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N,N'-Dialkyldiamide-type Phosphate Protecting Groups for Fmoc Synthesis of Phosphotyrosine-containing Peptides

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Abstract: Z-Tyr[P(O)(NHR)$_2$]-OBzl (R=*n*-Pr and *i*-Pr) were obtained by reactions of Z-Tyr-OBzl, lithiated with LDA, with *N,N'*-dipropyl- or diisopropylphosphorodiamidic chloride. Fmoc derivatives, Fmoc-Tyr[P(O)(NHR)$_2$]-OH, were obtained from them as stable crystals. The P-N bonds are stable toward 20% piperidine in DMF and were cleaved completely with 95% trifluoroacetic acid.

Synthesis of phosphotyrosine-containing peptides and peptide mimetics to study the cellular signal pathways and to develop potential antitumor agents is an active area of research.[1]

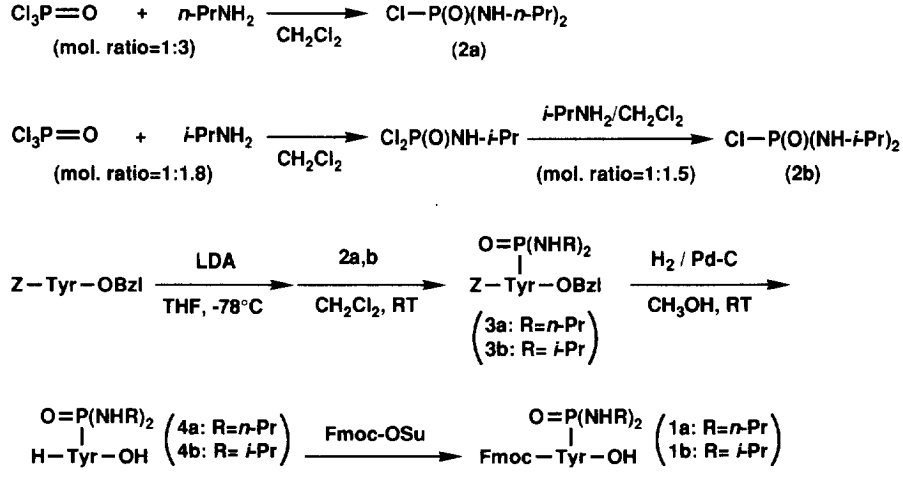
Two major strategies, postphosphorylation and prephosphorylation, have been adopted in phosphopeptide synthesis, both of which, however, still entail some problems. In the postphosphorylation consisting of phosphitylation and oxidation,[2] which may not be compatible with methionine, cysteine and tryptophan residues, incomplete oxidation has been indicated as a side reaction.[3] Lack of sufficient protecting groups for the phosphate group is the major problem in the prephosphorylation.[4] For this reason, use of a phosphotyrosine derivative with a free phosphate group has also been proposed,[3] but the instability of the free form restricts its versatile use. This paper describes our attempts to develop a new type of phosphate protecting group.

The basis of phosphate protection hitherto used is the ester formation. Phosphate esters have two cleavable sites at P-O and C-O bonds. Loss of one of the two ester groups, especially under acidic conditions in the Boc strategy synthesis, should be attributed to the acid-catalyzed P-O bond fission, which would occur independently of the alkyl groups used.[5] Removal of one of the two alkyl groups of Tyr[P(O)(OR)$_2$] (R=methyl and benzyl) by the strong nucleophile, piperidine, during Fmoc-solid phase synthesis was also reported.[6] Therefore, in this study we tried to develop amide-type protecting groups applicable to the Fmoc strategy. Since C-N bonds are stable under the acidic and basic conditions used in peptide synthesis, these protecting groups would have the possibility of cleavage under acidic conditions only at two P-N bonds.

There are three phosphoamino acids in nature. In developing a new phosphate protecting group, there is a great difference in conditions between aromatic and aliphatic phosphoamino acids. In the case of phosphoserine and phosphothreonine, stability of their protected form toward β-elimination would be one of the important factors in evaluating the protecting group. Thus, protection of the phosphate group of phosphotyrosine was pursued in this study.

