128. Alternative Strategies for the Fmoc Solid-Phase Synthesis of O^4-Phospho-L-tyrosine-Containing Peptides

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A number of biologically relevant O^4-phospho-L-tyrosine-containing peptides have been synthesized by either the global phosphorylation of the side-chain-unprotected L-tyrosine moiety in presynthesized resin-bound peptides or alternatively by the incorporation of suitably protected O^4-phospho-L-tyrosine building blocks in the continuous-flow method of Fmoc solid-phase peptide synthesis. Different phosphate-protecting groups have been applied.

1. Introduction. – Protein phosphorylation is regarded as an important post-translational modification which regulates enzymic functions within cells [1] [2]. Phosphorylation can occur at serine/threonine or at tyrosine residues. Tyrosine phosphorylation in proteins appears to be a major mechanism of cellular signal transduction. It is regulated by two classes of enzymes: the protein tyrosine kinases (PTK's) and the protein tyrosine phosphatases (PTPases). The family of the PTK's involves transmembrane receptors as essential elements for the response of cells to growth factors and hormones (*e.g.* insulin, EGF, PDGF receptor) and a number of transforming proteins encoded by oncogenes, lacking a transmembrane region and responsible for the control of normal and neoplastic cell growth [3]. The level of tyrosine phosphorylation is also regulated by the PTPases which remove the phosphate group from phosphorylated tyrosine [4]. Two classes of PTPases exist: the membrane bound and the cytosolic soluble enzymes. Whereas many PTK's [5] and a few PTPases [6] have been isolated and characterized, relatively little is known about the principles which govern the specificity of phosphorylation/dephosphorylation at particular tyrosine residues within proteins. Such questions can be best addressed by applying endogenous peptides as substrates for PTK's and PTPases. This requires the efficient synthesis of phosphotyrosine-containing peptides. They can be used either directly as substrates for PTPases or serve as reference compounds in phosphorylations by PTK's.

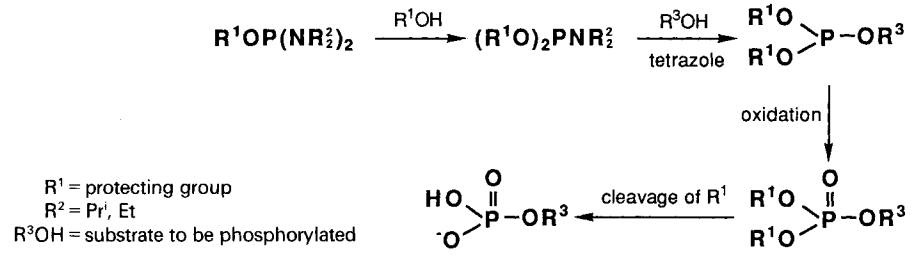
In principle, there are two synthetic strategies for the preparation of phosphotyrosine-containing peptides: synthesis of a phosphorylated tyrosine building block which can be incorporated in the course of solid-phase peptide synthesis or, alternatively, solid-phase synthesis of a peptide with the tyrosine side chain left unprotected followed by its phosphorylation on the solid support. The latter is referred to as global phosphorylation. Since this is carried out at the very end of the synthesis, the approach offers the application of different phosphoryl-protecting groups, even those which are not stable to the conditions of the elongation cycles. If both the phosphorylated and the corresponding

unphosphorylated peptides are needed, they can be obtained from the same peptide synthesis by phosphorylating part of the resin-bound peptide.

The building-block approach has the advantage that phosphotyrosine can be incorporated as a standard amino-acid building block, providing its synthesis is straightforward and the coupling proceeds with high efficiency. Further requirements are a reasonable shelf lifetime and the stability of phosphate protection during coupling cycles. Global phosphorylation circumvents the preparation of the phosphotyrosine building block, but steric constraints, especially due to secondary-structure formation, can hamper effective phosphorylation.

The most effective and versatile phosphorylation procedure for this purpose is based on P^{III} chemistry. Application of phosphoramidite chemistry, originally developed for rapid and effective solid-phase synthesis of oligodeoxynucleotides [7], leads to the preparation of both phosphorylated building blocks and reagents for the global phosphorylation procedure. The principle, outlined in *Scheme 1*, was used by us for the phosphorylation of the OH function in suitably protected serine, threonine, and tyrosine and for the preparation of a phosphoserine-containing peptide using solution chemistry [8]. A variety

Scheme 1

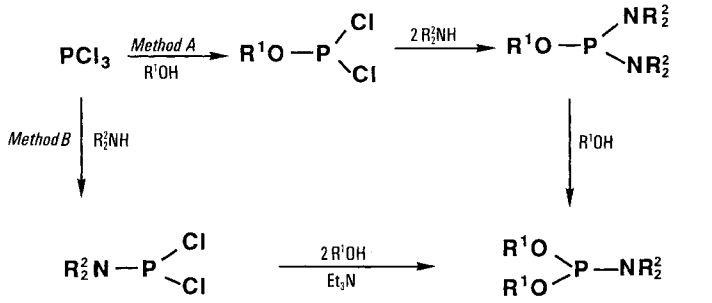


of different protecting groups was used (Bzl, *p*-ClBzl, cyanoethyl). Allyl protection has also been recently investigated [9]. A limitation of the phosphorylation of peptides is that an oxidation step has to be carried out which may lead to side reactions in tryptophan- and methionine-containing sequences [10].

The aim of this work is to compare the building-block approach and global phosphorylation for the preparation of O^4-phospho-L-tyrosine-containing peptides by the Fmoc strategy of solid-phase synthesis (Fmoc = [(9*H*-fluoren-9-yl)methoxy]carbonyl). This involves the preparation of phosphinylation reagents, appropriate phosphotyrosine building blocks with different protecting groups, and the synthesis of a number of biologically relevant phosphotyrosine-containing peptides employing both strategies. Global phosphorylation using P^{III} chemistry has not been reported for phosphotyrosine-containing peptides but has been successfully applied for the preparation of a phosphoserine/threonine-containing pentapeptide [11].

2. Results and Discussion. – 2.1. *Phosphinylation Reagents.* Key compounds for the synthesis of phosphotyrosine building blocks and for the global phosphorylation were the phosphinylating reagents **1a–g**. To give the system more flexibility, we selected a range of protecting groups cleavable under different conditions (R^1 = Me, Bzl, Bu', allyl). Compounds **1a–g** were obtained *via* alternative synthetic routes (*Scheme 2*). *Method A*,

Scheme 2

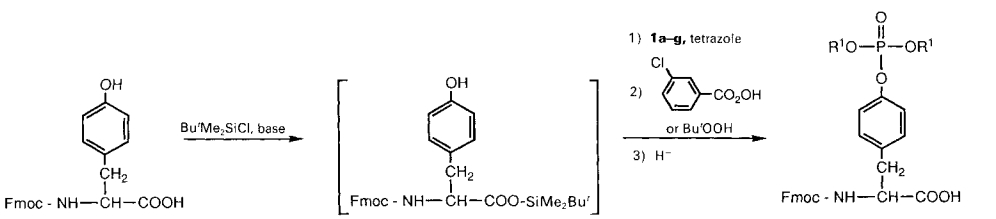


1a R^1 = Me, R^2 = Et
b R^1 = Bzl, R^2 = Pr^i
c R^1 = Bu^t, R^2 = Pr^i
d R^1 = allyl, R^2 = Pr^i
e R^1 = Bzl, R^2 = Et
f R^1 = Bu^t, R^2 = Et
g R^1 = allyl, R^2 = Et

although involving an additional step as compared to *Method B*, was usually preferred since the (alkoxy)dichlorophosphine was transformed without isolation to the corresponding (alkoxy)(diamino)phosphine which is easy to handle. Its reaction to the corresponding phosphoramidite **1** proceeded efficiently and in high yield. Reagents with R^2 = Pr^i are sufficiently stable to allow purification by silica-gel chromatography. Different amino substitutions (R^2 = Et, Pr^i) were selected to investigate if steric constraints hamper effective phosphinylation. This would be of concern to us when using the global phosphorylation strategy.

