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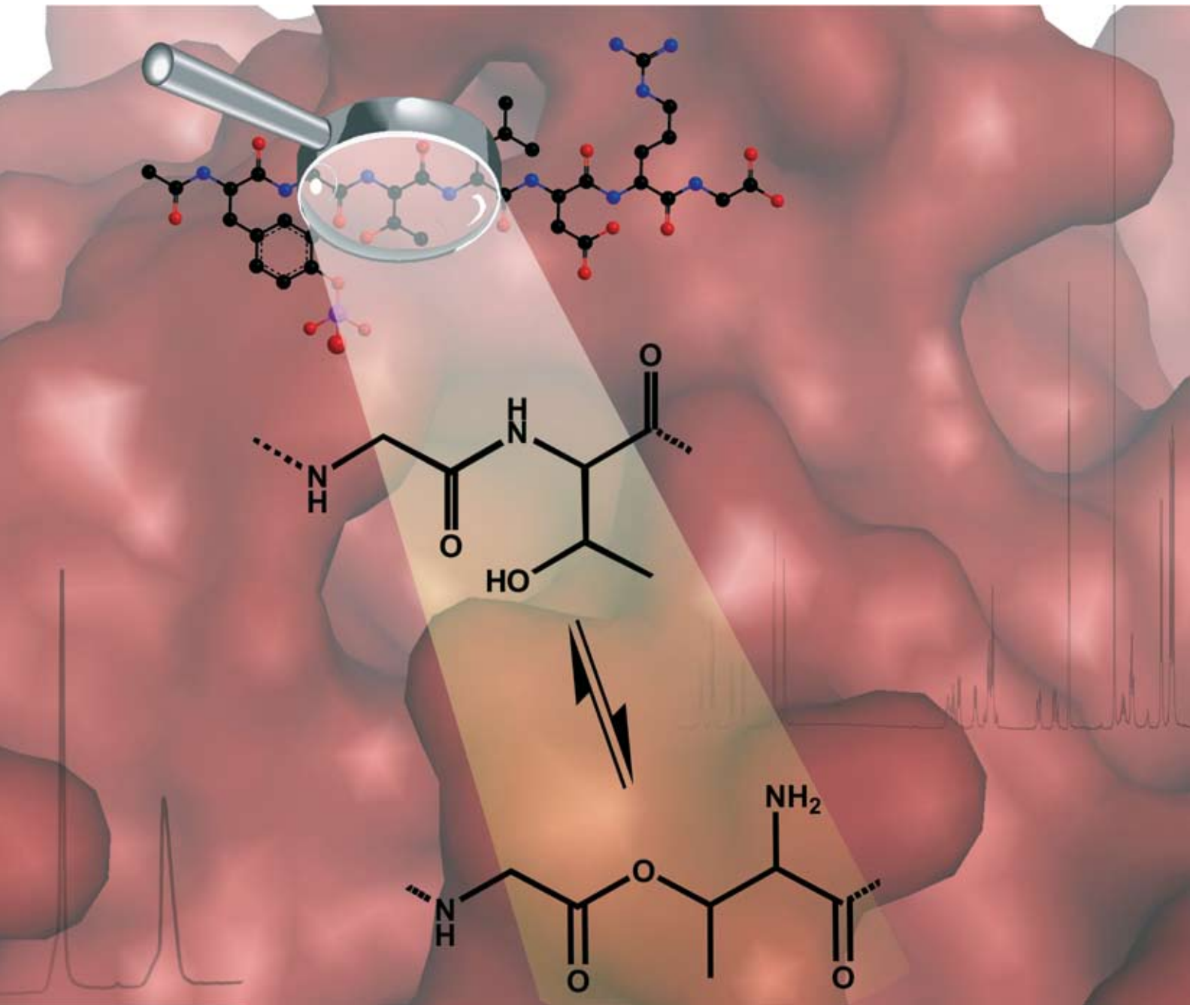

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N→*O*-Acyl shift in Fmoc-based synthesis of phosphopeptides†

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Synthetic phosphopeptides are frequently used as chemical probes to explore protein–protein interactions involved in cellular signal transduction. Most commonly, the solid-phase synthesis of phosphotyrosine-containing peptides is performed by applying the Fmoc-strategy and *N*-Fmoc-protected tyrosine derivatives bearing acid-labile phospho protecting groups. We observed a side-reaction, the isomerisation at threonine, which furnishes depsipeptides. It is shown that the rate of *N*→*O*-acyl migration depends on the sequence context. Depsipeptides were formed most rapidly when the phosphotyrosine was located in the +2 position. Furthermore, different phosphotyrosine building blocks were compared and a suitable method that provides phosphopeptides in enhanced purity and yield is suggested.

Introduction

Phosphorylation of proteins at serine, threonine and tyrosine hydroxyl groups is a ubiquitous intracellular event used for the regulation of protein–protein interactions in signal transduction. Synthetic phosphopeptides allow detailed studies of this key mechanism of cell regulation. For example, phosphopeptides have been used in investigations of the binding determinants,[1] as protein-diagnostic probes[2] and as target-specific inhibitors of protein–protein interactions.[3] Thus, methods that enable a rapid and efficient synthesis of phosphopeptides are of high interest.