Our preliminary experiments using linear and cyclic *N,N*-dialkylamides with the structure of PhOP(O)(NR$_2$)$_2$ as model revealed that their P-N bonds strongly resisted the acidic conditions used in the final deprotection step in the Fmoc/*t*-Bu strategy synthesis. However, a report suggesting a dif-

ference in the mechanism of alkaline hydrolysis of tetramethyl- and dipropylphosphorodiamidic chlorides[7] encouraged us to examine monoalkyl amides. Although no difference in rates of neutral and acid hydrolyses was reported for the above chlorides,[7] our results using $PhOP(O)(NHR)_2$ (R=Me, Et and *i*-Pr) showed that their P-N bonds could be cleaved almost completely within 1h by treating with a 95% trifluoroacetic acid (TFA) solution. This was in strong contrast with the fact that under the same conditions, 57% of $PhOP(O)(NMe_2)_2$ could be recovered without change. Our study next was focused on *N,N'*-dialkyldiamide-type protecting groups.



Preparation of Fmoc-phosphotyrosine derivatives protected as *N,N'*-dipropyl- and diisopropyldiamides (**1a** and **1b**) were performed according to the above equations. *N,N'*-dipropylphosphorodiamidic chloride, $(n\text{-PrNH})_2P(O)Cl$ (**2a**), was obtained in 41% yield as solids by the simple reaction of propylamine with $POCl_3$ in dichloromethane as reported.[7] *N,N'*-diisopropylphosphorodiamidic chloride, $(i\text{-PrNH})_2P(O)Cl$ (**2b**), was also prepared for easiness of assignment of ^{1}H-NMR spectral signals. For some unclear reason, a two-step reaction was necessary to prepare **2b**, which was obtained in a total yield of 43% as an oil.

N-Benzyloxycarbonyltyrosine benzyl ester (Z-Tyr-OBzl) was lithiated with lithium diisopropylamide (LDA) and successively treated with **2a, b** to give the protected phosphotyrosine derivatives **3a, b** in 94 and 71% yields, respectively. Their structures were ascertained by EI-MS and ^{1}H- and ^{13}C-NMR spectroscopy.[8] After removal of Z and Bzl ester groups by catalytic hydrogenolysis, an Fmoc group was introduced in the usual manner to give Fmoc-Tyr[$P(O)(NHR)_2$]-OH (**1a**: R=*n*-Pr; **1b**: R=*i*-Pr) as colorless crystals in 80 and 76% yields, respectively.[9]

Stability of the new amide-type phosphate protecting groups under the conditions for Fmoc deprotection was checked using **3a**. When **3a** was treated with 20% piperidine in DMF at RT for 72h, no change could be observed. On the other hand, when **3a** was treated with 2 equivalents of tetrabutylammonium fluoride (TBAF) hydrate[10] at RT for 30 min, 79% of **3a** was lost by decomposition. Since partial loss of diphenoxyphosphinyl, $(PhO)_2P(O)$-, group has also been reported,[11] use of TBAF for Fmoc deprotection should be avoided.

Deprotection of phosphorodiamidates could be carried out simply by acid hydrolysis. Compound **4a** was treated with 95% TFA solution for 1h to give phosphotyrosine, which showed the

same elution pattern with the authentic sample on an amino acid analyzer. Phosphorus 31 NMR also assured the complete conversion of **4a** (14.3ppm from H_3PO_4) to phosphotyrosine (-3.8ppm). As for stability and deprotection, the isopropylamide derivatives gave almost the same results.

When **1a** was coupled with H-Leu-OMe with water soluble carbodiimide-1-hydroxybenzotriazole in dichloromethane, Fmoc-Tyr[P(O)(NH*n*-Pr)$_2$-Leu-OMe (**5**) was obtained in 91% yield. Proton-NMR of the product showed that neither of the two amide bonds was lost during coupling. Compound **5** was treated with 95% TFA at RT for 4h to give Fmoc-Tyr(PO_3H_2)-Leu-OMe (**6**) in 96% yield,[12] which was coeluted on reversed phase HPLC[13] with the sample obtained by direct coupling of Fmoc-Tyr(PO_3H_2)-OH[3] with H-Leu-OMe. HPLC analysis also showed no formation of Fmoc-Tyr-Leu-OMe, the dephosphorylation product of **5** or **6**.[13]