2.2. *Phosphotyrosine Building Blocks.* Building blocks **2a–d** were prepared by applying different phosphinylating reagents to Fmoc-L-tyrosine as shown in *Scheme 3* [12]. Prior to phosphinylation, Fmoc-L-tyrosine was protected *in situ* as the (*t*-Bu)Me_2Si ester, thus, the whole synthesis comprises a 'one pot' procedure [13] [14]. Reagents **2a** and **2b** have been described earlier [14] [15], but without detailed procedures for their preparation. Reagents **2c** and **2d** are now reported for the first time. In using the original procedures for the preparation, yields varied considerably. Therefore, we tried to optimize the

Scheme 3

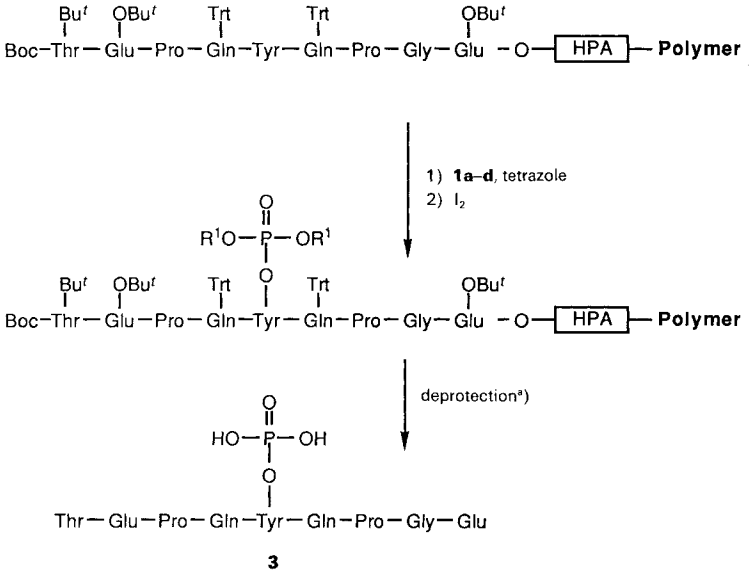


2a R^1 = Me (57%; δ(P) −2.89)
b R^1 = Bzl (71%; δ(P) −5.02)
c R^1 = Bu^t (71%; δ(P) −14.04)
d R^1 = allyl (63%; δ(P) −5.00)

synthesis. Although TLC analyses indicated virtually complete conversions in the phosphinylation/oxidation step, yields of up to 70% of pure product were isolated so far. The oxidation was carried out with 3-chloroperbenzoic acid or with *tert*-butyl hydroperoxide, since we had found earlier that both could be used for solution phosphorylation of tyrosine [8]. To obtain high quality building blocks, the compounds **2a–d** were purified by silica-gel chromatography. They are preferably stored as their salts, since the free acids are prone to partial deprotection of the phosphotriester moiety on storage (especially Bu^t derivative **2c**).

2.3. *Global Phosphorylation.* From solid-phase DNA synthesis, it is known that phosphinylation of the 5′-hydroxy function proceeds in excellent yields when using activated phosphoramidites. This suggests that these reagents may be appropriate for the phosphinylation of OH functions of peptides attached to a solid support and, therefore, worthy of investigation. The phosphorylation of the serine and threonine side chains in a resin-bound pentapeptide, using phosphinylation with bis[(4-chlorobenzyl)oxy](diisopropylamino)phosphine, has been recently reported [11]. However, owing to the length of the peptide, the effects of secondary structure on the phosphinylation step could not be assessed in those syntheses. Furthermore, phosphinylation of the tyrosine OH function on a solid support has not been previously reported. To this end, we prepared a number of different O^4-phospho-L-tyrosine-containing peptides (**3–9**) using global phosphorylation (*Table*). The principle of the phosphorylation is demonstrated for $O^{4,527}$-phospho-p60^{src}-(523–531)-nonapeptide (**3**, *Scheme 4*).

Scheme 4



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HPA = [4-(*H*ydroxymethyl)*p*henoxy]*a*cetic acid (–$CH_2C_6H_4OCH_2COO$–)

a) Reagent **1a** (R^1 = Me, R^2 = Et): deprotection with Me_3SiBr/CF_3COOH/thioanisole/*m*-cresol; **1b** and **1c** (R^1 = Bzl and Bu^t, resp., R^2 = Pr^i): with CF_3COOH/thiophenol; **1d** (R^1 = allyl, R^2 = Pr^i): with CF_3COOH, followed by $[Pd^0(PPh_3)_4]$.

Table. O^4-*Phosphotyrosine* (Tyr(*P*))-*Containing Peptides*

3 $O^{4,527}$-Phospho-p60^{src}-(523–531)-nonapeptide	Thr-Glu-Pro-Gln-**Tyr(*P*)**-Gln-Pro-Gly-Glu
4 $O^{4,394}$-Phospho-p56^{lck}-(390–398)-nonapeptide	Glu-Asp-Asn-Glu-**Tyr(*P*)**-Thr-Ala-Arg-Glu
5 $O^{4,505}$-Phospho-p56^{lck}-(501–509)-nonapeptide	Thr-Glu-Gly-Gln-**Tyr(*P*)**-Gln-Pro-Gln-Pro
6 $O^{4,1173}$-Phospho-EGFR-(1667–1177)-undecapeptide	Thr-Ala-Glu-Asn-Ala-Glu-**Tyr(*P*)**-Lys-Arg-Val-Ala
7 $O^{4,1146}$-Phospho-IR-(1142–1153)-dodecapeptide	Thr-Arg-Asp-Ile-**Tyr(*P*)**-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys
8 $O^{4,1150}$-Phospho-IR-(1142–1153)-dodecapeptide	Thr-Arg-Asp-Ile-Tyr-Glu-Thr-Asp-**Tyr(*P*)**-Tyr-Arg-Lys
9 $O^{4,1151}$-Phospho-IR-(1142–1153)-dodecapeptide	Thr-Arg-Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-**Tyr(*P*)**-Arg-Lys

Peptide synthesis was performed using the Fmoc continuous-flow strategy [16]. C-Terminal esterification was achieved using *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) [17] in the presence of 4-(dimethylamino)pyridine as a catalyst. For chain elongations, *O*-(1,2-dihydro-2-oxopyrid-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TPTU) was used, and Fmoc-L-tyrosine was incorporated with its side chain unprotected. As solvent for the chain elongation, we used 1,3-dimethylimidazolidin-2-one (= *N*,*N*′-dimethyl-*N*,*N*′-ethyleneurea; DMEU) because of the preferable properties of this solvent as compared to *N*,*N*-dimethylformamide (DMF) [18]. Comparative syntheses with side-chain-protected and unprotected Fmoc-L-tyrosine indicated that the phenolic OH function remained unaffected during acylations with TPTU. This judgement was made on the basis of the reversed-phase HPLC profiles of the crude p60^{src}-(523–531)-nonapeptide after deprotection (*Figs. 1a* and *1b*). Phosphorylations were carried out using reagents **1a–d**. No significant differences in reactivity could be observed in the preparation of all phospho-L-tyrosine-containing peptides **3–9**, except in the case of the EGFR sequence where the

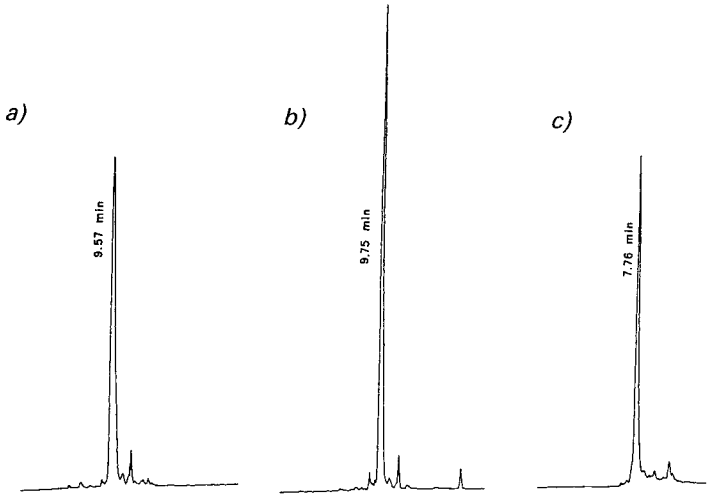


Fig. 1. *Reversed-phase HPLC of* a) *crude p60*^{src}*-(523–531)-nonapeptide, obtained with* O^4-Bu^t*-protected Tyr,* b) *crude p60*^{src}*-(523–531)-nonapeptide, obtained with side-chain-unprotected Tyr, and* c) *crude* $O^{4,527}$*-phospho-p60*^{src}*-(523–531)-nonapeptide* (**3**) *prepared by global phosphorylation.* Conditions: 0–100% MeCN in 0.05% CF_3COOH, 30 min, λ = 230 nm.

result was not as good as for the other peptides. This indicates that steric influences associated with the different reagents appear not to significantly hamper the reaction. As an example, *Fig. 1c* shows the reversed-phase HPLC profile of the crude $O^{4,527}$-phospho-p60^{src}-(523–531)-nonapeptide (**3**) obtained with reagent **1b**.

