52. Synthesis of Multi-O^4-phospho-L-tyrosine-Containing Peptides

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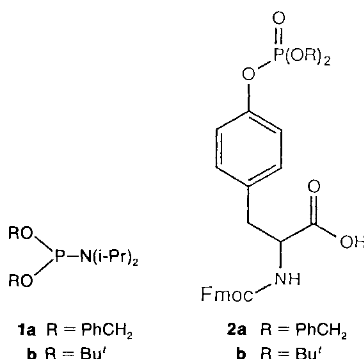
(6.II.92)

Multi-O^4-phospho-L-tyrosine-containing peptides can be synthesized by the global as well as the building-block approach. Thus, we prepared by both strategies the triphosphorylated and the three regioisomeric diphosphorylated insulin-receptor-(1142–1153)-dodecapeptide derivatives **7** and **4**–**6**, respectively, of the parent Thr-Arg-Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys (**3**). These phosphorylated peptides are valuable tools to study the regioselectivity of dephosphorylation by phosphatases.

1. Introduction. – Recently, we reported and compared two alternative strategies for the Fmoc solid-phase synthesis of phospho-L-tyrosine-containing peptides (Fmoc = [(9*H*-fluoren-9-yl)methoxy]carbonyl) [1]. Both approaches are based on P^{III} chemistry. In the global phosphorylation procedure, the free L-tyrosine side chain in pre-synthesized, resin-bound peptides was phosphorylated by applying different phosphinylation reagents and a variety of phospho-protecting groups (Me, Bzl, Bu^t, allyl; *e.g.* **1a**, **b**). In the second approach, protected phospho-L-tyrosine building blocks (*e.g.* **2a**, **b**) were incorporated in



stepwise solid-phase synthesis. Using both methods, we prepared a number of biologically relevant phospho-L-tyrosine-containing peptides. These peptides were used as substrates for a genetically engineered catalytic fragment of the human transmembrane leukocyte antigen related (LAR) protein tyrosine phosphatase (PTPase) [2] and the common antigen (CD45) PTPase [3] which were both expressed in *E. coli*.

We now report on the extension of both strategies to the synthesis of multi-O^4-phospho-L-tyrosine-containing peptides[1]). Such peptides are valuable tools to study the regioselectivity of dephosphorylation by phosphatases.

As an example, we chose the insulin-receptor(IR)-(1142–1153)-dodecapeptide Thr-Arg-Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys (**3**), containing three L-tyrosine residues as well as two L-threonine residues as potential phosphorylation sites. Since two of the L-tyrosines are at adjacent positions, the synthesis of multi-L-tyrosine-phosphorylated forms of this peptide should provide valuable information on the successive incorporation of sterically demanding phospho-L-tyrosine building blocks, as well as on the accessibility of the phosphinylating reagents to adjacent L-tyrosine OH functions during the global phosphorylation procedure. The L-tyrosine-phosphorylated IR-(1142–1193)-dodecapeptides are especially suited for investigations concerning the regioselectivity of dephosphorylation since the signals due to the three sets of aromatic protons corresponding to the three L-tyrosine residues in this sequence are known to be well resolved in the ^{1}H-NMR spectrum under conditions of high field strength [4]. This makes it possible to directly monitor the regioselectivity of dephosphorylation by PTPases using the NMR technique.

2. Results and Discussion. – 2.1. *Global Phosphorylation.* In our previous work [1], we observed that phosphinylation/phosphorylation of pre-synthesized resin-bound peptides proceeded well on side-chain-unprotected L-tyrosine. The nature of the phospho-protecting group that was applied (Me, Bzl, Bu^t, allyl) had only negligible effect on the reaction rate. Furthermore, we established that incorporation of the side-chain-unprotected L-tyrosine proceeded with efficiency by using *O*-(1,2-dihydro-2-oxopyrid-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TPTU) as the condensing agent. This result was confirmed by synthesizing the IR-(1142–1153)-dodecapeptide **3** with and without Bu^t side-chain protection for L-tyrosine. Both syntheses proceeded equally well.

