conjugate groups by phosphate elimination and subsequent Michael addition

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Received 21st April 2005, Accepted 17th June 2005
First published as an Advance Article on the web 13th July 2005

Kinetics of the β-elimination of the phosphate group from H–Tyr–Ser(PO_3H_2)–Phe–OH and H–Tyr–Thr(PO_3H_2)–Phe–OH and subsequent addition of thiols and amines to the dehydroalaninyl and β-methyldehydroalaninyl residues formed, were followed by RP HPLC under alkaline conditions in the absence and presence of Ba^{2+} ions. By this reaction sequence, the phosphoserinyl peptide was conjugated with mono-*N*-(2-mercaptoethyl)amide of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (**4**), a mercapto-functionalized pentapeptide, H–His–Gly–Gly–His–Gly–$NH(CH_2)_4SH$, and an amino-functionalized fluorescent dye, 5-dimethylaminonaphthalene-1-[*N*-(5-aminopentyl)]sulfonamide (dansyl cadaverine). The β-methyldehydroalanine residue was, in turn, observed to be a poor Michael acceptor.

Introduction

Reversible phosphorylation of serine, threonine and tyrosine residues in proteins is a post-translational modification which plays a major role in intracellular signaling.[1–3] Accordingly, enrichment and identification of phosphoproteins from the protein pool of the cell is one of the central issues of the development of chemical tools for cell biology.[4] Traditional ^{32}P-radiolabeling,[5] phosphospesific antibodies[6] and various MS-techniques combined with affinity chromatography,[7] while highly useful in numerous cases, still suffer from some limitations. In particular, quantification of low abundance phosphoproteins would benefit from effective enrichment to a solid support, which allows removal of non-phosphorylated proteins by simple washing. Recently, two tagging methods applicable to this purpose have been demonstrated.[8] One of these is based on phosphate group elimination from phosphoserine and phosphothreonine residues and subsequent nucleophilic addition of the affinity tag.[9–11] A similar approach has been applied to introduce mass spectroscopy tags[12] and to convert phosphoserine and phosphothreonine to lysine analogs that are recognized by lysine-specific proteases.[13] The other approach, in turn, consists of a multistep conversion of the phosphate group to a tagged phosphoramidate and it may, hence, be applied to enrichment of all phosphoproteins, including the tyrosine-containing ones.[14] Both approaches appear highly promising, but await thorough chemical analysis for evaluation of their full potential.

In fact, exploitation of sequential phosphate elimination and Michael addition for structural modification of peptides dates back to the mid 1980s. In 1986 Meyer *et al.*[15] converted phosphoserine-containing peptides to their *S*-ethylcysteine counterparts before sequencing by Edmans degradation. Recently, glycosyl thiolates have been introduced to dehydroalaninyl peptides by Michael addition.[16,17] In spite of this rather long history and several applications, quantitative data on the course of elimination and subsequent addition in aqueous solution is limited to the early studies of Byford, concerning the conversion of a phosphoserine residue to dehydroalanine in saturated barium hydroxide and subsequent addition of methylamine.[18] Even the data on addition of amines and thiols to monomeric dehydroalanine and its simple derivatives is surprisingly scarce and it usually refers to non-aqueous conditions.[19–22] Only recently, a more extensive study on Michael addition of amines and thiols to a dehydroalanine amide in various aqueous solvents has been reported.[23]

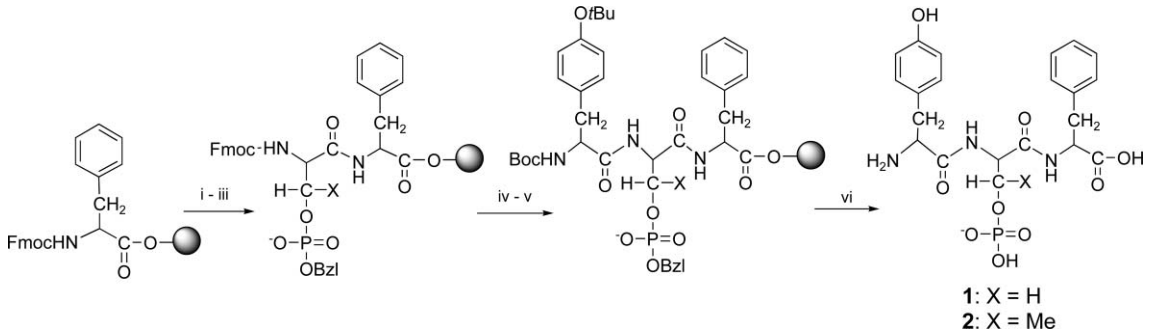
The present study is aimed at providing a solid chemical basis for the exploitation of base-catalyzed phosphate elimination in chemical tagging of phosphopeptides by intermediary formation of a dehydroalanyl peptide. For this purpose, the kinetics of phosphate elimination from two tripeptides, H–Tyr–Ser(PO_3H_2)–Phe–OH (**1**) and H–Tyr–Thr(PO_3H_2)–Phe–OH (**2**), and subsequent Michael addition of various mercapto and amino nucleophiles have been studied. As an application, mono-*N*-(2-mercaptoethyl)amide of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (**4**) has been prepared and introduced in **1** by sequential phosphate elimination and Michael addition. Since the 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) moiety may be expected to exhibit a reversible pH-dependent binding to metal-ion-loaded solid supports, conversion of phosphopeptides to DOTA-conjugates offers a viable method for their enrichment. In addition, they can possibly be used as element-coded tags in mass spectrometry.[24] Such conjugates may additionally find applications in biomedical research as carriers of radioactive or paramagnetic metal ions in various imaging techniques[25] or radiopharmaceutical applications.[26] In addition to DOTA group, the phosphoserinyl peptide (**1**) has been derivatized with a mercapto-functionalized pentapeptide, H–His–Gly–Gly–His–Gly–NH-$(CH_2)_4SH$ (**3**), a mimic of metal-ion chelating peptides,[27] and with a fluorescent dye, 5-dimethylaminonaphthalene-1-[*N*-(5-aminopentyl)]sulfonamide (dansyl cadaverine).