The oxidation step following phosphinylation was performed using an I_2 solution. This has been found in nucleotide chemistry to be superior in solid-phase approaches to 3-chloroperbenzoic acid and *tert*-butyl hydroperoxide, although the latter two reagents have been found to be very effective for oxidation of P^{III} to P^{V} in solution [8]. As expected, no electrophilic substitution of I_2 on the aromatic ring of L-tyrosine was observed due to the conversion of the L-tyrosine OH function into the phosphoric-acid triester prior to oxidation.

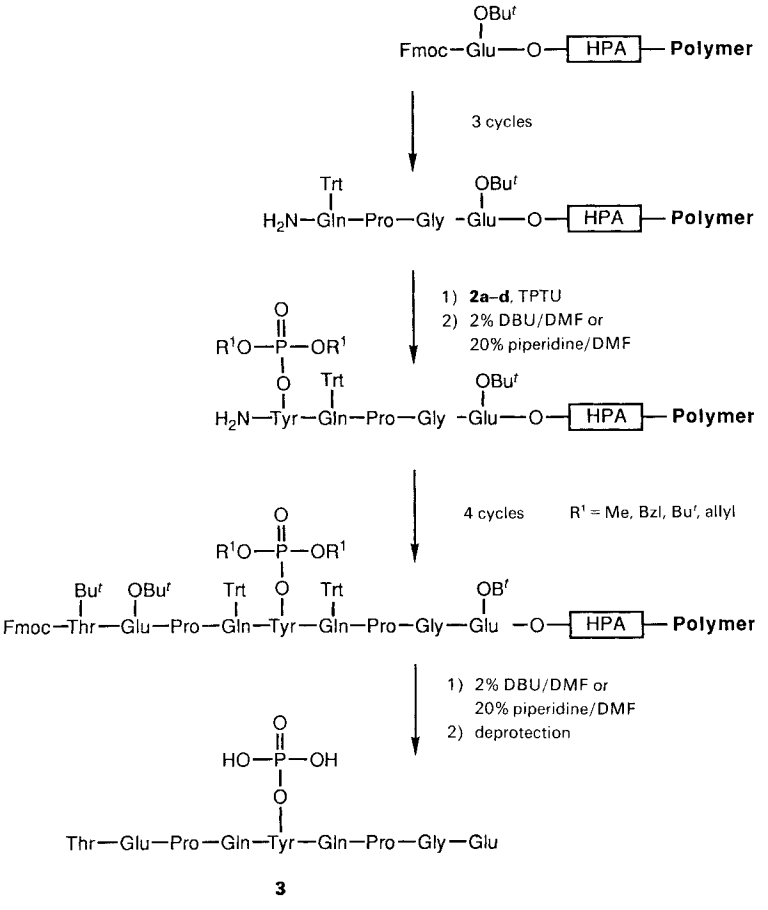
Where many tyrosine residues are present, selective phosphorylation can be achieved by incorporating side-chain protection (*e.g.* as *tert*-butyl ethers) for those OH-containing residues which are not to be modified. This strategy was used in the synthesis of insulin-receptor peptides **7**–**9**, selectively phosphorylated at positions Tyr^{1146}, Tyr^{1150}, and Tyr^{1151} (*Table*).

2.4. *Building-Block Approach.* Several problems have been encountered with the introduction of phosphorylated building units in peptide synthesis. The incorporation of Fmoc-Ser[$PO_3(Bzl)_2$]-OH as well as subsequent acylation steps have been observed to proceed with difficulty [19]. Partial loss of phosphoryl-protecting groups during the course of the synthesis and the possible dephosphorylation during the final deprotection step are also of concern. Nevertheless, it has been shown in two communications that building blocks **2a** and **2b** can be successfully applied in the Fmoc solid-phase synthesis of phosphotyrosine-containing peptides [14] [15]. Coupling reactions were achieved using [(1*H*-benzotriazol-1-yl)oxy]tris(dimethylamino)phosphonium hexafluorophosphate (BOP) [20] or [(1*H*-benzotriazol-1-yl)oxy]tris(pyrrolidin-1-yl)phosphonium hexafluorophosphate (PyBOP) [21].

The undesired removal of the methyl or benzyl groups when **2a** and **2b** were used, to give the respective phosphodiester during N^{α}-deprotection, could be decreased but not completely suppressed by employing 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in DMF instead of the customary piperidine/DMF solution [15]. A number of experiments have revealed that phosphodiesters resulting from partial deprotection cannot be activated by a possible excess of TPTU [22], which might lead to side reactions. However, it is still possible that the phosphodiester, formed on partial deprotection due to piperidine treatment, consumes part of the incoming amino acid and, therefore, decreases the actual excess of reagent for the acylation reaction. For this reason, we have extended the range of phosphate-protecting groups to allyl and Bu^t since these groups should not be susceptible to nucleophilic attack. Orthogonal cleavage procedures as, *e.g.*, with [$Pd^0(PPh_3)_4$] for allyl groups could also solve the potential problem of dephosphorylation in the final deprotection step. It has been demonstrated in DNA chemistry that allyl groups can be effectively removed from phosphate functions of protected oligonucleotides linked to a solid support [23].

Typically, the syntheses of the different O^4-phospho-L-tyrosine-containing peptides **3**–**9** were performed as shown for the $O^{4,527}$-phospho-p60^{src}-(523–531)-nonapeptide (**3**; *Scheme 5*). Syntheses were carried out essentially as described in *Sect. 2.3*. The p60^{src}-peptide was used as a test sequence for all phospho-L-tyrosine building blocks **2a**–**d**: from

Scheme 5



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the on-line UV monitoring of the synthesis cycles, which the continuous-flow method of synthesis permits, and from reversed-phase HPLC's of crude products (*Fig. 2*), no significant differences could be observed in their reactivities or in the incorporation of subsequent residues. The peptides **4**–**9** were synthesized by applying the dimethyl- or the dibenzyl-phosphate-protected derivatives **2a** and **2b**, respectively.

The synthesis of the $O^{4,505}$-phospho-p56^{lck}-(501–509)-nonapeptide (**5**) involved the incorporation of Fmoc-Pro-Gln(Trt)-OH (**10**) in the first acylation step to avoid diketopiperazine formation when C-terminal proline is present [10]. In the synthesis of $O^{4,394}$-phospho-p56^{lck}-(391–394)-tetrapeptide, we presumably obtained partial succinimide formation from cyclization of the aspartic-acid residue [10], as indicated by FAB-MS ([MH – 18]$^+$). This side reaction was prevented by reverting to piperidine for Fmoc deprotection in place of the DBU/DMF solution and taking into account that partial deprotection of the phosphate group could occur.