3	IR-(1142–1153)-dodecapeptide	Thr-Arg-Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys
4	$O^{4,1146}$,$O^{4,1150}$-Diphospho-IR-(1142–1153)-dodecapeptide	Thr-Arg-Asp-Ile-**Tyr(*P*)**-Glu-Thr-Asp-**Tyr(*P*)**-Tyr-Arg-Lys
5	$O^{4,1146}$,$O^{4,1151}$-Diphospho-IR-(1142–1153)-dodecapeptide	Thr-Arg-Asp-Ile-**Tyr(*P*)**-Glu-Thr-Asp-Tyr-**Tyr(*P*)**-Arg-Lys
6	$O^{4,1150}$,$O^{4,1151}$-Diphospho-IR-(1142–1153)-dodecapeptide	Thr-Arg-Asp-Ile-Tyr-Glu-Thr-Asp-**Tyr(*P*)**-**Tyr(*P*)**-Arg-Lys
7	$O^{4,1146}$,$O^{4,1150}$,$O^{4,1151}$-Triphospho-IR-(1142–1153)-dodecapeptide	Thr-Arg-Asp-Ile-**Tyr(*P*)**-Glu-Thr-Asp-**Tyr(*P*)**-**Tyr(*P*)**-Arg-Lys

For the synthesis of the three regioisomeric diphosphorylated IR-(1142–1153)-dodecapeptides **4**–**6**, the L-tyrosine residue not to be phosphorylated was incorporated with the side chain protected as *tert*-butyl ether, and the L-tyrosine residues to be phosphorylated were inserted as side-chain-free Fmoc-Tyr-OH derivative. The L-threonine residues were protected at the side chain also as *tert*-butyl ethers.

For the synthesis of the triphosphorylated peptide **7**, all L-tyrosine residues were introduced as side-chain-unprotected building blocks. Part of the resin was deprotected prior to phosphorylation to yield the unphosphorylated peptide **3** (for HPLC, see *Fig. 1a*).

[1]) Partly reported at the 2nd International Congress on Amino Acids and Analogues, August 5–9, 1991, Vienna, Austria.