Results and discussion

Preparation of peptides and mono-*N*-(2-mercaptoethyl)amide of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid

Phosphopeptides H–Tyr–Ser(PO_3H_2)–Phe–OH (**1**) and H–Tyr–Thr(PO_3H_2)–Phe–OH (**2**) were prepared by manual synthesis on a Wang resin applying the Fmoc-strategy. The phosphorylated amino acids were introduced as monobenzyl esters to avoid elimination of a fully protected phosphate group.[28] Activation by 1-*H*-benzotriazolium-1-[bis(dimethylamino)methylene)hexafluorophosphate *N*-oxide (HBTU) and 1-hydroxybenzotriazole (HOBt) in the presence of *N*,*N*-diisopropylethylamine (DIPEA) was used for coupling (Scheme 1). Treatment with aqueous trifluoroacetic acid (TFA) containing dithiotreitol resulted in final deprotection and cleavage from the resin. The crude peptides were purified by reversed-phase HPLC and characterized by NMR and mass spectroscopy.

DOI: 10.1039/b505573c



Scheme 1 *Reagents and conditions*: i) 20% piperidine–DMF; ii) Fmoc–Ser[PO(OBzl)OH]–OH; iii) HBTU, HOBt, DIPEA, DMF; iv) 20% piperidine–DMF; v) Boc–Tyr(*t*Bu)–OH, HBTU, HOBt, DIPEA, DMF; vi) TFA, DTT, H_2O.

H–His–Gly–Gly–His–Gly–NH$(CH_2)_4$SH (**3**), used for tagging of **1**, was assembled on a commercially available (4-aminobutanethio)-4-methoxytrityl support applying the Fmoc chemistry and 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo-[4,5-*b*] pyridinium hexafluorophosphate 3-oxide (HATU) coupling. Acidolytic cleavage from the resin, followed by HPLC purification, gave the mercapto functionalized peptide.

Commercially available 4,7,10-tris(2-*tert*-butoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1-acetic acid was converted to its *N*-(6-amino-3,4-dithiahexyl)amide by HBTU/*N*-hydroxysuccinimide (NHS) activated amidation with cystamine in DMF (Scheme 2). Reduction with tris(2-carboxyethyl)phosphine (TCEP) in aqueous dioxane, followed by acidolytic removal of the *tert*-butyl groups with aqueous TFA containing triisopropylsilane (TIPS) then gave the desired product (**4**).

Elimination of the phosphate group

According to the results of Byford,[18] elimination of the phosphate group from phosphoserinyl peptides in aqueous alkali (Scheme 3) is catalyzed by group II metal ions, the catalytic efficiency increasing in the order $Ca^{2+} < Sr^{2+} < Ba^{2+}$. To quantify the rate-accelerating effect of Ba^{2+} ion on elimination of the phosphate group from both phosphoserinyl and phosphothreoninyl residues and to examine the occurrence of side reactions at various hydroxide and barium ion concentrations, elimination of the phosphate group from **1** and **2** was followed as a function of pH (10.0–12.0) and barium ion concentration (0.005–0.050 mol dm^{-3}) at 60 °C. The composition of the aliquots withdrawn at appropriate intervals from the reaction mixture was determined by RP HPLC. The products were identified by a mass spectrometric analysis (HPLC–ESI-MS).

In 0.1 mol dm^{-3} aqueous sodium hydroxide containing barium nitrate 0.041 mol dm^{-3}, the conversion of phosphoserinyl peptide **1** ($t_R = 5.3$ min) to the respective dehydroalaninyl peptide **5a** ($t_R = 25.1$ min) was quantitative. When 80% of the starting material was disappeared, not even traces of any other products could be observed. In 0.01 mol dm^{-3} sodium hydroxide, the elimination product (**5a**) was still the only product detected during the first half-life of the disappearance of **1**, but, on prolonged treatment, slow hydration of **5a** to diasteromeric serinyl peptides **6a** ($[M + H]^+ = 414.4$, $t_R = 9.0$ and 9.6 min) and isomerization to several products having the same molecular mass as **5a** ($[M + H]^+ = 398.4$, $t_R = 13.2$, 14.7 and 27.0 min) took place (Fig. 1). Formation of the isomeric products may tentatively be attributed to intramolecular cyclizations by nucleophilic attack on the β-carbon of the dehydroalanine residue. The structures of these