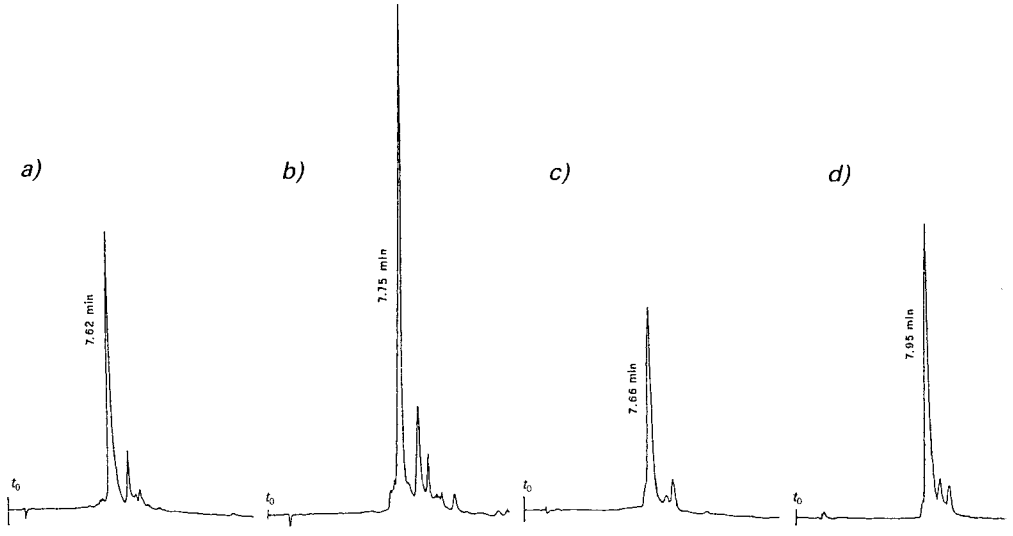

Fig. 2. a) *Reversed-phase HPLC of crude* $O^{4,527}$*-phospho-p60*^{src}*-(523–532)-nonapeptide* (**3**) *prepared by the building-block approach* a) *inserting* **2a**, b) *inserting* **2b**, c) *inserting* **2c**, *and* d) *inserting* **2d**. Conditions as in *Fig. 1.*

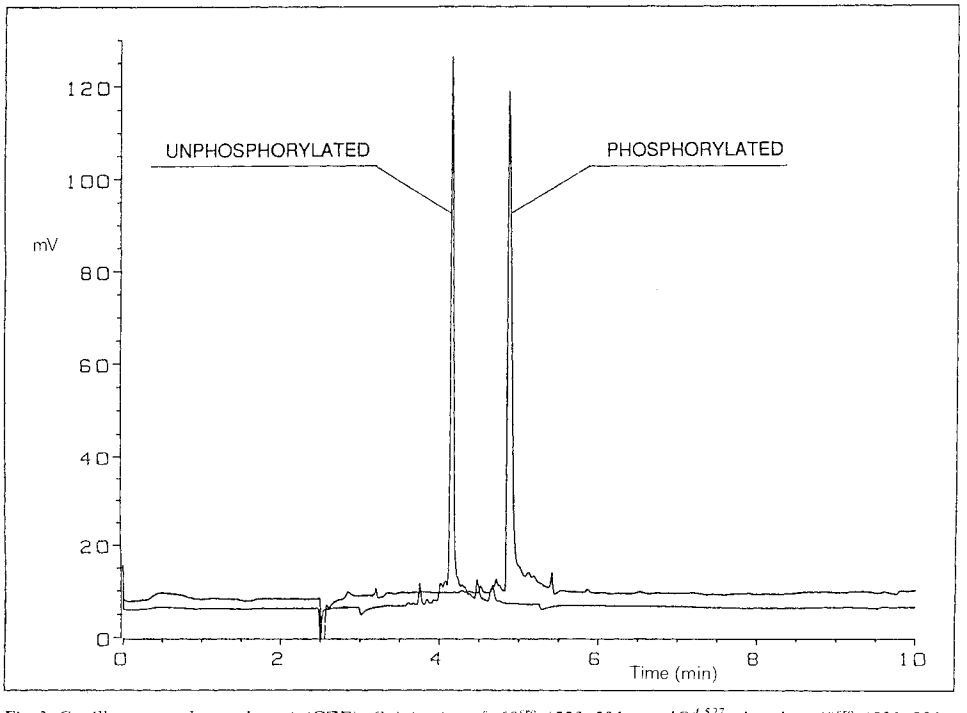

Fig. 3. Capillary zone electrophoresis (CZE): *Coinjection of p60*^{src}*-(523–531)- and* $O^{4,527}$*-phospho-p60*^{src}*-(523–531)-nonapeptide* (**3**). Conditions: 20 mM 3-[(cyclohexyl)amino]propanesulfonic acid (CAPS), pH 11.0, 25 kV.

The deprotection of the phosphorylated peptide attached to the support was carried out depending on the phosphate protecting groups, as already indicated for the global phosphorylation method (*Scheme 4*). When **2a** was incorporated, the cleavage was performed with 1M Me_3SiBr/thioanisole/CF_3COOH. When **2b** and **2c** were used in synthesis, deprotection was achieved with CF_3COOH/5% thiophenol. When applying **2d**, the peptide-resin was first treated with CF_3COOH to cleave the peptide from the support and the side-chain protecting groups and then with $[Pd^0(PPh_3)_4]$ to remove the allyl

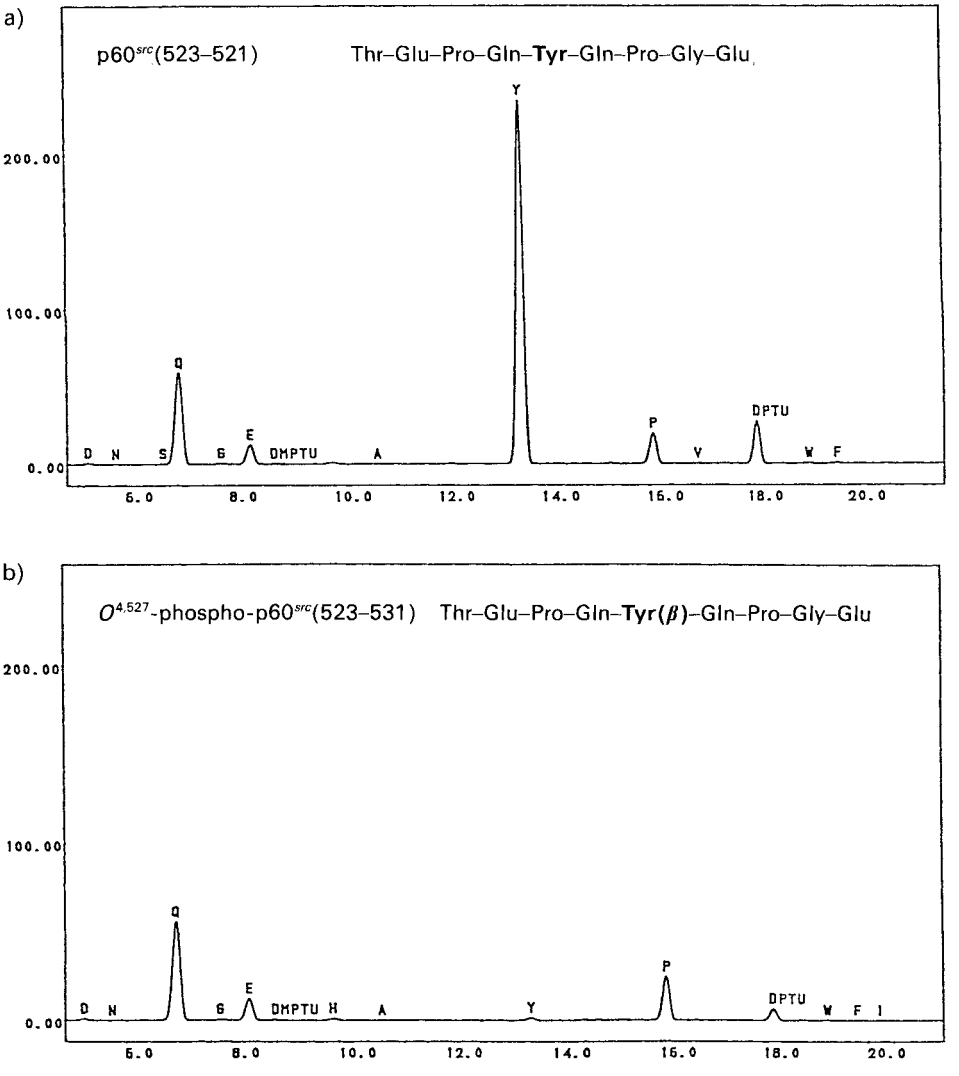

Fig. 4. *Gas-phase sequencing of* a) *residue 527* (Tyr) *in p60*^{src}*-(523–531)-nonapeptide and* b) *residue 527* (Tyr(*P*)) *in* $O^{4,527}$*-phospho-p60*^{src}*-(523–531)-nonapeptide* (**3**). Detection by reversed-phase HPLC at 269 nm as phenylthiohydantoines (PTH); D = Asp, N = Asn, S = Ser, Q = Gln, G = Gly, E = Glu, H = His, A = Ala, Y = Tyr, P = Pro, V = Val, W = Trp, F = Phe, I = Ile; DMPTU = *dim*ethyl*p*henyl*t*hio*u*rea, DPTU = *dip*henyl*t*hio*u*rea.