In the synthesis of phosphotyrosine-containing peptides the reactivity of phosphoric acid phenyl esters has to be considered. Phosphotyrosine peptides are mainly prepared by two different approaches: a) global phosphorylation, in which the phosphate-group is introduced by a phosphorylating agent (*e.g.* diarylphosphorochloridate or phosphoramidite) after coupling of the amino acid, and b) the use of protected, phosphorylated tyrosine building blocks in the solid phase peptide synthesis (SPPS) known as the synthon method.[4,5] The global approach allows peptide phosphorylation without alteration of the SPPS-method, but problems may occur owing to side reactions during the oxidation step of P^{III} (*e.g.* oxidation of cysteine, methionine or tryptophan residues) or the formation of H-phosphonates.[6] The use of preformed phosphorylated amino acid building blocks avoids these problems. Most commonly, the electrophilic reactivity of phospho groups in these synthons is masked by means of protecting groups in order to avoid pyrophosphate formation and dephosphorylation reactions.[7,8] Phosphotyrosine containing peptides have been synthesised most successfully by applying the Fmoc-strategy and *N*-Fmoc-protected tyrosine derivatives bearing acid-labile phospho protecting groups.[9–11] However, we observed yet another problem that has previously not been reported to occur in phosphopeptide synthesis; the rearrangement at threonine residues to depsipeptides. We herein report our investigations into the identification of parameters that might affect this side-reaction and suggest an improved method that provides phosphopeptides in enhanced purity and yield. It is shown that both sequence context and duration of acid-induced global deprotection critically affect the extent of peptide isomerisation. While the former can not be changed in a given target sequence we demonstrate that the latter can be minimised through a careful choice of phosphate protecting groups.

Results and discussion

In a project directed to the regulation of signal transducing kinases we attempted the solid phase synthesis (Scheme 1) of phosphopeptide **1**, a part of the immuno-tyrosine-activation-motif (ITAM) of the SH2-domain of Syk-kinase.[12–14] For introduction of the phosphotyrosine, the commercially available bisdimethylamino-protected building block was used. However, HPLC analysis of the crude material obtained after acidolytic cleavage revealed two products with identical mass in HPLC-MS (Fig. 1).

Identification of the by-product

High-resolution mass spectrometry and fragmentation of both compounds exposed no difference (Fig. S1†) and racemisation seemed unlikely because of the relatively large difference in retention times. For closer examination we carried out a glycine scan in which each amino acid in phosphopeptide **1** was substituted by glycine (peptides **2–5**, Table 1). The HPLC showed single peaks for peptides **3** and **5** and two peaks for peptides **1**, **2** and **4** (Fig. 2). Thus, the formation of two products required the usage of both phosphotyrosine and threonine building blocks.

NMR-Measurements of both isolated products **4a** and **4b** were performed to obtain structure information. HMQC and HMBC measurements (Fig. S2†) enabled a complete assignment of amino acid protons and carbons in peptides **4a** and **4b** (Scheme 2). The chemical shift values of threonine protons and carbons were most informative. The unusual low field shift of the β-threonine carbon from 67.2 ppm to 70.2 ppm suggested the presence of

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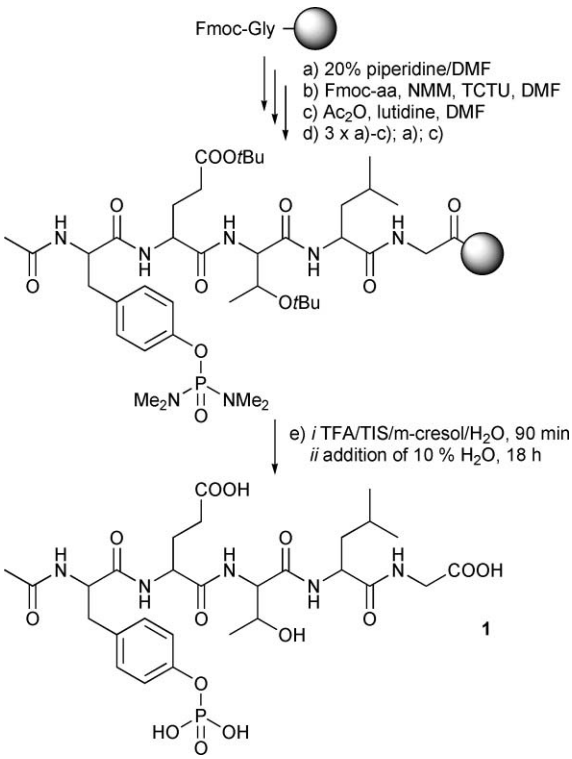
† Electronic supplementary information (ESI) available: High-resolution mass spectra of **1a** and **1b**. HMQC- and HMBC-spectra of **4a** and **4b**, HPLC-traces of **8–11** and **12a**. See DOI: 10.1039/b718568e



Scheme 1 Solid phase peptide synthesis of phosphopeptide **1**.

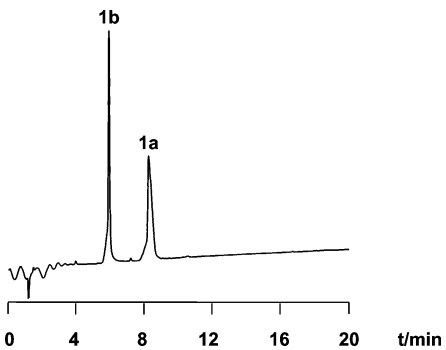


Fig. 1 HPLC trace of crude peptides **1** obtained after solid phase synthesis as described in Scheme 1. (Nucleodur-Gravity C18, 1 ml min^{-1}, 3%–50% buffer B [CH_3CN, 1% water, 0.1% formic acid].)