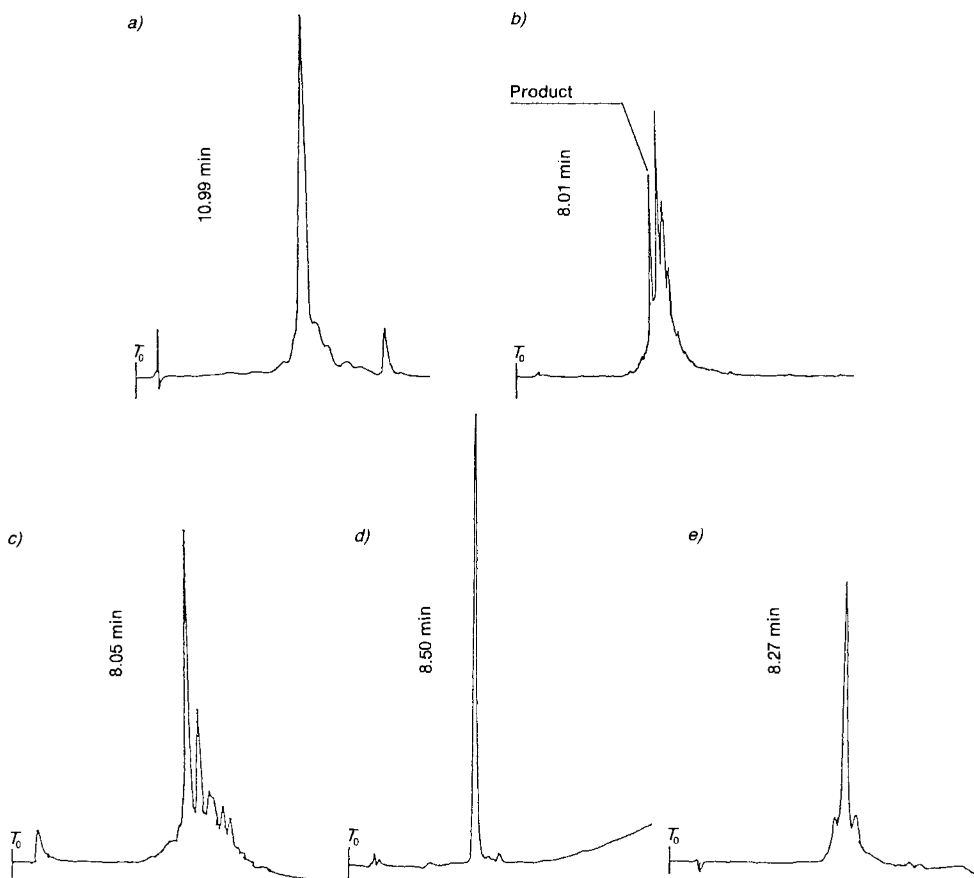


Fig. 1. *Reversed-phase HPLC of* a) *crude* **3** *after synthesis with side-chain-unprotected Fmoc-L-tyrosine*, b) *of crude* **7** *synthesized by the global approach*, c) *of crude* **7** *synthesized by the building-block approach using* **2a**, d) *of purified* **7**, *and* e) *of crude* **7** *synthesized by the building-block approach using* **2b**. Conditions: 0–100% MeCN in 0.05% CF_3COOH, 30 min, 230 nm.

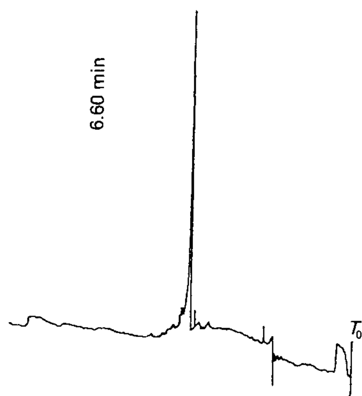
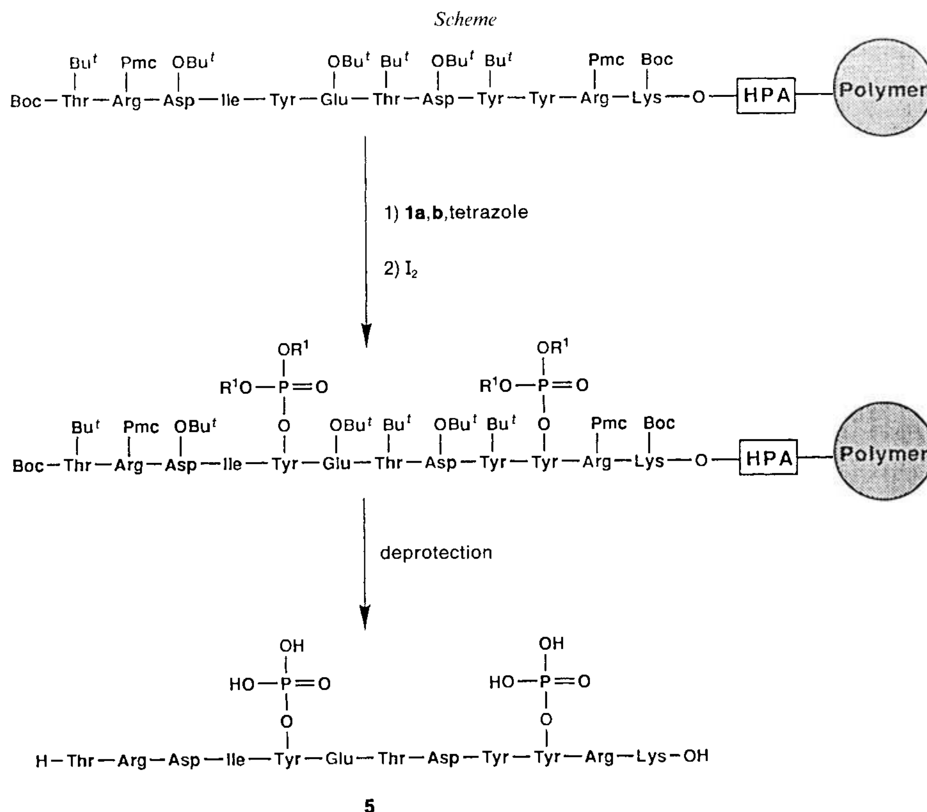