protecting groups. Alternatively, the reverse order of deprotection can be used, cleaving the allyl groups while the peptide is attached to the solid support. This allows for a washing step to remove excess reagents before the $CF_3COOH/5\%$ thiophenol treatment, which cleaves both the side-chain protecting groups and the peptide from the support. The incorporation of **2a–d** in solid-phase synthesis and the effectiveness of the deprotection method can be judged from the reversed-phase HPLC analysis of the crude $O^{4,527}$-phospho-p60^{src}-(523–531)-nonapeptide (**3**) (*Fig. 2*).

The peptides were purified by prep. reversed-phase HPLC and their purity was ascertained by anal. reversed-phase HPLC and capillary zone electrophoresis (CZE). A clear difference in HPLC as well as in CZE retention times was observed for the phosphorylated and non-phosphorylated forms of each peptide (see, *e.g.*, *Figs. 1* and *3*). The identity of the sequence was confirmed by FAB-MS, standard gas-phase as well as solid-phase protein sequencing. Using gas-phase sequencing, we obtained the expected blank for the cycle corresponding to the O^4-phospho-L-tyrosine residue (*Fig. 4*). With the solid-phase sequencing technique, a positive signal for the O^4-phospho-L-tyrosine residue was obtained in the CZE electropherogram under the conditions of reversed polarity and at pH 2.5 (*Fig. 5*). The phosphorylated peptides **3–9** were tested as substrates for a human transmembrane protein tyrosine phosphatase obtained from an *E. coli* expression system, and the results of this investigation will be reported elsewhere [24].

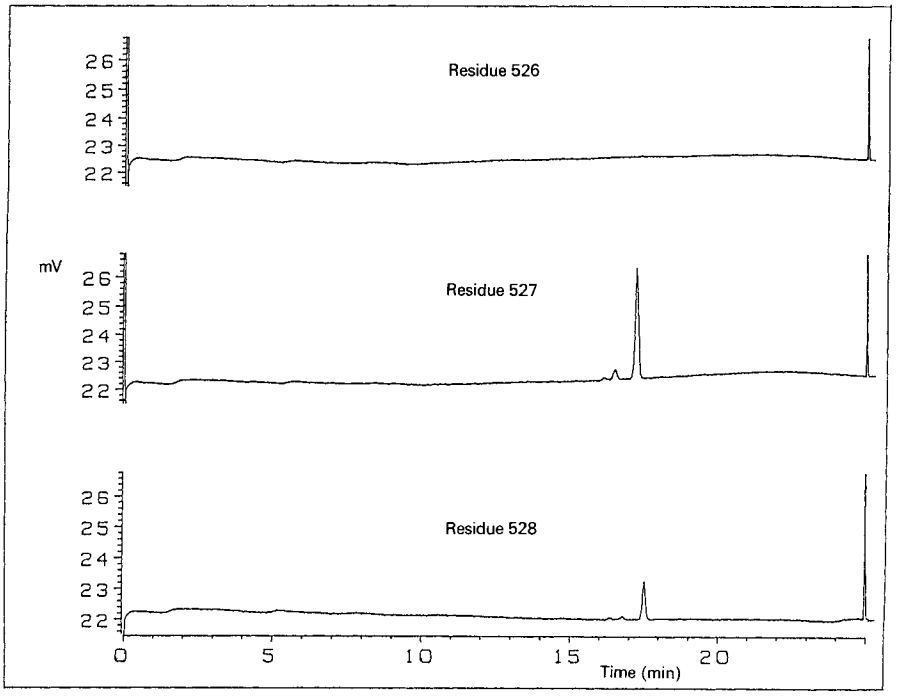

Fig. 5. *Covalent sequencing of residues 526–528 in* $O^{4,527}$*-phospho-p60*^{src}*-(523–531)-nonapeptide* (**3**). Detection by CZE.

3. Conclusion. – O^4-Phospho-L-tyrosine-containing peptides were prepared by either the global phosphorylation of side-chain-unprotected L-tyrosine present in resin-bound peptides or by inserting phosphorylated building blocks for this amino acid in the Fmoc continuous-flow method of solid-phase synthesis. Both procedures yielded comparable results. Different phosphate-protecting groups were applied to gain more flexibility in our synthetic procedures, but no significant differences in reactivity were observed.

Overall, in our hands, the global-phosphorylation procedure appears to be the simpler approach since it omits the preparation of the O^4-phospho-L-tyrosine building block and allows also the preparation of the corresponding unphosphorylated peptide which is often required as a reference. If steric constraints due to secondary structure hamper the effective global phosphorylation or if L-methionine- or L-tryptophan-containing peptides have to be prepared in their phosphorylated version, then the incorporation of the synthons for phospho-L-tyrosine is the better approach. Steric effects are a significant parameter to consider where multiphosphorylations in a peptide are required, and this is currently under investigation.

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Experimental Part

1. *General.* DMF, piperidine, Et(i-Pr)$_2$N, DMEU (all *purum; Fluka*). Fmoc-Amino acids were from *Novabiochem* and *Bachem*, Switzerland. HBTU and TPTU were from *Fluka*. Tetrazole (*Fluka*) was sublimed. 'Kieselgur'-supported poly(dimethylacrylamide) was prepared according to [25]. Peptide syntheses were performed on a *Milligen-9050* continuous-flow synthesizer using an adapted software package. The Fmoc group was used for N^{α}-protection throughout, and side-chain protection was afforded by the following: N^{ω}-(2,2,5,7,8-pentamethylchroman-6-sulfonyl) (Pmc) for arginine [26], trityl (Trt) for glutamine and asparagine [27], (*tert*-butoxy)carbonyl (Boc) for lysine, and *tert*-butyl (Bu^t) for serine, threonine, and glutamic and aspartic acid. At the completion of each synthesis, the peptide-resin was removed from the reaction column and transferred to a sintered funnel, washed with DMF, MeOH, CH_2Cl_2, and Et_2O, and dried under vacuum. Desalting of crude peptide mixtures for anal. HPLC was performed on small *Sephadex* columns (*NAP 25*; *Pharmacia*). Peptides were purified on *Delta-Prep-3000* HPLC (*Waters*). ^{1}H-NMR: at 250 MHz, chemical shifts in δ (ppm) rel. to TMS. Capillary zone electrophoresis (CZE): *270 A* (*ABI*). Protein gas-phase and solid-phase sequencing: *475 A* (*ABI*); in the former, a blank is obtained in the HPLC detection for PTH-O^4-phospho-L-tyrosine and in the latter, a positive signal by CZE detection in 200 mM phosphate buffer (pH 2.5).

2. *Phosphinylation Reagents. (Diethylamino)dimethoxyphosphine* (**1a**) was prepared as described in [28]. The reported b.p. 38–40°/2 Torr should read 38–40°/20 Torr. The latter is in agreement with our observed b.p.