an electron-withdrawing group at the threonine hydroxy group. The β-threonine proton experienced a similar low field shift from 4.00 ppm to 4.96 ppm. In contrast the α-threonine carbon and proton exhibited a high field shift from 58.4 ppm to 55.8 ppm and from 4.26 ppm to 3.8 ppm, respectively. Furthermore, the glycine carboxyl group had a higher chemical shift ($\delta = 170.2$ ppm) in peptide **4a** than in peptide **4b** ($\delta = 165.7$ ppm). An *O*-acyl structure in peptide **4b** formed upon an acid-induced $N \rightarrow O$-acyl shift (Scheme 2) was considered as a plausible cause. In this case, the chemical shift of the threonine amino group should provide valuable information. There would be two amine protons in depsipeptide **4b** whereas integration would yield only one threonine amide proton for peptide **4a**. Indeed, the ^{1}H-NMR resonance of the threonine amino group in **4b** integrated as two protons. Unfortunately, HMBC spectra failed to provide unambiguous proof as the coupling of the quaternary acyl carbon

Table 1 Synthesised phosphopeptides

Peptide	Sequence
1a/1b	pTyr-Glu-Thr-Leu-Gly/depsipeptide
2a/2b	pTyr-Glu-Thr-Gly-Gly/depsipeptide
3	pTyr-Glu-Gly-Leu-Gly
4a/4b	pTyr-Gly-Thr-Leu-Gly/depsipeptide
5	Gly-Glu-Thr-Leu-Gly
6a/6b	Tyr-Glu-Thr-Leu-Gly/depsipeptide
7a/7b	Gly-Glu-Thr-Leu-Gly/depsipeptide
8	pTyr-Thr-Ala-Ala-Gly
9	pTyr-Ala-Thr-Ala-Gly
10	pTyr-Ala-Ala-Thr-Gly
11	pTyr-Ala-Ala-Ala-Thr-Gly
12a/12b	Asp-Ile-pTyr-Glu-Thr-Asp-Gly/depsipeptide

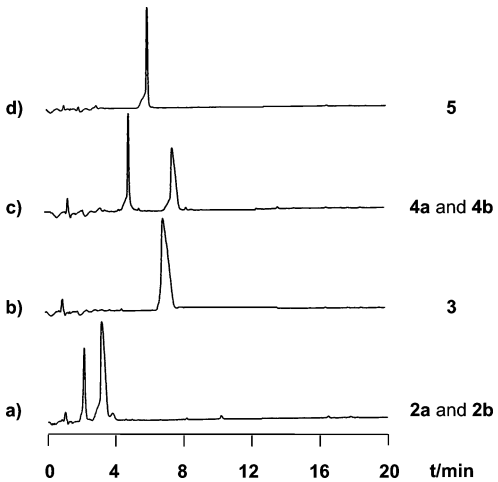
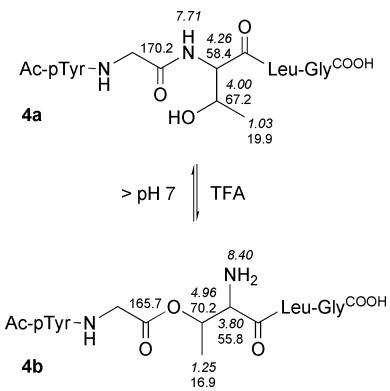


Fig. 2 HPLC traces of crude glycine scan peptides **2–5**. All peptides were prepared using building block Fmoc-TyrPO(NMe_2)$_2$. (Conditions: see Fig. 1.)



Scheme 2 Peptide **4a** and depsipeptide **4b**. Numbers in italic and plain style denote ^{1}H- and ^{13}C-NMR resonances, respectively.

from the N-terminal glycine with the β-threonine proton in **4b** was not detected.

To validate the assumed $N \rightarrow O$-acyl shift reaction we synthesised the depsipeptide **4b** as an authentic reference by coupling Boc-Thr instead of Fmoc-Thr(*t*Bu) according to published procedures.[15] Analysis of authentic **4b** by HPLC-MS (Fig. 3a) and co-injection of the mixture of peptide **4a** and **4b** (Fig. 3b) obtained after solid-phase synthesis suggested the identity. The $N \rightarrow O$-acyl shift reaction is reversible and *O*-acyl peptides have been reported

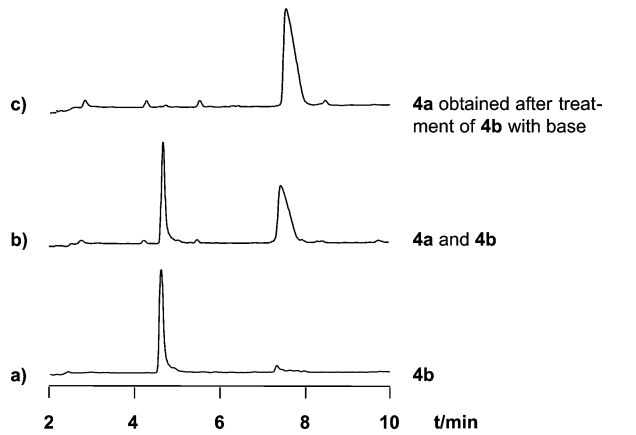


Fig. 3 HPLC analysis of a) authentic depsipeptide **4b**; b) co-injection of authentic **4b** and the mixture **4a**–**4b** obtained after solid phase synthesis of **4a** and c) peptide **4a** obtained after treatment of depsipeptide **4b** with base.

to be amenable to an $O \rightarrow N$-acyl shift when exposed to pH > 7.4. Thus, base-induced conversion of *O*-acyl peptide **4b** to the desired *N*-acyl peptide **4a** can serve as an additional means of validation. Indeed, both the authentic depsipeptide **4b** and the peptide in the mixture could be converted to the desired peptide **4a** by adding base (Fig. 3c).