Fig. 2. *Capillary zone electrophoresis* (CZE) *of purified* **7**. Conditions: field 417 V/cm, current 55 μA, buffer 100 mM 3-[(cyclohexyl)amino]propanesulfonic acid (CAPS), pH 11.0, capillary length 72 cm, capillary diameter 50 μm, temp. 30°, analysis time 15 min, 200 nm.

Since the benzyl group as phospho-protecting group can be cleaved under various conditions and is compatible with the Fmoc solid-phase peptide chemistry, we first concentrated on bis(benzyloxy)(diisopropylamino)phosphine (**1a**) for the evaluation of the global multi-phosphorylation and the corresponding phospho-L-tyrosine building block **2a** for the stepwise synthesis (see below) of multi-phospho-L-tyrosine-containing IR-peptides. The phosphinylation step was performed with a 6.5-fold excess of reagent **1a** per L-tyrosine residue which was then followed by the oxidation step using I_2 (see *Scheme* for the preparation of **5**). After removal of the protecting groups and cleavage from the support with CF_3COOH/thiophenol/H_2O, the crude reaction mixtures were analyzed by reversed-phase HPLC. The diphosphorylated regioisomers **4** and **5** were clearly formed as the main product, whereas the synthesis of the regioisomer **6** was incomplete and, furthermore, repeated phosphorylation of the peptide resin yielded no improvement in the quality of the crude product as ascertained by analytical reversed-phase HPLC. From analytical HPLC's we could not deduce which position is phosphorylated first due to the small difference in the retention times of the monophosphorylated peptides.

Scheme



HPA = [4-(*H*ydroxymethyl)*p*henoxy]*a*cetyl ($-CH_2C_6H_4OCH_2CO-$)
Pmc = (2,2,5,7,8-Pentamethylchroman-6-sulfonyl)

The results suggest that steric interactions may be involved in subsequent phosphinylation of adjacent tyrosine residues. The phosphinylation is the first step of our phosphorylation approach which is followed by an oxidation step to yield the corresponding phosphorylated species. Another possibility is that secondary structure formation by phosphinylation of one tyrosine residue may be impeding subsequent phosphinylation.

This was also observed during the preparation of the triphosphorylated peptide **7** by the global approach. The major components in the product mixture were the mono- and diphosphorylated regioisomers (*Fig. 2b*), and only a minor amount of the desired compound **7** had been formed. Repeating the phosphorylation with the peptide-resin used in the first attempt did not result in a better yield of the desired compound **7**. Changing the phosphinylation reagent to the less bulky **1b** also failed to improve the degree of phosphorylation. Thus, the global phosphorylation approach appears to have its limitation when considering multiphosphorylations.

The peptides were purified by reversed phase HPLC and purity was again ascertained by analytical reversed-phase HPLC as well as by capillary zone electrophoresis (CZE). Characterization was achieved by FAB-MS and ion-spray MS in the cases where no fast-atom-bombardment (FAB)-MS could be obtained due to the high polarity of the phosphorylated peptide. The identity of the peptides was further confirmed by solid-phase protein sequencing where a positive signal was obtained for phospho-L-tyrosine residues [5].

2.2. *Building-Block Approach.* Previously we reported [1] that O^4-phospho-L-tyrosine building blocks can be efficiently incorporated in stepwise Fmoc solid-phase peptide synthesis with TPTU for activation. The extent of incorporation was not influenced by the nature of the phosphate-protecting groups (Me, Bzl, Bu^t, allyl). Thus, we had prepared a number of biologically relevant monophospho-L-tyrosine-containing peptides. The results of that study were compared with the syntheses of the same phosphorylated peptides by the global approach. Generally, no significant differences in the performances of the syntheses by either approach were observed.