Bis(benzyloxy)(diisopropylamino)phosphine (**1b**) *and Bis(allyloxy)(diisoproplyamino)phosphine* (**1d**) were prepared according to [8] and [9], resp.

Bis(benzyloxy)(diethylamino)phosphine (**1e**) *and Di(*tert*-butoxy)(diethylamino)phosphine* (**1f**) were synthesized according to [29] and [30], resp.

*Di(*tert*-butoxy)(diisopropylamino)phosphine* (**1c**). The intermediate dichloro(*tert*-butoxy)phosphine was prepared from PCl_3 and *t*-BuOH as described in [8]. To 0.44 mol (77.3 g) of this compound in 600 ml of dry Et_2O, 4 mol (562 ml) of (i-Pr)$_2$NH were slowly added over 1 h at −10 to −20°. Stirring was continued at r.t. overnight. The precipitate was filtered under Ar and washed with dry Et_2O and the combined Et_2O soln. evaporated: 104.5 g (78%) of (*tert*-butoxy)bis(diisopropylamino)phosphine. To 108 mmol (32.7 g) of this material, we added 54 mmol (9.45 g) of diisopropylammonium tetrazolide and 40 mmol (12 ml) of *t*-BuOH. After 3 h, the mixture was diluted with 1.2 l of Et_2O and extracted with sat. $NaHCO_3$ soln. (3 × 400 ml). The org. layer was dried (Na_2SO_4) after a

small volume of Et_3N had been added and evaporated. The crude material was purified by short-column chromatography (150 g of silica gel pentane/Et_2O 3:1 (*v/v*) containing 4% of Et_3N; TLC monitoring): 17.36 g (58%) of pure **1c**. ^{1}H-NMR ($CDCl_3$): 1.18 (*d*, 2 $(CH_3)_2CH$); 1.34 (*s*, 2 $(CH_3)_3C$); 3.54–3.69 (*m*, 2 $(CH_3)_2CH$). ^{31}P-NMR ($CDCl_3$): 130.1 (*s*). Anal. calc. for $C_{14}H_{32}NO_2P$ (277.39): C 60.62, H 11.63, N 5.05; found: C 60.89, H 11.73, N 5.03.

Bis(allyloxy)(diethylamino)phosphine (**1g**). A soln. of allyl alcohol (143 mmol, 10 ml) and Et_3N (158 mmol, 22 ml) in dry Et_2O (50 ml) was slowly added to a soln. of dichloro(diethylamino)phosphine (72 mmol, 12.4 ml) in dry Et_2O (90 ml) under Ar such that the reaction temp. was maintained at −10°. After complete addition, stirring was continued for another 2 h at r.t. The mixture was transferred to a separating funnel using 70 ml of sat. $NaHCO_3$ soln., and the aq. phase was discarded. The org. layer was washed with 70 ml of sat. $NaHCO_3$ soln., dried (Na_2SO_4), and evaporated and the crude liquid residue purified by short-column chromatography (100 g of silica gel, CH_2Cl_2/AcOEt/Et_3N 70:25:5): 5.8 g (75%) of **1g**. Colourless oil. ^{1}H-NMR ($CDCl_3$): 1.06 (*t*, 2 CH_3); 3.03–3.17 (*m*, 2 CH_2); 4.13–4.25 (*m*, 2 CH_2=CHCH_2); 5.10–5.36 (*m*, 2 CH_2=$CHCH_2$); 5.86–6.03 (*m*, 2 CH_2=$CHCH_2$). ^{31}P-NMR ($CDCl_3$): 148.27 (*s*). Anal. calc. for $C_{10}H_{20}NO_2P$ (217.25): C 55.29, H 9.28, N 6.45, P 14.26; found: C 54.83, H 9.30, N 6.36, P. 13.69.

3. *O⁴-Phospho-L-tyrosine Building Blocks.* *N²-{[(9H-Fluoren-9-yl)methoxy]carbonyl}-O⁴-(dimethoxyphosphoryl)-L-tyrosine* (**2a**). To a stirred soln. of 7.44 mmol (3.00 g) of Fmoc-Tyr-OH in 20 ml of anh. THF were added 2 ml of (*t*-Bu)Me_2SiCl (8.18 mmol, 1.27 g) in anh. THF, followed by 7.44 mmol (0.84 ml) of *N*-methylmorpholine (→white suspension). After 10 min, 14.88 mmol (2.46 g) of **1a** and 22.32 mmol (1.56 g) of tetrazole were added, and stirring was continued for 3 h at r.t. The mixture was cooled to −40°, and 14.88 mmol (2.04 ml) of 70% *tert*-butyl hydroperoxide were added. Then the cold bath was removed, and after 1 h, the mixture was again cooled to −40°, and 15 ml of 10% $Na_2S_2O_5$ soln. were added. The mixture was transferred for separation using 100 ml of Et_2O, and the org. layer was washed with 30 ml of 10% $Na_2S_2O_5$ soln., 30 ml of H_2O, and 30 ml of a sat. NaCl soln. The org. layer was evaporated and the residue vigorously stirred in AcOH/H_2O/THF 2:1:1 (30 ml) for 1 h at r.t. The mixture was concentrated to a smaller volume, and 5% $NaHCO_3$ soln. (50 ml) was added. After 5 min vigorous stirring, the aq. layer was washed with Et_2O (2 × 50 ml), then chilled, and carefully acidified to pH 3 using 3M HCl. The product was extracted with CH_2Cl_2 (2 × 50 ml) and the combined extract dried (Na_2SO_4), filtered, and evaporated to yield a foam. Purification by short-column chromatography in two lots (60 g of silica gel, $CHCl_3$/MeOH 9:1) gave 2.20 g (57%) of anal. pure **2a**. TLC (silica gel, $CHCl_3$/MeOH/H_2O 65:25:4): R_f 0.63. $[\alpha]_D^{20} = 42.3$ ($c = 0.7$, $CHCl_3$). ^{1}H-NMR ((D_6)DMSO): 2.80–3.14 (*m*, CH_2(3) of Tyr); 3.77 (*d*, *J*(P,H) = 11.25 , 2 MeO); 4.10–4.26 (*m*, CH(2) of Tyr, $COOCH_2CH$); 7.30–7.92 (*m*, arom. H); 7.21 (*AA'XX'*, C_6H_4); 7.77 (*d*, NH); 12.80 (br. *s*, COOH). ^{31}P-NMR ((D_6)DMSO): −2.89 (*s*). FAB-MS (Xe, positive mode): 512 (50, MH^+). Anal. calc. for $C_{26}H_{26}NO_8P$ (511.47): C 61.06, H 5.12, N 2.74, P. 6.06; found: C 60.94, H 5.12, N 2.82, P 6.15.

N²-{[(9H-Fluoren-9-yl)methoxy]carbonyl}-O⁴-[bis(benzyloxy)phosphoryl]-L-tyrosine (**2b**) was prepared essentially as described for **2a** and according to [15] starting with the same amounts but using **1e** for the phosphinylation. Chromatography (100 g of silica gel) as described for **2a** gave 3.50 g (71%) of anal. pure amorphous **2b**. TLC (silica gel, $CHCl_3$/MeOH/H_2O 65:25:4): R_f 0.75. M.p. 57–67°. $[\alpha]_D^{20} = 35.1$ ($c = 0.6$, $CHCl_3$) ([15]: $[\alpha]_D^{18} = 53.8$ ($c = 1.2$, $CHCl_3$)). ^{1}H-NMR ((D_6)DMSO): 2.78–3.14 (*m*, CH_2(3) of Tyr); 4.10–4.26 (*m*, CH(2) of Tyr, $COOCH_2C$); 5.14 (*d*, *J*(P,H) = 8.38, 2 PhCH_2O); 7.36 (br. *s*, 2 C_6H_5); 7.19 (*AA'XX'*, C_6H_4); 7.25–7.93 (*m*, arom. H); 7.78 (*d*, NH); 12.80 (br. *s*, COOH). ^{31}P-NMR ((D_6)DMSO): −5.02 (*s*). FAB-MS (Xe, positive mode): 686 (60, $[M + Na]^+$). Anal. calc. for $C_{38}H_{34}NO_8P$ (663.66): C 68.77, H 5.16, N 2.11, P 4.67; found: C 68.35, H 5.22, N 2.04, P 4.52.