Recently a paper appeared on evaluation of several Fmoc-Tyr(PO_3R_2)-OH derivatives through synthesis of two model compounds.[14] To evaluate the new protecting group, Gly-Val-Tyr(PO_3H_2)-Ala-Ala-Ser-Gly (**7**), one of the model compounds, was synthesized using **1a**. Solid phase synthesis was performed on a semiautomatic continuous flow instrument using 20% piperidine in DMF for deprotection and diisopropylcarbodiimide for coupling. Clear deprotection, together with release of the peptide chain from the resin, was accomplished with 95% TFA within 4h (Figure A).[15] After gel chromatography on Sephadex LH-20 using methanol for elution **7** was obtained in 52% yield as TFA salt, which was further purified with preparative HPLC to homogeneity (Figure B).[15] The identity of the product was established by FAB-MS and amino acid analysis.[16]

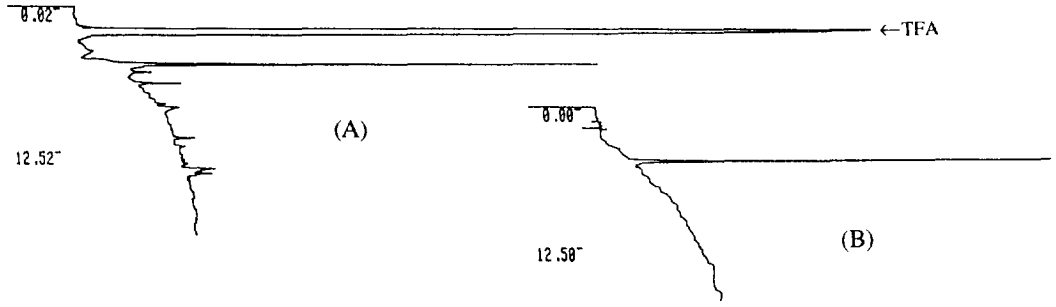


Figure. HPLC profiles of the deprotection mixture (A) and the purified **7** (B)

In conclusion, the new monoalkylamide-type protecting groups are suitable as a phosphate protecting group for the Fmoc strategy synthesis. Fmoc-Tyr[P(O)(NHR)$_2$]-OH (R=*n*-Pr and *i*-Pr) as stable materials with good crystallinity could serve as new building blocks for synthesis of phosphotyrosine-containing peptides. No significant difference between *n*-Pr and *i*-Pr derivatives has so far been observed. The only minor difference is that **2a** is more stable in storage and more convenient in introduction to the tyrosine moiety. Further applications are now under way.