Sequence dependence of the $N \rightarrow O$-acyl shift

The $N \rightarrow O$-acyl shift in peptides, which leads to depsipeptides, is a possible side reaction that has been reported to occur in Boc-based solid-phase peptide synthesis.[16] This reaction was first reported by Bergmann *et al.* in γ-benzamido-β-hydroxy-butanoic acid.[17] Usually, strong acids such as H_2SO_4, HF or HCl are required to promote the $N \rightarrow O$-acyl transfer.[18–20] Mild acids such as trifluoroacetic acid (TFA) used in the final deprotection in Fmoc-based solid-phase peptide synthesis have not been regarded as troublesome as far as $N \rightarrow O$-acyl shift reactions at serine and/or threonine are concerned. However, a recent report by Carpino and co-workers demonstrated that the incorporation of D-amino acids can increase the susceptibility to depsipeptide formation.[21] An influence of the primary structure on the $N \rightarrow O$-acyl migration was proposed.

We considered the feasibility of the phosphotyrosine residue affecting the rate of the $N \rightarrow O$-acyl shift. Addressing this issue, the phosphotyrosine in peptide **1** was substituted by tyrosine (peptide **6a**) and glycine (peptide **7a**). Thereupon, peptides **1**, **6a** and **7a** were exposed to TFA and the conversion into depsipeptides **1b**, **6b** and **7b**, respectively, was monitored by HPLC-analysis. It became apparent that the phosphotyrosine containing peptide **1** was more susceptible to $N \rightarrow O$-acyl migration than the glycine-containing analogue **6a** (Fig. 4). For example, only 7.9% depsipeptide was formed when **6a** was exposed to a 6 hours TFA treatment while **1** furnished 14.5% depsipeptide. This kinetic analysis revealed that both the amino acid residue and the phospho group affect the $N \rightarrow O$-acyl migration rates.

Next, the distance dependence of the $N \rightarrow O$-acyl shift was examined. Four model peptides (**8**–**11**) comprising alanine, threonine and phosphotyrosine were synthesised. In these peptides the number of alanine units between phosphotyrosine and threonine

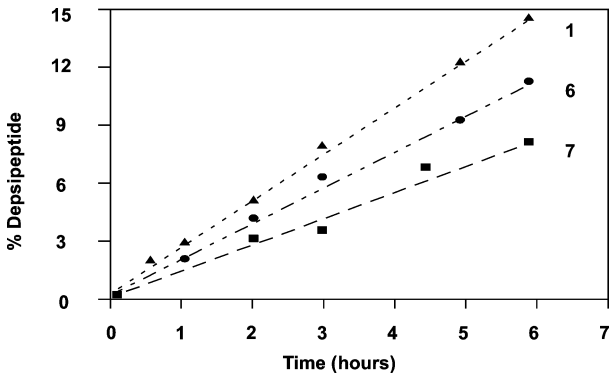


Fig. 4 Time course of depsipeptide formation for peptides **1**, **6** and **7** in TFA.

was varied while keeping the length of the peptide constant. The analysis of the time course of depsipeptide formation (Fig. 5, Fig. S3†) clearly showed that there is an influence of the position of the phosphotyrosine residue on the rate of $N \rightarrow O$-acyl migration. The shift reaction was slowest, furnishing only 8% depsipeptide after 6 hours of TFA treatment, when phosphotyrosine was positioned in the direct N-terminal neighbourhood (see **8**). A remarkably fast $N \rightarrow O$-acyl migration occurred when pY was located in the Thr+2-position. In this case, almost 50% of depsipeptide was obtained after 6 hours exposure to TFA. We speculate that the protonated tyrosine phosphate acts as an internal acid that activates acyl groups for migration. Following this notion, it would be difficult to transfer a proton *via* a cyclic intermediate from tyrosine phosphate to the tyrosine carboxyl group which may explain the low migration rate found for **8**.

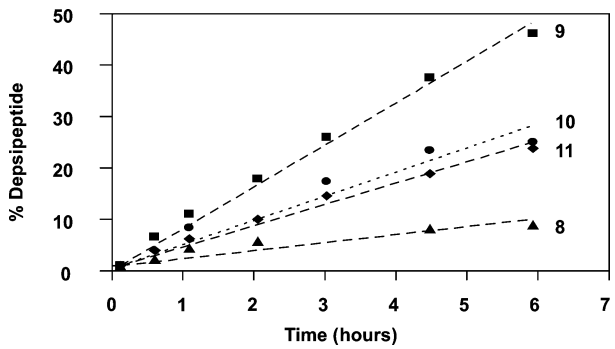


Fig. 5 Time course of depsipeptide formation for peptides **8**–**11** in TFA.

Minimising $N \rightarrow O$-acyl migration in solid phase synthesis

In most cases, phosphopeptide synthesis is performed in order to rapidly and efficiently provide homogeneous material for subsequent biological studies. We sought for a method that minimises the problem of $N \rightarrow O$-acyl migration, thereby avoiding the need to reverse potential $N \rightarrow O$-acyl shift reactions by a precautionary exposure to basic conditions. We supposed that refinement of the TFA treatment required to remove the phospho protecting groups may allow the desired improvements of product purity. The initial synthesis of phosphopeptide **1a** was performed by coupling the bisdimethylamino-masked phosphotyrosine **13** (Fig. 6). For hydrolysis of the phosphoramidate prolonged treatments in

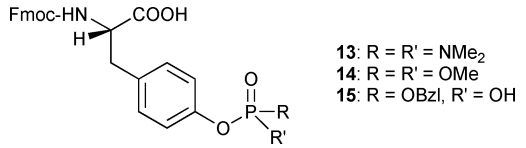


Fig. 6 Protected phosphotyrosine building blocks for Fmoc-based solid phase peptide synthesis.