We now demonstrate that building block **2a** can also be applied to the synthesis of multi-O^4-phospho-L-tyrosine-containing peptides. As examples, we chose again the diphosphorylated regioisomers **4**–**6** of the parent IR-(1142–1153)-dodecapeptide (**3**) as well as the corresponding triphosphorylated peptide **7** so that the results could be compared to the preparation of **4**–**7** by the global approach.

From previous work [6] it is known that piperidine treatment during Fmoc cleavage may lead to partial deprotection of a benzyl group from the phosphotriester after insertion of building block **2a**. The resulting phosphodiester can then consume part of the incoming amino acid and, therefore, decrease the actual excess of acylating reagent. To minimize this reaction, we employed 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in DMF for the Fmoc-cleavage step. However, by applying these conditions to Asp-containing peptides, we observed in the FAB-MS of the HPLC-purified product an $[M-18]^+$ peak besides the correct molecular-ion peak. We suspect this is due to succinimide formation [7]. When using 2% DBU in DMF and building block **2a** for the syntheses of the di- and the triphosphorylated peptides **4**–**7**, which contain two Asp residues, we observed a similar result. In FAB-MS or ion-spray MS of the crude material, we observed a main peak corresponding to the desired molecular weight together with a relatively strong $[M-18]^+$ peak and a weak $[M-36]^+$ peak. The relatively strong $[M-18]^+$ peak was also observed in the FAB-MS and ion-spray MS of the purified peptide, although homogeneity of the material was indicated by reversed-phase HPLC and CZE. For this reason, we reverted to the original 20% piperidine/DMF treatment for Fmoc-group cleavage. As a result, no $[M-18]^+$ peak was observed in the purified product.

Satisfactory results were obtained for the synthesis of all three regioisomers **4–6**. Efficient coupling was observed even when adjacent, protected phospho-L-tyrosine residues were incorporated by TPTU as in the synthesis of **6**. The triphosphorylated peptide **7** was also found to be accessible by incorporating **2a** as indicated by the HPLC profile of the crude material after deprotection (*Fig. 1c*). A better HPLC profile was obtained when the synthesis of **7** was repeated under the same conditions but incorporating building block **2b** (*Fig. 1e*; see *Fig. 1d* and *2* for purified **7**). This is most likely due to the resistance of the *t*-Bu group in phosphotriesters towards base attack. At the same time, this proved that the steric demands of the phospho-protecting groups have a negligible effect on the acylation reaction. After deprotection, the purified peptides were shown to be identical to the corresponding peptides synthesized by the global-phosphorylation approach.

The incorporation of **2b** has its limitation in that this building block is not stable during storage which prevents it from routine application. Building block **2b** was also recently used by another group for the synthesis of a O^4-phospho-L-tyrosine-containing peptide [8].

3. Conclusion. – Our results show that, in principle, multi-O^4-phospho-L-tyrosine-containing peptides can be prepared by the global-phosphorylation approach or the building-block strategy. In the preparation of the mono-O^4-phospho-L-tyrosine-containing peptides, the global approach and the building-block strategy proceeded equally well [1]. Here, we observed that for the syntheses of the multiphosphorylated peptides **4–7**, the building-block approach yielded better results. The nature of the protecting group of the phosphinylation reagent had no influence on the global phosphorylation. To avoid possible succinimide formation in the building-block approach with **2a** where Asp-containing peptides are to be synthesized, we recommend 20% piperidine/DMF instead of 2% DBU/DMF for Fmoc deprotection. Best results were obtained in the synthesis of **7** when **2b** was applied as the building block for phospho-L-tyrosine, but this unit is prone to decomposition on storage.

The peptides were used to study by ^{1}H-NMR the regioselectivity of dephosphorylation by the catalytic part of the LAR phosphatase, an important enzyme involved in signal transduction in T-cells [9].