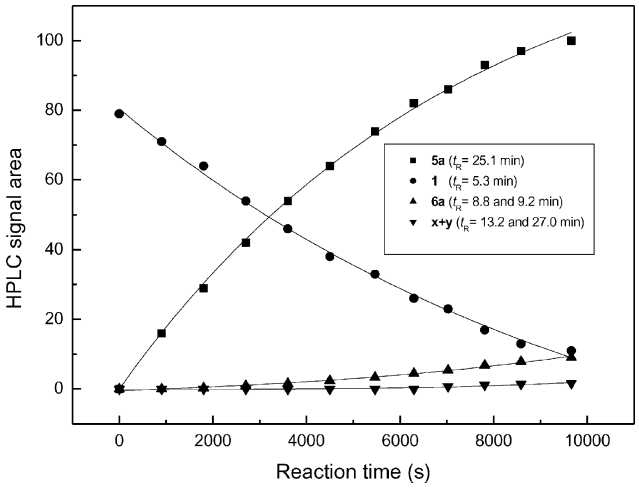
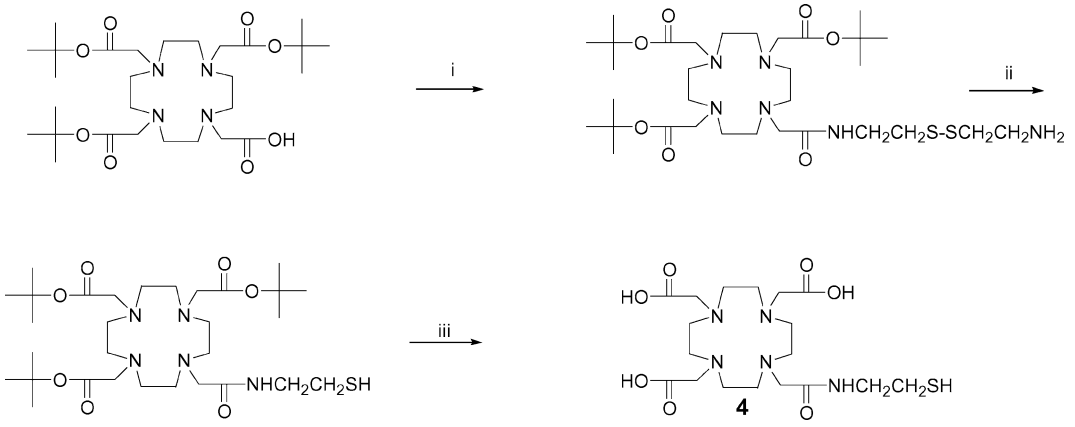
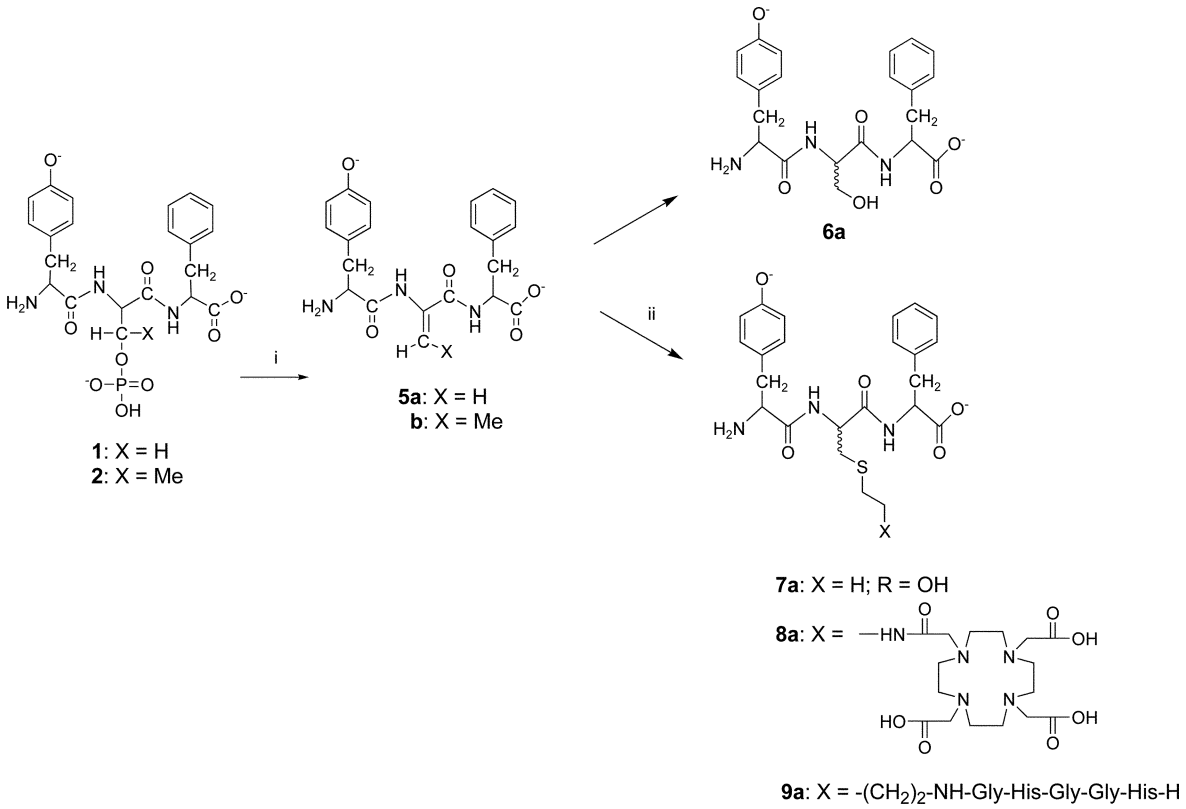


Fig. 1 Time dependent product distribution for the conversion of phosphoserinyl peptide **1** to dehydroalaninyl peptide **5a** in 0.010 mol dm^{-3} aqueous sodium hydroxide containing 0.041 mol dm^{-3} $Ba(NO_3)_2$ at 60 °C. **6a** refers to diastereomeric serinyl peptides. The two other products, exhibiting retention times of 13.2 and 27.0 min, are unidentified isomers of **5a**.