aqueous TFA solution are required. It has been recommended to firstly detach the phosphotyrosine containing peptide from the resin and to secondly add 10% of H_2O to the TFA–TIS–*m*-cresol–H_2O (85 : 5 : 5 : 5, TIS is triisopropylsilane) filtrate and extend the reaction time to 18 hours.[22–24] We first explored whether the reaction time can be shortened. However, the formation of the depsipeptide **1b** was detected already after 90 minutes (Fig. 7a). After this short reaction time deprotection of the phospho group was not complete and, as a result, the crude product contained only 64% of the desired phosphopeptide **1a** (entry 2, Table 2). As an alternative, the use of the dimethyl-protected building block **14** was examined. The removal of the methyl protecting groups can be accomplished *via* an S_N2-like mechanism under non-aqueous conditions. The reported procedure for deprotection of the dimethylphosphotyrosine moiety involved a 5 hour treatment with 1 M trimethylsilylbromide–thioanisole in TFA (0.05% *m*-cresol).[9,25] The HPLC analysis of the crude product revealed an unacceptably high extent (40%) of depsipeptide formation (entry 3, Table 2). Again, shortening of the deprotection time resulted in the expected decrease of the depsipeptide content (compare Fig. 7b), however, at the cost of incomplete protecting group removal which lead to a rather low content (77%) of phosphopeptide **1** (entry 4, Table 2). It was concluded that the $N\rightarrow O$-acyl migration proceeded equally fast regardless of the water content of the cleavage solution. Thus, an augmentation of the acid lability of the phospho protecting groups was considered necessary. The commercially available monobenzyl-protected phosphotyrosine **15** allows the application of milder cleavage conditions.[26–28] Indeed, a 90 minute treatment with a mixture of TFA–TIS–EDT–H_2O (90 : 2.5 : 2.5 : 5, EDT is 1,2-ethanedithiol) was sufficient to accomplish quantitative deprotection and to deliver phosphopeptide **1a** in high 98% purity of the crude material (Fig. 7c, entry 5 Table 2).

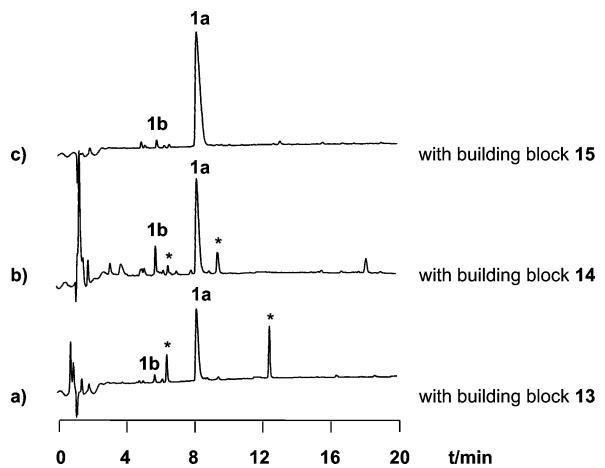


Fig. 7 HPLC-Analysis of crude product **1** obtained after 90 min deprotection by using a) building block **13** and method A; b) building block **14** and method B and c) building block **15** and method C. The peaks marked with an asterisk denote protected products.

The use of the monobenzyl-protected phosphotyrosine building block **15** may give rise to reasons for concern. It is conceivable that coupling reactions that are performed after incorporation of the monoprotected phosphotyrosine building block may be impeded by concomitant reactions at the remaining, potentially nucleophilic phospho oxygen. The solid phase synthesis of phosphopeptide **12a**, which is part of the human insulin receptor,[29,30] addressed this issue. The HPLC-analysis of crude materials confirmed the rather low content of desired **12a** (<65%) in crude materials that was obtained when bisdimethylamino- and dimethyl-protected phosphotyrosine derivatives **13** and **14**, respectively, were incorporated (Fig. 8a–c, entries 6–8 in Table 2). The usage of Fmoc-Tyr(PO(OBzl)OH) **15** furnished, again, the highest 98% purity of crude products (Fig. 8d, entry 9 in Table 2). HPLC-MS analysis showed minor peaks corresponding to truncation products (Fig. S4†). However, the application of double couplings (2 × 6 eq.) completely solved this problem and provided the fully deprotected phosphopeptide **12a** in high purity and an isolated overall yield of 52%.

Conclusions

In summary, we have shown that $N\rightarrow O$-acyl migration can occur at threonine residues during the Fmoc-based solid-phase synthesis of phosphotyrosine-containing peptides. The phosphotyrosine residues were introduced by means of *N*-Fmoc-protected tyrosine derivatives bearing acid-labile phospho protecting groups typically used in phosphopeptide synthesis. HPLC-MS-Data,

Table 2 Purity and yield of synthesised phosphopeptides

	Building block	Method	Crude product	Isolated yield
1	**13**	A, 18 h	69% **1a**, 31% **1b**	n.d.
2	**13**	A, 1.5 h	64% **1a**, 3% **1b**, 33% protected	n.d.
3	**14**	B, 5 h	60% **1a**, 40% **1b**	10%
4	**14**	B, 1.5 h	77% **1a**, 10% **1b**, 13% protected	n.d.
5	**15**	C, 1.5 h	98% **1a**, 2% **1b**	29%
6	**13**	A, 18 h	59% **12a**, 30% **12b**	12%
7	**13**	A, 1.5 h	65% **12a**, 3% **12b**, 33% protected	n.d.
8	**14**	B, 1.5 h	65% **12a**, 24% **12b**, 11% protected	n.d.
9	**15**	C, 1.5 h	98% **12a**, 2% **12b**	52%

A: i) TFA–TIS–*m*-cresol–H_2O (85 : 5 : 5 : 5), 60 min; ii) addition of 10% H_2O; B: 1 M trimethylsilylbromide–thioanisole in TFA (0.05% *m*-cresol); C: TFA–TIS–EDT–H_2O (90 : 2.5 : 2.5 : 5). n.d., not determined.