We would like to thank *P. Armbruster, B. Hennequin, E. Küng,* and *S. Oehler* for their skilled technical assistance and Dr. *H. W. Lahm* and *U. Röthlisberger* for providing the sequencing data. We also thank Dr. *J. Mezger*, University of Tübingen, for ion-spray MS and our colleagues from PRT for C,H,N analysis (Dr. *S. Müller*), NMR spectra (Dr. *G. Englert*, Dr. *W. Arnold*), and FAB-MS (Dr. *W. Vetter, W. Meister*), and *A. Iff* for typing the manuscript.

Experimental Part

1. *General.* See [1]. In addition: Phosphinylation reagent **1a** and **1b** were prepared according to [1] and [10]. The phospho-L-tyrosine building blocks **2a** and **2b** were synthesized by the 'one-pot' procedure [1]. Prep. reversed-phase HPLC purifications: *Delta Prep-3000* (*Waters*) with an EtOH gradient in 0.1% CF_3COOH.

2. *Global Phosphorylation.* 2.1. *$O^{4,1146}$,$O^{4,1150}$,$O^{4,1151}$-Triphospho-IR-(1142–1153)-dodecapeptide* (**7**): *General Procedure.* To poly(dimethylacrylamide) on 'Kieselgur' (15 g; corresponding to 2.08 mmol functionalization) equipped with a [4-(hydroxymethyl)phenoxy]acetyl (HPA) linker in DMF, 9.1 mmol (4.26 g) of Fmoc-Lys-(Boc)–OH, 8.97 mmol (3.4 g) of *O*-(1*H*-benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU), 14.05 mmol (9 ml) of $(i\text{-}Pr)_2EtN$ and 1.6 mmol (0.22 g) of 4-(dimethylamino)pyridine were added and

reacted overnight. The support was washed with DMF and Et_2O and dried (loading: 0.084 mmol/g). Of this support, 2.67 g were applied to the stepwise synthesis of the L-tyrosine side chain unprotected IR-(1142–1153)-dodecapeptide to be used in the global phosphorylation procedure. Elongations were performed in the following coupling cycles: 1. wash (DMF), 30 s, average flow rate 15 ml/min; 2. deprotection (20% piperidine/DMF), 10 min; 3. wash (DMF), 11 min; 4. coupling: 4-fold excess of activated amino acid, 30 min (Fmoc–Amino acids were predissolved in 2 ml of *N,N'*-dimethyl-*N,N'*-ethyleneurea [11] (DMEU)/MeCN 3:1 (*v*/*v*) per mmol together with 1 equiv. of TPTU and 2 equiv. of $(i\text{-}Pr)_2EtN$ (1 ml; 2M in DMEU; Fmoc–L-Tyrosine was incorporated side-chain-unprotected); 5. wash (DMF), 8 min. After synthesis, the N-terminal Fmoc group was removed and replaced with Boc by adding 10 equiv. of Boc_2O in DMF for 30 min.

Of this peptide-resin, 0.5 g were deprotected with CF_3COOH/thiophenol/H_2O 95:2.5:2.5 (*v*/*v*) for 30 min at r.t. After filtration, AcOH was added and the soln. concentrated to a small volume. The crude peptide was precipitated with Et_2O and purified by reversed-phase HPLC: 24 mg of pure **3**. Purity was assessed by anal. reversed-phase HPLC and CZE. FAB-MS: 1623.0 ($C_{72}H_{107}N_{19}O_{24}$, calc. 1622.8).

For comparison, the synthesis of peptide **3** was repeated using side-chain-protected O^4-(*tert*-butyl)-N_α-Fmoc-L-tyrosine.