*N²-{[(9H-Fluoren-9-yl)methoxy]carbonyl}-O⁴-[di(*tert*-butoxy)phosphoryl]-L-tyrosine* (**2c**) was prepared as described for **2a** and **2b**, but with 1.98 mmol (0.80 g) of Fmoc-Tyr-OH, 1.98 mmol (0.30 g) of (*t*-Bu)Me_2SiCl 1.90 mmol (0.22 ml) of *N*-methylmorpholine, 4.95 mmol (0.22 ml) of **1f**, and 6.93 mmol (0.49 g) of tetrazole. Oxidation was performed with 4.95 mmol (0.95 g) of 3-chloroperbenzoic acid. Workup as described for **2a** and **2b** and chromatography (55 g of silica gel, $CHCl_3$/MeOH 9:1) yielded 0.84 g (71%) of pure **2c** as a foam. TLC (silica gel, $CHCl_3$/MeOH/H_2O 65:25:4): R_f 0.71. Sodium salt (more stable): $[\alpha]_D^{20} = 3.38$ ($c = 0.8$, $CHCl_3$). ^{1}H-NMR ((D_6)DMSO): 1.32, 1.33 (2*s*, 2 $(CH_3)_3C$); 2.85–3.24 (*m*, CH_2(3) of Tyr); 4.05–4.37 (*m*, CH(2) of Tyr, $COOCH_2CH$); 7.06–7.95 (*m*, arom. H); 7.14 (*AA'XX'*, C_6H_4); 7.82 (br. *d*, NH). ^{31}P-NMR ((D_6)DMSO): −14.04 (*s*). FAB-MS (Xe, positive mode): 522 (20, $[MH - Bu^tOH]^+$). Anal. calc. for $C_{32}H_{37}NNaO_8P$ (617.61): C 62.23, H 6.04, N 2.27, P 5.02; found: C 62.13, H 6.15, N 2.44, P 4.83.

N²-{[(9H-Fluoren-9-yl)methoxy]carbonyl}-O⁴-[bis(allyloxy)phosphoryl]-L-tyrosine (**2d**) was prepared as described for **2c** starting from 2.48 mmol (1.00 g) of Fmoc-Tyr-OH. Solvent: 10 ml of dry MeCN/THF 1:1. Oxidation, workup, and short-column chromatography as above yielded anal. pure **2d** (880 mg, 63%). TLC (silica gel, $CHCl_3$/MeOH/H_2O 65:25:4): R_f 0.66. $[\alpha]_D^{20} = -18.00$ ($c = 0.1$, DMF). ^{1}H-NMR ((D_6)DMSO): 2.76–3.16 (*m*,

CH_3(3) of Tyr); 4.08–4.26 (*m*, CH(2) of Tyr, $COOCH_2CH$); 4.54–4.67 (*m*, 2 CH_2=$CHCH_2$); 5.26 (*ddd*, 2 CH_2=$CHCH_2$); 5.94 (*m*, 2 CH_2=$CHCH_2$); 7.26 (*AA'XX'*, C_6H_4); 7.36–7.96 (*m*, arom. H); 7.78 (*d*, NH). ^{31}P-NMR ((D_6)DMSO): −5.00 (*s*). FAB-MS (Xe, positive mode): 564 (35, MH^+). Anal. calc. for $C_{30}H_{29}NNaO_8P$ (585.52): C 61.54, H 5.00, N 2.39; found: C 61.21, H 5.23, N 2.37.

4. *Fmoc-Pro-Gln(Trt)-OH* (**10**). *Z-Pro-Gln(Trt)-OH* (**11**). To 40 ml of H_2O/DMF 1:1 containing 4.00 mmol (1.52 g) of H-Gln(Trt)-OH were added 12 mmol (12 ml) of 1M $NaHCO_3$ followed by the dropwise addition of 4.40 mmol (1.52 g) of Z-Pro-OSu [31] in 40 ml of DMF. The mixture was stirred for 16 h at r.t. and then filtered. The filtrate was concentrated to one-third of the original volume, diluted with 150 ml of H_2O, acidified to pH 3 with 1M $KHSO_4$, and extracted using AcOEt (2 × 100 ml). The combined extracts were dried (Na_2SO_4), filtered, and concentrated several times from MeCN to yield a foam which was crystallized from AcOEt/hexane: 2.25 g (90%) of **11**. M.p. 158.0–158.4°. $[\alpha]_D^{20} = -20.9$ (*c* = 0.6, DMF). FAB-MS (Xe, positive mode): 642 (50, $[M + Na]^+$), 620 (30, MH^+). Anal. calc. for $C_{37}H_{37}N_3O_6$ (619.72): C 71.71, H 6.02, N 6.78; found: C 71.41, H 5.89, N 6.74.

HCl·H-Pro-Gln(Trt)-OH (**12**). A soln. of 12.00 mmol (7.78 g) of **11** in MeOH/H_2O 95:5 (50 ml) was hydrogenated over 10% Pd/C for 6 h (→precipitate which was resolubilized by addition of 13 mmol (1.50 g) of Py·HCl). After a total of 8 h, the catalyst was filtered off and the filtrate evaporated. The white solid was crystallized from MeOH/petroleum ether: 5.79 g (92%) of **12**. M.p. 229° (dec.). $[\alpha]_D^{20} = -26.8$ (*c* = 0.7, DMF). FAB-MS (Xe, positive mode): 486 (30, MH^+), 243 (100, $[MH - Trt]^+$). Anal. calc. for $C_{29}H_{31}ClN_3O_4$ (521.04): C 66.85, H 6.00, N 8.06; found: C 66.47, H 6.08, N 7.96.

Fmoc-Pro-Gln(Trt)-OH (**10**). The pH of a suspension of 9.10 mmol (3.40 g) of **12** in 100 ml of H_2O/1,4-dioxan 1:1 was adjusted to 9.5 before 9.10 mmol (3.05 g) of Fmoc-OSu were added. The mixture was vigorously stirred for 2 h at pH 9.5, and then 100 ml of a 5% $KHSO_4$/10% K_2SO_4 soln. was added. A white gum settled, and after exhaustive trituration, a solid was collected, washed with H_2O, and dried under vacuum over P_2O_5. Attempts to crystallize it failed: 5.00 g (80%) of anal. pure **10**. TLC (silica gel, $CHCl_3$/MeOH/H_2O 65:25:4): R_f 0.63. M.p. 163.7–164.3°. $[\alpha]_D^{20} = -20.0°$ (*c* = 0.6, DMF). FAB-MS (Xe, positive mode): 746 (50, $[M + K]^+$), 730 (25, $[M + Na]^+$). Anal. calc. for $C_{44}H_{41}N_3O_6$ (707.83): C 74.66, H 5.84, N 5.94; found: C 74.70, H 6.01, N 5.84.

5. *Peptide Synthesis for Global Phosporylation: p60^{src}-(523–531)-nonapeptide*. To poly(dimethylacrylamide) on 'Kieselgur' (3.18 g; corresponding to 0.44 mmol functionalization), equipped with a [4-(hydroxymethyl)-phenoxy]acetic (HPA) linker, in DMF, 1.2 mmol (510 mg) of Fmoc-Glu(OBu^t)-OH, 1.2 mmol (455 mg) of HBTU, 2.4 mmol (0.41 ml) of Et(i-Pr)$_2$N, and 0.2 mmol (25 mg) of 4-(dimethylamino)pyridine were added and reacted for 12 h at r.t. The support was washed with DMF and Et_2O and dried. The loading was estimated by cleaving the Fmoc group and reacting the free amino-group with 2,2'-thiobis[acetic acid] anhydride followed by elemental analysis of the S-content. The degree of esterification was determined as 90% and corresponds to 0.14 mmol/g. 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: 2.5-fold excess of activated amino acid, 30 min; Fmoc-amino acid derivatives were predissolved in 2 ml of DMEU/MeCN 3:1 (*v*/*v*) per mmol together with 1 equiv. of TPTU and activated immediately before coupling by addition of 2 equiv. of Et(i-Pr)$_2$N (1 ml; 2M in DMEU); Fmoc-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. Part of the peptide-resin was deprotected with CF_3COOH/H_2O/thiophenol 95:2.5:2.5 (*v*/*v*/*v*) for 30 min at r.t. After filtration, AcOH was added, the soln. concentrated to a small volume, and the crude peptide precipitated with Et_2O. The peptide was dissolved in H_2O and desalted in two lots using a small *NAP-25* column (*Pharmacia*). Reversed-phase HPLC analysis of the product is shown in *Fig. 1b*. The remaining crude peptide was purified by prep. reversed-phase HPLC and identified by FAB-MS.