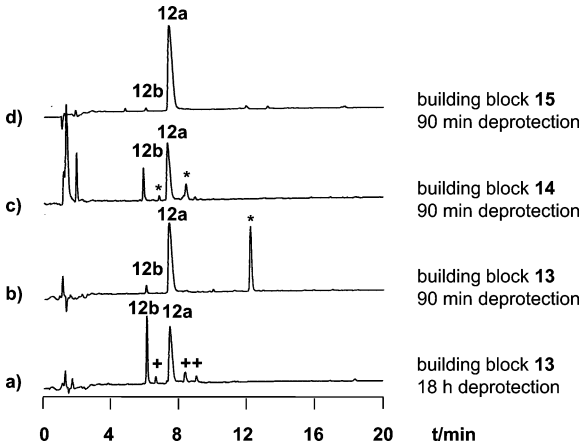


Fig. 8 HPLC-Analysis of crude peptide **12** obtained by using a) building block **13** and method A, 18 h; b) building block **13** and method A, 90 min; c) building block **14** and method B and d) building block **15** and method C. The peaks marked with an asterisk and with a plus denote protected products and aspartamide by-products, respectively.

NMR-spectroscopy and the reversibility of the reaction at basic conditions have provided ample evidence for this acid-induced side reaction which results in the formation of depsipeptides. This side reaction occurred during the acidolytic cleavage required to remove the dimethylamino- or methyl protecting groups of the tyrosine phosphate group. Furthermore, the analysis of initial kinetics suggested an influence of the phosphotyrosine residue. The $N \rightarrow O$-acyl shift proceeded most rapidly when the phosphotyrosine was located in the +2 position. In contrast, rather slow $N \rightarrow O$-acyl migration was observed for peptides in which the phosphotyrosine was the N-terminal neighbour of threonine. The duration of the acid-induced global deprotection was found to most affect the extent of peptide isomerisation. It is demonstrated that the use of the more acid-labile monobenzyl-protected phosphotyrosine building block Fmoc-Tyr(PO(OBzl)OH) allows improvements of both purity and yield of phosphopeptides. We wish to note that the formation of depsipeptides also occurs at serine residues (data not shown). The findings are expected to be of interest for those who are involved in the synthesis of depsipeptides (*e.g.* as precursors to switch peptides)[31–33] as well as for those who seek methods that enable rapid and efficient access to homogeneous phosphopeptides for subsequent biological studies.

Experimental

General

All organic starting materials were purchased in analytically pure grade and used without further purification. The amino acids were purchased from SennChemicals. Building block **13** was synthesised using methods described in the literature.[20] Building blocks **14** and **15** were purchased from Bachem and Novabiochem, respectively. HPLC-Grade acetonitrile was purchased from Acros, DMF from Biosolve. The solid phase peptide synthesis was performed using a Respep Synthesizer from Intavis Bioanalytical Instruments AG. Cleavage of the peptide resins was performed in 2 ml PET-syringes from MultsynTech/Witten, which were equipped with Teflon filters (pore size 50 µm). The combined cleavage filtrates were concentrated by rotary evaporation and the crude product was isolated by ether precipitation. Analytical HPLC-MS was performed on an Agilent 1100 HPLC-MS system equipped with a UV–Vis-detector and a VL-quadrupole mass spectrometer using a thermostated (55 °C) analytical CC 125/4 Nucleodur-C18 gravity, 3 µ column (Macherey-Nagel) and detection wavelength $\lambda = 210$ nm. Eluents A (H_2O : MeCN : HCOOH = 98.9 : 1 : 0.1 [v/v/v]) and B (MeCN : H_2O : HCOOH = 98.9 : 1 : 0.1 [v/v/v]) were used in a linear gradient (gradient 1: 0–20 min, 3–50% B in A or gradient 2: 0–20 min, 3–20% B in A) at a flow rate of 1 mL min^{-1}. ^1H- and ^{13}C-NMR spectra were recorded on a Bruker spectrometer at 300 MHz and 75 MHz, respectively.

General procedure for the synthesis of the phosphopeptides

Preloaded resin was suspended in DMF for 1 hour and treated with 25% piperidine–DMF (1 ml) for 2 min and washed with 25% piperidine–DMF, DMF, dichloromethane and DMF again. The automated synthesis was commenced with a deprotection step, which included treatment with 1 ml DMF–piperidine (4 : 1 [v/v]) for 2 min and subsequent washing with DMF. For coupling 6 eq. amino acid (0.2 M) in DMF were preactivated using 12 eq. *N*-methylmorpholine and 6 eq. 1-[bis(dimethylamino)methylene]-5-chloro-1*H*-benzotriazolium-3-oxide tetrafluoroborate (TCTU). This solution was added to the resin. Double couplings were performed after the incorporation of the fourth amino acid. The resin was capped with 1 ml of a solution of DMF–2,6-lutidine–acetic anhydride (90 : 5 : 5 [v/v/v]). The terminal capping was performed twice. Prior to final cleavage the resin was washed ten times with dichloromethane.

Cleavage method A: in the case of building block **13** the resin was shaken for 90 min with 1 ml of a solution that contained 850 µl TFA, 50 µl triisopropylsilane, 50 µl *m*-cresol, 50 µl water and 5 mg cysteine methyl ester. The resin was washed with 200 µl TFA. Then, 120 µl water were added to the combined filtrates. After 18 h shaking the solution was concentrated to 1/5 of its volume. The crude product precipitated upon addition of cold diethylether. The pellet obtained after centrifugation and disposal of the supernatant was washed with cold diethylether and collected by centrifigation.

Cleavage method B: when building block **14** was used the resin was shaken for 5 h with 1 ml of a 1 M solution of trimethylsilylbromide–thioanisol and 50 µl *m*-cresol in TFA at 5 °C. The crude product was concentrated, precipitated with cold ether and collected by centrifugation.