Scheme 2 *Reagents and conditions*: i) cystamine, HBTU, NHS, DMF; ii) TCEP, dioxane–H_2O; iii) TFA–TIPS–H_2O.



Scheme 3 *Reagents and conditions*: i) NaOH, DMSO, EtOH, $Ba(NO_3)_2$; ii) RCH_2CH_2SH.

products were not, however, unambiguously determined. In less basic solutions, the subsequent intramolecular reactions became so fast compared to the elimination that several products were observed already during the early stage of the disappearance of **5a**.

With phosphothreoninyl peptide (**2**) (t_R = 11.6 min), the elimination was almost one order of magnitude slower than with **1**. Despite this, no additional products appeared during the first two half-lives of the disappearance of **2** at hydroxide ion concentrations higher than 0.010 mol dm^{-3}. Evidently the subsequent reactions of the elimination product (**5b**) ($[M + H]^+$ = 412.4; t_R = 33.0 min) are retarded by the presence of the methyl group even more markedly than the elimination. This is expected, since the methyl group protects the β-carbon against a nucleophilic attack. Only at $[OH^-] < 0.001$ mol dm^{-3} were traces of additional products observed.

The elimination of both **1** and **2** was observed to be first-order in hydroxide ion concentration at 0.001 mol dm^{-3} < $[OH^-]$ < 0.100 mol dm^{-3} (Fig. 2) and first-order in barium ion concentration at 0.005 mol dm^{-3} < $[Ba^{2+}]$ < 0.050 mol dm^{-3} (Fig. 3). In other words, one proton is removed on going to the transition state and one metal ion is involved in this process. One may tentatively assume that the rate-limiting step involves abstraction of the phosphoserine α-proton by a hydroxo ligand of the phosphate bound Ba^{2+}.

To quantify the rate-accelerating effect of Ba^{2+} ion, the elimination was additionally studied in the absence of the metal ion. The elimination product (t_R = 25.1 min) was still clearly accumulated in 0.1 mol dm^{-3} aqueous sodium hydroxide at 90 °C, but its subsequent hydration to the diastereomeric serinyl peptides (**6a** t_R = 8.8 and 9.2 min) and isomerization to unidentified products mentioned above (t_R = 13.2 and 27.6 min) were so fast that the product mixture became complicated at a rather early stage of the disappearance of the phosphopeptide (Fig. 4). In 0.01 mol dm^{-3} aqueous sodium hydroxide, the elimination product **5a** was not any more markedly accumulated.

Comparison of the data in Fig. 2 and Fig. 3 indicates that Ba^{2+} ion at a concentration 0.050 mol dm^{-3} accelerates the elimination of the phosphate group by two orders of magnitude. With the phosphothreoninyl peptide (**2**) for example, the rate constants observed at $[OH^-]$ = 0.1 mol dm^{-3} and 60 °C are 2.5×10^{-4} s^{-1} and 2.1×10^{-6} s^{-1} in the presence and absence of Ba^{2+} ion (0.050 mol dm^{-3}), respectively. The subsequent transformation reactions of the elimination products are not similarly accelerated and, hence, the elimination product is quantitatively accumulated in the presence of Ba^{2+} ion, but not in its absence.

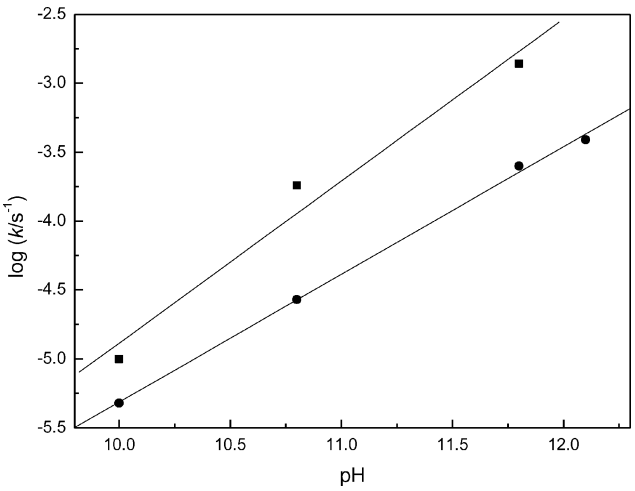


Fig. 2 The effect of pH on the first-order rate constant of the conversion of phosphoserinyl **1** (■) and phosphothreoninyl peptide **2** (●) to the corresponding dehydropeptides **5a** and **5b** in the presence of Ba^{2+} (0.040 and 0.050 mol dm^{-3} with **1** and **2**, respectively) at 60 °C. The pH was adjusted with sodium hydroxide and a triethylamine buffer (I = 0.25 mol dm^{-3} with $NaNO_3$). The pK_a value of water is 12.82 under the experimental conditions.[29]