For the global phosphorylation, 1 g of the residual material of the former synthesis (N-terminal Boc-protected) was placed on a frit and washed several times with MeCN. The frit was sealed with a septum and the support dried by several additions of anh. MeCN, which was pushed through the frit by a slight overpressure of Ar. Then 7.5 ml of anh. MeCN containing 3.57 mmol (250 mg) of tetrazole and 2.7 mmol (945 mg) od **1a** were added *via* syringe and reacted for 10 min. The phosphinylating mixture was removed by suction, and 2.5 ml of 1M I_2 in THF/2,6-dimethylpyridine/H_2O 40:10:1 (*v*/*v*) were added and removed by suction. This was repeated several times. The resin was then washed with MeCN and dried. Of this material, 100 mg were deprotected as outlined above, and the crude peptide was investigated by reversed-phase HPLC indicating a relatively low yield of the desired **7** (*Fig. 1b*). Repetition of the global phosphorylation with the same excess of phosphinylation reagent as described above did not improve the yield (anal. reversed-phase HPLC following deprotection).

Global phosphorylation under the same conditions but employing **1b** for the phosphinylation showed no improvement either. Purification was performed by reversed-phase HPLC yielding 1.1 mg (7% overall) of **7**. Purity was assessed by anal. reversed-phase HPLC and CZE (*Fig. 1d* and *2*). Ion-spray MS: 1862.5 ($[M+H]^+$, $C_{72}H_{110}N_{19}O_{33}P$, calc. 1862.7). Solid-phase protein sequencing [5]: positive signals in CZE for positions 1146, 1150, and 1151.

2.2. *Diphosphorylated IR-(1142–1153)-dodecapeptides* **4–6** were synthesized according to *2.1* using **1a** for the global phosphorylation.

$O^{4,1146}$,$O^{4,1150}$-*Diphospho-IR-(1142–1153)-dodecapeptide* (**4**): 25 mg (16.7% overall) of pure material. FAB-MS: 1783.1 ($[M+H]^+$, $C_{72}H_{109}H_{19}O_{30}P_2$, calc. 1782.7). Solid-phase protein sequencing: positive signal in CZE for residues 1146 and 1150.

$O^{4,1146}$,$O^{4,1151}$-*Diphospho-IR-(1142–1153)-dodecapeptide* (**5**): 40 mg (26.7% overall) of pure material. FAB-MS: 1783.2 ($[M+H]^+$, $C_{72}H_{109}H_{19}O_{30}P_2$, calc. 1782.7). Solid-phase protein sequencing: positive signal in CZE for residues 1146 and 1151.

$O^{4,1150}$,$O^{4,1151}$-*Diphospho-IR-(1142–1153)-dodecapeptide* (**6**): 23 mg (15.4% overall) of pure material. FAB-MS: 1782.1 ($[M+1]^+$, $C_{72}H_{109}H_{19}O_{30}P_2$, calc. 1782.7). Solid-phase protein sequencing: positive signal in CZE for residues 1150 and 1151.

3. *Building-Block Strategy*. Solid-phase syntheses were performed by incorporating building blocks **2a** and **2b** using the protocol previously defined in *Section 2* and [1]. Unambiguous MS data was obtained when 20% piperidine/DMF was employed in the N^α-deprotection step.

Diphospho-IR-(1143–1153)-dodecapeptides were prepared starting with 0.21 mmol of Fmoc-Lys(Boc)-esterified resin. Deprotections were carried out with CF_3COOH/thiophenol/H_2O as before, yielding 18 mg (8.6% overall) of pure **4** (FAB-MS: 1783.1 ($[M+H]^+$)), 41 mg (19.6% overall) of pure **5** (FAB-MS: 1782.5 ($[M+H]^+$)), and 41 mg (19.6% overall) of pure **6** (FAB-MS: 1783.5 ($[M+H]^+$)), resp.

Triphospho-IR-(1142–1153)-dodecapeptide **7** was prepared by using **2a** and **2b** starting with 0.12 mmol of esterified resin. Deprotection by either 1M Me_3SiBr/thioanisole/CF_3COOH or CF_3COOH/thiophenol/H_2O yielded comparable results as judged by anal. reversed-phase HPLC. The syntheses using **2a** or **2b** both yielded 8 mg (10.3%) of pure **7** (from 0.5 g of resin). FAB-MS: 1862.4 ($[M+H]^+$).

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