Cleavage method C: in the case of building block **15** the resin was shaken for 90 min with a mixture of 950 µl TFA, 25 µl water, 12.5 µl 1,2-ethanedithiol and 12.5 µl triisopropylsilane. Further work-up was performed as described for method A.

Synthesis of Ac-pTyr-Glu-Thr-Leu-Gly-OH (1)

Method A: Fmoc-Gly-Wang-resin (12.5 mg, 10 µmol) and Fmoc-Tyr($PO(NMe_2)_2$) **13** were used. Analytical HPLC-MS (gradient 1) exposed two major products at $r_t = 5.1$ min (30.8% of integral area at $\lambda = 210$ nm) and $r_t = 8.2$ min (69.2%). $[M + H]^+$: *m/z* 704.2 for peptide **1b** and **1a** ($C_{28}H_{42}N_5O_{14}P_1$: 703.25 g mol^{-1}).

Method B: Fmoc-Gly-Wang-resin (2 µmol) and building block **14** were used. Analytical HPLC-MS (gradient 1) exposed two major products at $r_t = 5.1$ min (40.3%) and $r_t = 8.2$ min (59.7%)

([M + H]$^+$: m/z 704.2). Purification by prep. HPLC resulted in 10.4% overall yield of peptide **1a** determined with an extinction coefficient for phosphotyrosine at $\lambda = 260$ nm of 652 M^{-1} cm^{-1}.[34]

Method C: Fmoc-Gly-Wang-resin (2 μmol) and building block **15** were used. Analytical HPLC-MS (gradient 1) revealed 97.9% peptide **1a** and 2.1% **1b**. Purification by prep. HPLC furnished peptide **1a** in 29.1% overall yield.

Synthesis of peptides 2–7

Fmoc-Gly-TGR-resin (42 mg, 5 μmol) and Fmoc-Tyr($PO(NMe_2)_2$) **13** were used. HPLC-MS analysis (gradient 1) exposed two peaks for peptide **2** (1.9 min and 3.0 min, [M + H]$^+$: m/z 647.2), one peak for peptide **3** (6.8 min, [M + H]$^+$: m/z 659.2), two peaks for peptide **4** (4.4 min and 7.2 min, [M + H]$^+$: m/z 631.2), one peak for peptide **5** (5.9 min, [M + H]$^+$: m/z 517.3), two peaks for peptide **6** (gradient 2, 5.6 min and 8.1 min, [M + H]$^+$: m/z 623.3) and two peaks for peptide **7** (2.9 min and 5.9 min, [M + H]$^+$: m/z 517.3).

Synthesis of Ac-pTyr-Gly-Thr-Leu-Gly-OH (4a/4b)

Fmoc-Gly-Wang-resin (230 mg, 150 μmol) and Fmoc-Tyr($PO(NMe_2)_2$) **13** were used. HPLC-MS analysis (gradient 2) exposed two major peaks at $r_t = 7.5$ min and $r_t = 13.4$ min ([M + H]$^+$: m/z 704.2) for peptide **4a** and **4b** ($C_{28}H_{42}N_5O_{14}P_1$: 703.25 g mol^{-1}). The crude product was purified by prep. HPLC to yield 16.1 mg peptide **4a** (15.3%) and 35 mg depsipeptide **4b** (33.2%) after lyophylisation.

^{1}H-NMR for **4a** (DMSO-d^6), 300 MHz, δ 0.83–0.89 (dd, J = 6.5 Hz, J = 12.4 Hz, 6 H, 2 × CH_3-Leu); 1.03 (d, J = 6.3 Hz, 3 H, CH_3-Thr); 1.50 (t, J = 7.2 Hz, 3 H, CH_2-Leu); 1.63 (m, 1 H, CH-Leu); 1.78 (s, 3 H, Ac-CH_3); 2.85 (ddd, J = 7.2 Hz, J = 14.0 Hz, J = 24.0 Hz, 2 H, CH_2-pTyr); 3.65–3.86 (m, 4 H, 2 × CH_2-Gly); 3.97–4.04 (m, 1 H, CH-OH-Thr); 4.26 (dd, J = 4.1 Hz, J = 8.3 Hz, 1 H, CH-Thr); 4.31–4.37 (m, 1 H, CH-Leu); 4.39–4.48 (m, 1 H, CH-pTyr); 7.13 (dd, J = 8.1 Hz, J = 50.3 Hz, 4 H, $CH_{arom.}$-pTyr); 7.71 (d, J = 8.3 Hz, 1 H, NH-Thr); 7.89 (d, J = 8.4 Hz, 1 H, NH-Leu); 8.17–8.23 (m, 2 H, NH-pTyr, NH-Gly); 8.39 (t, J = 5.6 Hz, 1 H, NH-Gly) ppm.

^{13}C-NMR: (DMSO-d^6), 75 MHz, δ 19.9 (1 C, CH_3-Thr); 22.0, 22.9 (2 C, 2 × CH_3-Leu); 23.6 (1 C, CH-Leu); 24.5 (1 C, CH_3-acetyl); 37.0 (1 C, CH_2-pTyr); 42.7 (1 C, CH_2-Gly); 51.3 (1 C, CH-Leu); 54.8 (1 C, CH-pTyr); 58.4 (1 C, CH-Thr); 67.2 (1 C, CH-OH-Thr); 120.1 (2 C, CH-pTyr-arom.); 130.5 (2 C, CH-pTyr-arom.); 134.1 (1 C, Cq-pTyr); 150.4 (1 C, Cq-O-pTyr); 169.4 (1 C, Gly-C=O); 170.0 (1 C, Thr-C=O); 170.2 (1 C, acetyl-C=O); 171.5 (1 C, Leu-C=O); 172.3 (1 C, pY-C=O); 172.8 (1 C, $Gly_{term.}$-C=O) ppm.