As suggested previously,[10,13] addition of DMSO and ethanol to aqueous alkali accelerates the elimination of the phosphate group. When the elimination was carried out in an 8 : 3 : 1 (v/v/v) mixture of water, DMSO and ethanol saturated with $Ba(OH)_2$,

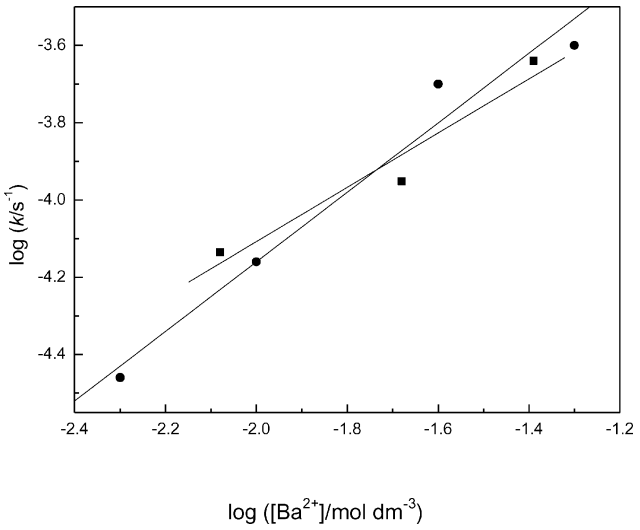


Fig. 3 The effect of Ba^{2+} ion concentration on the first-order rate constant of the conversion of phosphoserinyl peptide **1** (■) and phosphothreoninyl peptide **2** (●) to their dehydroalanine analogs **5a** and **5b** at 60 °C. The data referring to **1** and **2** was obtained at pH 10.8 and 11.8 ($I = 0.25$ mol dm^{-3} with $NaNO_3$), respectively.

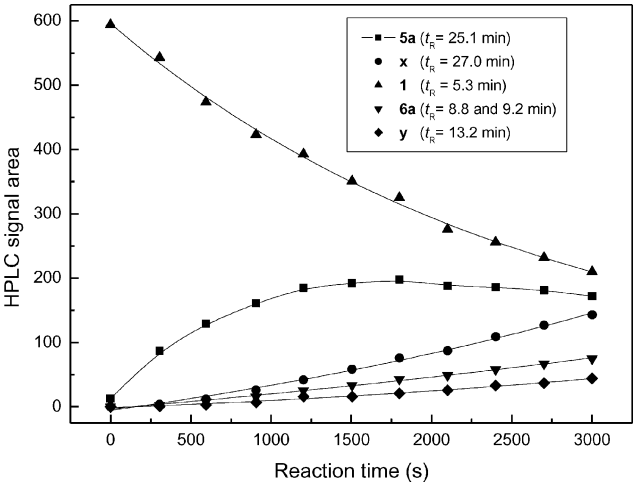


Fig. 4 Time dependent product distribution for the conversion of phosphoserinyl peptide **1** to dehydroalaninyl peptide **5a** in 0.10 mol dm^{-3} aqueous sodium hydroxide at 90 °C. **6a** refers to diastereomeric serinyl peptides. The two other products, exhibiting retention times of 13.2 and 27.0 min, are unidentified isomers of **5a**.

the first-order rate constant for the elimination reaction of **1** was 2.1×10^{-2} s^{-1} at 60 °C and 3.0×10^{-4} s^{-1} at 25 °C, *i.e.* 10-fold faster than in 0.1 mol dm^{-3} aqueous NaOH containing 0.040 mol dm^{-3} $Ba(NO_3)_2$. To avoid possible precipitation of $BaCO_3$ during the reaction, the Ba^{2+} ion concentration was in subsequent studies adjusted to 0.040 mol dm^{-3} with $Ba(NO_3)_2$ and the hydroxide ion concentration to 0.13 mol dm^{-3} with NaOH in the same solvent mixture. In this solution, the first-order rate constant for the elimination of **1** was $(1.36 \pm 0.06) \times 10^{-3}$ s^{-1} at 37 °C, a temperature used in the subsequent conjugation studies. Under these conditions, the elimination proceeded to completion without formation of any other products.

Addition of thiols to the dehydroalaninyl peptide 5a

Conversion of phosphoserinyl peptide **1** to diastereomeric *S*-(2-hydroxyethyl)cysteinyl peptides **7a** was first studied by carrying out the elimination in an equimolar mixture of hydroxide and 2-hydroxyethyl sulfide ions ($[OH^-] = [HOCH_2CH_2S^-] =$ 0.1 mol dm^{-3}) in the absence of Ba^{2+} ions. The 2-hydroxyethyl sulfide ion did not markedly affect the rate of the disappearance of **1**. The first-order rate constant was 6.5×10^{-4} s^{-1} at 90 °C, *i.e.* approximately double to the value obtained in the absence of the sulfide ion. This moderate rate acceleration may, however, result from the fact that addition of the sulfide ion increased the ionic strength from 0.1 to 0.2 mol dm^{-3}. No elimination product (**5a**) was accumulated, but the starting material ($t_R =$ 5.3 min) was quantitatively converted to diastereomeric *S*-(2-hydroxyethyl)cysteinyl peptides ($[M + H]^+ = 476.2$, $t_R = 11.6$ and 14.8). In other words, the attack of the sulfide ion on the β-carbon of dehydroalaninyl residue was sufficiently fast as to prevent all the transformation reactions of **5a** that took place in its absence.