For comparison, we synthesized this peptide using side-chain-protected (Bu^t ether) Fmoc-L-tyrosine using the above procedure (*Fig. 1a*).

6. *Global Phosphorylation: O^{4,527}-Phospho-p60^{src}-(523–31)nonapeptide* (**3**). The support (1.5 g, 0.21 mmol) from the p60^{src} synthesis with side-chain-unprotected L-tyrosine 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. Then, 15 ml of anh. MeCN containing 500 mg (7.14 mmol) of tetrazole and 1.75 g (5 mmol) of **1b** were added *via* syringe and reacted for 10 min. The mixture was removed by suction and 5 ml of 1M I_2 in THF/2,6-dimethylpyridine/H_2O 40:10:1 were added and removed by filtration under vacuum. This was repeated several times. The support was then washed with MeCN and dried. Deprotection was performed with CF_3COOH/H_2O/thiophenol 95:2.5:2.5 as described before: crude **3**. HPLC: *Fig. 1c*. Purification was achieved using prep. reversed-phase HPLC: 61 mg of pure **3**. The purity was also assessed by CZE (*Fig. 3*). FAB-MS: 1128.6 ($C_{45}H_{66}N_{11}O_{21}P$, calc. 1128.1). Gas-phase and solid-phase protein sequencing: *Fig. 4* and *5*, resp.

Phosphorylated peptide **3** was prepared in the same way as described above with the other phosphinylation reagents **1b–d**. Deprotection was then identical with the ones described in *Sect. 7*.

All other peptides **4–9** (*Table*) were prepared in the same way as **3** with reagent **1b** for the global phosphorylation and with identical deprotection. The peptides were all purified by prep. reversed-phase HPLC and their purity confirmed by anal. reversed-phase HPLC and CZE. Characterization was performed by FAB-MS and protein sequencing.

$O^{4,394}$-*Phospho-p56*^{lck}-*(390–398)-nonapeptide* (**4**): From 1.5 g of resin, 72 mg of HPLC-purified material. FAB-MS: 1206.4 ($C_{45}H_{68}N_{13}O_{24}P$, calc. 1206.1). Protein sequencing: blank for residue 394.

$O^{4,505}$-*Phospho-p56*^{lck}-*(501–509)-nonapeptide* (**5**): From 0.87 g of resin, 15 mg of pure **5**. FAB-MS: 1127.5 ($C_{45}H_{67}N_{12}O_{20}P$, calc. 1127.1). Protein sequencing: blank for residue 505.

$O^{4,1173}$-*Phospho-EGFR-(1167–1177)-undecapeptide* (**6**): From 0.8 g of resin, 5.7 mg of pure **6**. FAB-MS: 1316.0 ($C_{53}H_{86}N_{15}O_{22}P$, calc. 1316.3). Protein sequencing: blank for residue 1173.

$O^{4,1146}$-*Phospho-IR-(1142-1153)-dodecapeptide* (**7**): From 1.34 g of resin, 19 mg of pure **7**. FAB-MS: 1702.6 ($C_{72}H_{108}N_{19}O_{27}P$, calc. 1702.7). Protein sequencing: blank for residue 1146.

$O^{4,1150}$-*Phospho-IR-(1142–1153)-dodecapeptide* (**8**): From 1.34 g of resin, 15 mg of pure **8**. FAB-MS: 1702.7 ($C_{72}H_{108}N_{19}O_{27}P$, calc. 1702.7). Protein sequencing: blank for residue 1150.

$O^{4,1151}$-*Phospho-IR(1142–1153)-dodecapeptide* (**9**): From 1.34 g of resin, 31 mg of pure **9**. FAB-MS: 1702.7 ($C_{72}H_{108}N_{19}O_{27}P$, 1702.7). Protein sequencing: blank for residue 1151.

7. *Incorporation of* O^4-*Phospho-*L-*tyrosine Building Blocks* **2a–d**. Syntheses were carried out as described in *Sect. 5* but starting from 0.10 to 0.15 mmol of functionalized support. Esterification of the first amino acid and chain elongations were performed with the same excess of amino-acid building blocks and under the same conditions as described in *Sect. 5*. For the synthesis of **3**, all different building units **2a–d** were applied. No significant differences could be observed (*Fig. 2a–d*). All other phospho-L-tyrosine-containing peptides **4–9** were prepared by inserting **2b** into the solid-phase synthesis. The cleavage of the Fmoc group was performed with 2% DBU-DMF to minimize phosphate deprotection. In Asp-containing peptides, 20% piperidine was used instead, to avoid succinimide formation [10].

For the synthesis of **5**, we incorporated building block **2b** and dipeptide **10** starting from 0.80 g (0.15 mmol/g) of resin to which the first amino acid Fmoc-Pro-OH had already been esterified.

The deprotection was carried out according to the building blocks used: *Building Block* **2a**: The dried peptide-resin (0.90 g, 0.15 mmol/g) was treated overnight (16 h) at 4° with a 10-ml mixture containing Me_3SiBr (1.29 ml), thioanisole (1.18 ml), and CF_3COOH (7.50 ml), and *m*-cresol (0.08 ml) was used as an additional scavenger. The resin was filtered off and washed with a small volume of CF_3COOH. The filtrate was concentrated and the crude peptide precipitated with chilled Et_2O, washed with Et_2O (2×) and Et_2O/AcOEt, then dissolved in little H_2O, and desalted on a *NAP-25* column. Lyophilization of the eluant yielded 13 mg of crude **3**. Reversed-phase HPLC: *Fig. 2a*. Coinjection with an authentic sample indicated the product to be predominantly the desired **3**.

Building Blocks **2b** *and* **2c**: Cleavage and deprotection of the peptide-resins resulting from the incorporation of **2b** and **2c** in solid-phase syntheses was achieved using a 10-ml mixture of CF_3COOH/5% thiophenol for 1–2 h at r.t. Crude product isolation as above yielded the desired **3** whose homogeneity was assessed by anal. reversed-phase HPLC (*Figs. 2b, c*).

Building Block **2d**: A sample of the peptide-resin (400 mg) prepared by incorporating building block **2d** was first treated with CF_3COOH/5% thiophenol (10 ml) for 2 h. The cleaved resin was filtered and washed with a small volume of CF_3COOH and the filtrate concentrated. The peptide salt was isolated as above (20 mg). Reversed-phase HPLC: product of high homogeneity. FAB-MS: 1208.7 (intense M^+ of [bis-(allyloxy)phosphoryl]-protected peptide). In the second step, 14 mg of this protected peptide salt was treated overnight under Ar with 0.4 ml of Et_3N, 0.4 ml of HCOOH, 6 mg of PPh_3, and 4 mg of $[Pd^0(PPh_3)_4]$ in 1 ml of 1,4-dioxan/THF/H_2O 1:1:1. The mixture was evaporated and the residue taken up in H_2O and purified using a short *Sephadex* (*NAP-25*) column. Lyophilization yielded **3** as a foam (10 mg). Reversed-phase HPLC: *Fig. 2d*. FAB-MS: 1128.6 (intense MH^+), $[M + Na]^+$.

Deprotection of all phosphorylated peptides **4–9** when **2b** had been applied was performed as indicated in *Sect. 6* for the preparation of **3** with global phosphorylation using **1b**. The products proved to be identical with those obtained by global phosphorylation.

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