^{1}H-NMR for **4b** (DMSO-d^6), 300 MHz, δ 0.83–0.92 (m, 6 H, 2 × CH_3-Leu); 1.25 (d, J = 6.4 Hz, 3 H, CH_3-Thr); 1.50 (t, J = 7.2 Hz, 3 H, CH_2-Leu); 1.63 (m, 1 H, CH-Leu); 1.78 (s, 3 H, Ac-CH_3); 2.85 (ddd, J = 7.2 Hz, J = 14.0 Hz, J = 24.0 Hz, 2 H, CH_2-pTyr); 3.68–3.81 (m, 2 H, CH_2-Gly); 3.82–4.28 (m, 3 H, CH_2-Gly, CH-Thr); 4.34–4.63 (m, 2 H, CH-Leu, CH-pTyr); 4.96 (p, J = 6.3 Hz, 1 H, CH-O-Thr); 7.13 (dd, J = 8.1 Hz, J = 50.3 Hz, 4 H, $CH_{arom.}$-pTyr); 8.19–8.23 (m, 2 H, NH-Gly, NH-Leu); 8.37–8.49 (m, 3 H, NH-pTyr, NH_2-Thr); 8.82 (d, J = 8.1 Hz, 1 H, NH-Gly) ppm.

^{13}C-NMR (DMSO-d^6), 75 MHz, δ 16.8 (1 C, CH_3-Thr); 22.0, 22.9 (2 C, 2 × CH_3-Leu); 23.6 (1 C, CH-Leu); 24.5 (1 C, CH_3-acetyl); 37.0 (1 C, CH_2-pTyr); 40.1 (1 C, CH_2-Gly); 51.3 (1 C, CH-Leu); 54.8 (1 C, CH-pTyr); 55.8 (1 C, CH-Thr); 70.2 (1 C, CH-O-Thr); 120.1 (2 C, CH-pTyr-arom.); 130.5 (2 C, CH-pTyr-arom.); 134.1 (1 C, Cq-pTyr); 150.4 (1 C, Cq-O-pTyr); 165.7 (1 C, Gly-C=O); 170.0 (1 C, Thr-C=O); 170.2 (1 C, acetyl-C=O); 171.5 (1 C, Leu-C=O); 172.3 (1 C, pTyr-C=O); 172.8 (1 C, $Gly_{term.}$-C=O) ppm.

Synthesis of depsipeptide Ac-pTyr-Gly-Thr-Leu-Gly-NH_2 (4b)

Fmoc-Gly-TGR-resin (17 mg, 2 μmol) and Fmoc-Tyr(PO_3H_2) were used. The first amino acids were coupled according to the general protocol. After coupling of Boc-threonine the resin was washed three times with DMF and dichloromethane. Subsequently, the resin was submitted to a double coupling with 2 mg Fmoc-glycine (297.3 g mol^{-1}, 3 eq.) in 50 μl dichloromethane and DMF with 0.3 eq. DMAP and 1.1 μl diisopropylcarbodiimide (3 eq.). The synthesis was continued as described in the general method. HPLC-MS analysis (gradient 1) showed one major product at $r_t = 4.7$ min ([M + H]$^+$: m/z 631.2) for depsipeptide **4b** ($C_{25}H_{39}N_6O_{11}P_1$: 630.24 g mol^{-1}).

Synthesis of peptides 8–11

Fmoc-Gly-Wang-resin (3 mg, 2 μmol) was used. HPLC-MS analysis (gradient 1) showed two major peaks for peptide **8** (r_t = 3.8 min and 5.6 min, [M + H]$^+$: m/z 604.2), for peptide **9** (r_t = 7.4 min and 8.1 min, [M + H]$^+$: m/z 604.2), for peptide **10** (r_t = 3.1 min and 5.8 min, [M + H]$^+$: m/z 604.2) and for peptide **11** (r_t = 6.4 min and 8.8 min, [M + H]$^+$: m/z 675.2).

Synthesis of peptide Asp-Ile-pTyr-Glu-Thr-Asp-Gly 12

Fmoc-Gly-Wang-resin (3 mg, 2 μmol) was used.

Method A: analytical HPLC-MS (gradient 1) showed two major products at $r_t = 5.9$ min (30.0%) and $r_t = 8.2$ min (58.5%) ([M + H]$^+$: m/z 934.3) for peptides **12b** and **12a** ($C_{36}H_{52}N_7O_{20}P_1$: 933.30 g mol^{-1}), respectively, and minor peaks for aspartamide by-products (11.5% at r_t = 6.4 min, 8.1 min and 8.9 min, [M + H]$^+$: m/z 916.2). After 90 min deprotection 64.8% **12a**, 2.6% **12b** and 32.6% protected peptide (9.8 min and 12.0 min, [M + H]$^+$: m/z 988.3) were found. Purification by prep. HPLC furnished 11.9% overall yield.

Method B: HPLC-MS analysis of crudes obtained after 90 min deprotection time revealed products (gradient 1) at $r_t = 5.9$ min (23.8%, **12b**), $r_t = 7.2$ min (65.2%, **12a**) ([M + H]$^+$: m/z 934.3) and 11.0% methyl-protected peptide (r_t = 7.3 min and 8.4 min [M + H]$^+$: m/z 948.3).

Method C: HPLC-MS analysis of crudes obtained after 90 min deprotection time showed one major peak for **12a** (98%) and one minor peak for **12b** (2.0%). Purification by prep. HPLC furnished peptide **12a** in 52.2% overall yield.

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