The addition of thiols to the dehydroalaninyl peptide **5a** was then studied at 37 °C. The phosphoserinyl peptide **1** was first allowed to undergo complete elimination to **5a** in an 8 : 3 : 1 (v/v/v) mixture of water, DMSO and ethanol, containing sodium hydroxide 0.13 mol dm^{-3} and $Ba(NO_3)_2$ 0.040 mol dm^{-3}. After 90 min, *i.e.* when **1** (1.5×10^{-4} mol dm^{-3}) was entirely converted to **5a**, β-mercaptoethanol was added to give the final concentration of 0.1 mol dm^{-3}. In other words, the hydroxide ion concentration was decreased from 0.13 mol dm^{-3} to 0.03 mol dm^{-3}, owing to deprotonation of β-mercaptoethanol to 2-hydroxysulfide ion. Under these conditions, the addition reaction proceeded smoothly giving the diastereomeric *S*-(2-hydroxyethyl)cysteinyl peptides (**7a**) as the only products. The pseudo first-order rate constant for the disappearance of **5a** was $(1.31 \pm 0.02) \times 10^{-2}$ s^{-1}. Similar experiment with 2-aminoethanethiol gave the rate constant $(1.30 \pm 0.02) \times 10^{-2}$ s^{-1}.

The mono-*N*-(2-mercaptoethyl)amide of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (**4**) was efficiently conjugated to phosphoserinyl peptide **1** in a similar manner. A 0.0077 mol dm^{-3} solution of **1** in an 8 : 3 : 1 mixture of H_2O–DMSO–EtOH (100 mm^3) containing sodium hydroxide (0.13 mol dm^{-3}) and $Ba(NO_3)_2$ (40 mmol dm^{-3}) was allowed to undergo elimination to **5a** at 37 °C. After completion of the elimination, **4** dissolved in 0.5 mol dm^{-3} aqueous sodium hydroxide (120 mm^3) was added, giving a final concentration of 0.055 mol dm^{-3}. Disappearance of **5a** (t_R 27.0 min) was accompanied by formation of two products (t_R 22.8 and 23.8 min) exhibiting the expected molecular ion mass of the desired conjugate **8a** ($[M + H]^+ = 861.7$). In addition, only a minor amount (< 3%) of disulfide of **4** (t_R 15.0 min) was formed. At this 15-fold excess of **4** compared to **5a**, the disappearance of **5a** obeyed first-order kinetics, the pseudo first-order rate constant being $(1.69 \pm 0.02) \times 10^{-3}$ s^{-1}. At a one order of magnitude lower concentration of **4** (0.0055 mol dm^{-3}) and **5a** (7.7×10^{-4} mol dm^{-3}), the addition was one order of magnitude slower ($k = 1.8 \times 10^{-4}$ s^{-1}). Under these conditions, oxidative dimerization of **4** was, however, more marked; about 15% of **4** was converted to a disulfide.

Mercapto-functionalized peptide, H–His–Gly–Gly–His–Gly–$NH(CH_2)_4SH$ (**3**), was conjugated similarly to the dehydroalaninyl peptide **5a**. Upon addition of **3** (t_R 16.2 min) in 15-fold excess compared to **5a**, gradual disappearance of **5a** was accompanied by appearance of the diastereomeric peptide conjugates **9a** at t_R 23.4 and 23.7 min ($[M + H]^+ = 949.0$). In addition, the disulfide dimer of **3** was formed (t_R 19.2 min, $[M + H]^+ = 1100.1$).

The β-methyldehydroalanininyl peptide (**5b**) was much less susceptible than **5a** to addition of thiols. It was not observed to act as a Michael acceptor for 2-mercaptoethanol under conditions where **5a** was readily attacked. 1,2-Ethanedithiol was observed to attack **5b**, but 50 times less readily than **5a**. Conjugation with **4** failed entirely under conditions where the dehydroalaninyl peptide was quantitatively converted to a DOTA conjugate.

Addition of amines to the dehydroalaninyl peptide 5a

Addition of an amino-functionalized fluorescent dye, 5-dimethylaminonaphthalene-1-[*N*-(5-aminopentyl)]sulfonamide (dansyl cadaverine), to the dehydroalaninyl peptide **5a**, obtained

as described above, was also attempted. Disappearance of **5a** was accompanied by formation two new products at 5.1 and 5.6 min longer retention times, respectively. Both products exhibited the expected mass of $[M + H]^+ = 733.9$ of the dansyl conjugate. Unfortunately, the reaction was not quantitative, but a marked side product having the same mass as **5a** appeared at a 2.5 min longer retention time than **5a**. On using the same 15-fold excess of the attacking nucleophile over **5a**, as used for preparing the DOTA conjugate, the first-order rate constant for disappearance of **5a** was $(3.4 \pm 0.2)\ 10^{-5}\ s^{-1}$ at 37 °C. In other words, the reaction was 50 times slower than with the mercapto-functionalized DOTA, which explains the appearance of additional reaction products formed by intramolecular reactions of **5a**.