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7. P. S. Traylor and F. H. Westheimer, *J. Am. Chem. Soc.*, **87**, 553-559(1965).
8. **3a**: colorless oil, $[\alpha]_D^{31}$ 4.3° (c1.0, $CHCl_3$); EI-MS (m/z) 567 ($[M]^+$); ^{1}H-NMR (270MHz, $CDCl_3$) δ=0.89(t, J=7.3Hz, 6H, $CH_2CH_2C\underline{H}_3$), 1.42-1.56(m, 4H, $CH_2C\underline{H}_2CH_3$), 2.73-2.86(m, 2H, P(O)N$\underline{H}$), 2.89-2.94(m, 4H, $C\underline{H}_2CH_2CH_3$), 3.05(dd, J=4.6, 5.6Hz, 2H, Tyr βCH$_2$), 4.64-4.67(m, 1H, Tyr αCH), 5.08(s, 2H, ArCH$_2$), 5.13(d, J=5.6Hz, 2H, ArCH$_2$), 5.38-5.41(m, 1H, TyN$\underline{H}$), 6.93(d, J=8.2Hz, 2H, Tyr 2, 6H), 7.06(d, J=8.2Hz, Tyr 3, 5H), 7.32-7.36(m, 10H, ArH); ^{13}C-NMR δ=11.21($CH_2CH_2\underline{C}H_3$), 24.98(d, J=7.3Hz, $CH_2\underline{C}H_2CH_3$), 37.25(Tyr βC), 43.07 ($\underline{C}H_2CH_2CH_3$), 54.82(Tyr αC), 66.94(Ar$\underline{C}$H$_2$), 67.24(Ar$\underline{C}$H$_2$), 120.43(d, J=4.9Hz, Tyr C3, C5), 128.06, 128.16, 128.50, 128.56, 128.62(Ar), 130.44(Tyr C2, C6), 131.46 (Tyr C1), 135.04, 136.21(Ar), 150.31(d, J=7.3Hz, Tyr C4), 155.67(urethane CO), 171.30(Tyr CO). **3b**: colorless oil, $[\alpha]_D^{29}$ 3.6° (c1.0, $CHCl_3$); EI-MS (m/z) 567 ($[M]^+$); ^{1}H-NMR (270MHz, $CDCl_3$) δ=1.12-1.16(m, 12H, CH(C$\underline{H}_3$), 2.56(d, J=9.6Hz, 1H, P(O)N$\underline{H}$), 2.60(d, J=9.6Hz, 1H, P(O)N$\underline{H}$), 3.05 (dd, J=5.9, 6.3Hz, 2H, Tyr βCH$_2$), 3.40-3.47(m, 2H, C$\underline{H}$(CH$_3$)$_2$), 4.63-4.66(m, 1H, Tyr αCH), 5.07 (s, 2H, ArCH$_2$), 5.12(d, J=5.3Hz, 2H, ArCH$_2$), 5.46-5.49(m, 1H, TyrN$\underline{H}$), 6.94(d, J=8.2Hz, 2H, Tyr 2, 6H), 7.08(d, J=8.2Hz, 2H, Tyr 3, 5H), 7.31-7.33 (m, 10H, ArH); ^{13}C-NMR δ=25.28 (d, J=6.1Hz, CH($\underline{C}$H$_3$)$_2$), 25.37(d, J=6.1Hz, CH($\underline{C}$H$_3$)$_2$), 37.07 (Tyr βC), 43.56($\underline{C}$H(CH$_3$)$_2$), 54.74 (Tyr αC), 66.76(Ar$\underline{C}$H$_2$), 67.06(Ar$\underline{C}$H$_2$), 120.16(d, J=4.8Hz, Tyr C3, C5), 127.91,128.00, 128.16, 128.37, 128.48(Ar), 130.24(Tyr C2, C6), 131.16(Tyr C1), 134.93, 136.10(Ar), 150.41 (d, J=6.1Hz, Tyr C4), 155.56(urethane CO), 171.19(Tyr CO).
9. **1a**: m.p. 147°C, $[\alpha]_D^{25}$ -8.3°(c 0.75, DMF); **1b**: m.p. 150-151°C, $[\alpha]_D^{27}$ -15.7°(c 0.75, DMF).
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12. After submission of this paper a communication that recommended *N,N*-dimethylamide as a versatile tyrosine phosphate protecting group appeared (H.-G. Chao, *et al.*, *J. Org. Chem.*, **60**, 7710-7711 (1995)), though, under the present conditions, Fmoc-Tyr[P(O)(NMe$_2$)$_2$]-Leu-OMe took more than 10h for complete phosphate deblocking.
13. Column: 5μm μBONDASPHERE C18 (3.9mm x 150mm). Elution: 0.1%TFA-acetonitrile (3:2 to 1:4 over 20min). Flow rate: 1ml/min. Detection: 280nm. Retention times: 16.1min (**5**), 7.6 min (**6**) and 17.7min (Fmoc-Tyr-Leu-OMe).
14 R. M. Valerio, A. M. Bray, N. J. Maeji, P. O. Morgan and J. W. Perich, Lett. Pept. Sci., **2**, 33-40 (1995).
15 Column: 5μm μBONDASPHERE C18 (3.9mm x 150mm). Elution: 0.1%TFA-acetonitrile (5: 95 to 40:60 over 20min), Flow rate: 1ml/min. Detection: 220nm. Retention time: 5.0min.
16 FAB-MS (m/z) 704 ($[M+H]^+$), 726($[M+Na]^+$); Amino acid ratios (4%HSCH$_2$CO$_2$H in 6M HCl 110°C 24h): Ser(0.95), Gly(2.06), Ala(2.01), Val(1.00), Tyr(0.90).

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