Conclusions

In summary, both phosphoserinyl and phosphothreoninyl peptides quantitatively undergo elimination to the corresponding dehydroalaninyl and β-methyldehydroalaninyl peptides under alkaline conditions in the presence of Ba^{2+}. In the absence of Ba^{2+}, dehydroalaninyl residue undergoes subsequent hydration and isomerization reactions so rapidly that side products may be expected to be formed if the enone structure is not efficiently trapped by an external nucleophile. The dehydroalaninyl residue serves as a good Michael acceptor, which allows efficient attachment of even relatively large conjugate groups, such as mercapto-functionalized DOTA or pentapeptide, to the peptide backbone. By contrast, β-methyldehydroalaninyl residue is not attacked by thiols sufficiently to readily allow quantitative introduction of conjugate groups. Attack of amino-functionalized conjugate groups on a phosphoserinyl residue is 50 times slower than the attack of a thiol of comparable size. Accordingly, side products accumulate concurrent with formation of the peptide conjugate.

Experimental

The Fmoc-protected amino acids, HOBt and HBTU were commercial products of Nova BioChem and 4,7,10-tris(2-*tert*-buto'xy-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1-acetic acid a product of Macrocyclics. Wang resin purchased from Nova BioChem was used for the solid-phase peptide synthesis. The NMR spectra were recorded on a Bruker AM 200 or JEOL 400 spectrometer. The ^{1}H NMR chemical shifts (400 MHz, 300 K) were referred to internal TMS and ^{31}P NMR shifts (162 MHz, 300 K) to external orthophosphoric acid. The mass spectra were acquired using a Perkin Elmer Sciex API 365 triple quadrupole LC/MS/MS spectrometer.

The progress of the phosphate elimination was followed by RP HPLC (UV detection at 215 nm). The reaction solutions in stoppered tubes were immersed in a thermostated water bath. Ionic strength was adjusted to 0.1 M with sodium chloride or sodium nitrate. Aliquots were withdrawn at appropriate intervals, neutralized with acetic acid and cooled in an ice bath. The initial peptide concentration was *ca.* 0.15 mmol dm^{-3}. In the absence of metal ions, the separations were carried out on a ODS Hypersil column (4 × 250 mm, 5 μm), using water containing 18% (v/v) acetonitrile and 0.1% (v/v) TFA as an eluent. In the presence of metal ions, the samples were analyzed on a Hypersil HyPURITY C 18 column (4.6 × 150 mm, 5 μm). A 10 min isocratic eluation with 0.1% aqueous TFA was followed by a linear gradient (30 min) up to 35% (v/v) of acetonitrile (flow rate 1 $cm^3\ min^{-1}$). The first-order rate constants were calculated by applying the integrated first-order rate equation to the diminution of the peak area of the starting material.

H–Tyr–Ser(PO_3H_2)–Phe–OH (1)

The synthesis was carried out starting from Fmoc-protected phenylalanine linked to Wang resin (2.0 g, 1 mmol g^{-1}). Free amine groups on the support were capped by acetic anhydride in DMF (1 : 1). The Fmoc group was removed by using 20% piperidine in DMF (20 cm^3) for 15 min. The treatment was repeated (35 min), followed by washing with DMF, DMF–dioxane (1 : 1, v/v) and DMF. The first coupling was achieved by suspending the resin in DMF, then adding Fmoc–Ser[PO(OBzl)OH)–OH (3.98 g, 8 mmol), HBTU, (3.03 g, 8 mmol), HOBt (1.23 g, 8 mmol) and DIPEA (3.4 cm^3). After 3 h shaking, the resin was filtered and washed with DMF, isopropanol and DMF. After removal of the Fmoc group with the conventional piperidine treatment, the second coupling with Boc-protected tyrosine (2.70 g, 8 mmol) was carried out following the same procedure described above. The deprotection and coupling was followed by Kaiser's test. The peptide was cleaved from support and deprotected with a mixture of TFA–DTT–H_2O (38 cm^3: 1 mg : 1 cm^3) for 2 h at rt. The cleavage mixture was filtered and evaporated to one third of the original volume. The crude product was precipitated from cold diethyl ether (300 cm^3), collected by centrifugation and washed with cold ether (5 × 20 cm^3). The peptide was dissolved in water and lyophilized. The crude peptide was purified by reversed-phase HPLC on a Supelco LC-18 column (21.2 × 250 mm, 12 μm), using a mixture of water and acetonitrile (18%) as an eluent (flow rate 6 $cm^3\ min^{-1}$), containing 0.1% TFA. The combined fractions were lyophilized to give 265 mg (27%) of pure product. The authenticity of the product was verified by NMR and mass spectroscopically. ^{31}P NMR (δ_P) (162 MHz, D_2O): 0.20. ^{1}H NMR (δ_H) (400 MHz, D_2O): 7.11–7.25 (m, 5H, C_6H_5), 6.91 (d, 2H, $J = 8.3$ Hz, H3 and H5 of 4-OH–C_6H_4), 6.66 (d, 2H, $J = 8.3$ Hz, H3 and H6 of 4-OH–C_6H_4), 4.45–4.52 (m, 2H, α-H of Phe and Tyr), 4.07 (t, 1H, $J = 7.3$ Hz, α-H of Ser), 3.82–3.92 (m, 2H, β-H of Ser), 3.07 (dd, 1H, $J = 5.4$ and 8.3 Hz, β-H of Phe or Tyr), 2.90 (dd, 1H, $J = 8.3$ and 8.0 Hz, β-H of Phe or Tyr), 2.85 (d, 2H, $J = 7.2$ Hz, β-H of Phe or Tyr). ^{13}C NMR (δ_C) (100.5 MHz, DMSO-d_6): 174.3, 169.5, 169.2, 155.1, 136.3, 130.8, 129.3, 128.8, 127.2, 125.3, 115.9, 64.2, 54.3, 54.1, 53.7, 36.4, 35.9. ESI^--MS: m/z 494.4 $[M–H]^-$.

H–Tyr–Thr(PO_3H_2)–Phe–OH (2)

The phosphothreoninyl peptide **2** was prepared as described for **1**. Yield (37%). ^{31}P NMR (δ_P) (162 MHz, DMSO): −0.96. ^{1}H NMR (δ_H) (400 MHz, DMSO-d_6): 9.32 (br s, 2H, NH_2), 8.72 (d, 1H, $J = 11.6$ Hz, NH of Ser or Phe), 8.44 (d, 1H $J = 12.6$ Hz, NH of Ser or Phe), 7.17–7.24 (m, 5H, C_6H_5), 7.01 (d, 2H, $J = 8.3$ Hz, H3 & H5 of 4-OH–C_6H_4), 6.66 (d, 2H, $J = 8.3$ Hz, H2 and H6 of 4-OH–C_6H_4), 4.43–4.49 (m, 2H, α-H of Phe and Tyr), 4.33–4.49 (m, 1H, β-H of Thr), 4.00 (t, 1H, $J = 11.6$ Hz, α-H of Thr), 3.40 (br s, HDO), 3.05 (dd, 1H, $J = 14.1$ and 5.7 Hz, β-H of Phe or Tyr), 2.89–2.97 (m, 2H, β-H of Phe and Tyr), 2.70 (dd, 1H, $J = 14.5$ and 8.1 Hz), 1.19 (d, 3H, $J = 6.2$ Hz, CH_3). ^{13}C NMR (δ_C) (100.5 MHz, DMSO-d_6): 172.8, 168.8, 168.4, 156.9, 137.7, 130.9, 129.6, 128.7, 126.9, 125.4, 115.8, 72.9, 58.3, 54.1, 54.0, 37.2, 36.2, 18.9. ESI^--MS: m/z 508.5 $[M–H]^-$.

H–His–Gly–Gly–His–Gly–$NH(CH_2)_4SH$ (3)

Peptide **3** was assembled on a commercially available (4-aminobutanethio)-4-methoxytrityl support applying the Fmoc chemistry and HATU coupling. Acidolytic cleavage from the resin, followed by HPLC purification, gave the mercapto-functionalized peptide. The purification was carried out on a LiChrospher RP-18 column (10 × 250 mm,10 μm), using a linear gradient from 0.1% aqueous TFA to MeCN in 30 min. The flow rate was 3.5 $cm^3\ min^{-1}$. ESI–MS: m/z 551.5 $[M–H]^-$.

Mono-*N*-(2-mercaptoethyl)amide of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (4)

4,7,10-Tris(2-*tert*-butoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1-acetic acid (0.5 g, 0.87 mmol) and cystamine were dissolved in 7 cm^3 of DMF, and *N*-hydroxysuccinimide (NHS)

(0.3 g, 2.6 mmol) and HBTU (0.55 g, 1.5 mmol) were added. After the solution was stirred for 24 h at rt, the crude product was isolated by a conventional aqueous workup. The product was dissolved in a mixture of dioxane and H_2O (10 cm^3; 1 : 1, v/v) and TCEP hydrochloride (0.49 g, 1.7 mmol) was added to reduce the disulfide linkage. The pH of the reaction solution was adjusted to 7 with 1.0 mol dm^{-3} sodium hydroxide. The reaction mixture was stirred overnight and the solution was concentrated *in vacuo* to a syrup. The product was dissolved in water and extracted with 3 × 10 cm^3 of CH_2Cl_2. The organic layer was washed with brine and evaporated to dryness. *Tert*-butyl protecting groups were removed with TFA–H_2O–TIPS (95 : 2.5 : 2.5, v/v/v). After the solution was stirred for 20 h, the product was isolated by precipitation from diethyl ether to yield **3** (0.2 g, 10%). ^{1}H NMR (δ_H) (400 MHz, DMSO): 3.10 (8H, br s), 3.24 (4H, m), 3.32 (4H, br s), 3.59 (4H, br s), 3.86 (4H, br s), 4.02 (4H, br s), 8.59 (1H, s). ^{13}C NMR (δ_H) (50.3 MHz, DMSO-d_6): 171.6, 169.0, 158.3, 54.8, 53.8, 52.7, 50.6, 50.4, 48.6, 48.3, 42.2, 23.3. ESI$^+$-MS: m/z 464.7 $[M + H]^+$.

H–Tyr–dhAla–Phe–OH (5a)

^{1}H NMR (δ_H) (400 mHz, 0.1 mol dm^{-3} NaOD): 7.17–7.28 (m, 5H, C_6H_5), 6.87 (d, 2H, J = 8.4 Hz, H3 and H5 of 4-OH–C_6H_4), 5.53 (s, 1H, β-H of dhAla), 5.36 (s, 1H, β-H of dhAla), 4.43–4.46 (m, 2H, α-H of Phe), 3.49 (dd, 1H, J = 6.8 and 6.8 Hz, α-H of Tyr), 3.13 (dd, 1H, J = 14.0 and 4.8 Hz, β-H of Phe), 2.93 (dd, 1H, J = 14,0 and 8.4 Hz, β-H of Phe), 2.68 (dd, 2H, J = 6.8 and 2.8 Hz, β-H of Tyr).

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