Preparations of N^{α}-Fmoc-O-[(Benzyloxy)hydroxyphosphinyl] β-Hydroxy α-Amino Acid Derivatives[1)]

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(Received September 29, 1995)

N^{α}-Fmoc-O-[(benzyloxy)hydroxyphosphinyl]serine and N^{α}-Fmoc-O-[(benzyloxy)hydroxyphosphinyl]threonine were prepared in order to be utilized for the synthesis of phosphoserine- or phosphothreonine-containing peptides based on the Fmoc-mode pre-phosphorylation strategy. These derivatives were obtained as crystalline compounds, which are favorable for use as building blocks for an automated peptide synthesis by a solid-phase method.

In previous papers we presented a practical procedure for the solid-phase synthesis of phosphopeptides based on the Boc-mode pre-phosphorylation strategy.[2—7)] Although the Boc strategy is well established as a conventional method for peptide synthesis, the Fmoc strategy is presently also a very common methodology. In particular, the Fmoc-mode solid-phase method seems to be rather advantageous for investigators who are not so familiar with peptide synthesis. It is therefore quite valuable to establish a synthetic method for phosphopeptides by the Fmoc strategy in terms of further progress in biochemical studies concerning protein-phosphorylation; biological chemists investigating this field are required to prepare many kinds of phosphopeptides related to natural phosphoproteins. However, the Fmoc-mode pre-phosphorylation strategy has so far been believed to be inadequate for synthesizing phosphoserine- or phosphothreonine-containing peptides, since β-elimination of phosphate in the phosphoamino acid residues readily occurs during a standard cycle for the deprotection of the Fmoc group with piperidine.[8,9)]

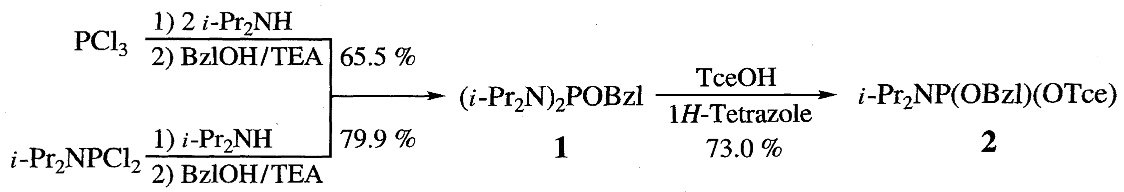
We recently precisely examined the base stability of the bis-protected O-phosphono group in the phosphoserine residue, and found the following very interesting facts: 1) a ready β-elimination of phosphate was observed in the O-(diphenoxyphosphinyl)serine derivative; 2) a partial cleavage of the protecting groups for the O-phosphono group by aminolysis with piperidine occurred much faster than β-elimination of phosphate in O-[di(4-nitrobenzyloxy)-, dibenzyloxy-, and dimethoxyphosphinyl]serine derivatives; and 3) the thus-formed O-[(alkoxy)hydroxyphosphinyl]serine residue was totally stable to piperidine.[1b,1e)]

As a result of an examination concerning the base stability of phosphate, we succeeded to propose an efficient methodology for synthesizing phosphopeptides using N^{α}-Fmoc-O-[(alkoxy)hydroxyphosphinyl] β-hydroxy α-amino acid derivatives.[1b,1e)] In the present paper we describe the synthesis of novel and valuable phosphoamino acid derivatives, i.e., N^{α}-Fmoc-O-[(benzyloxy)hydroxyphosphinyl]serine (Fmoc–Ser[PO(OBzl)(OH)]–OH) and N^{α}-Fmoc-O-[(benzyloxy)hydroxyphosphinyl]threonine (Fmoc–Thr[PO(OBzl)(OH)]–OH).

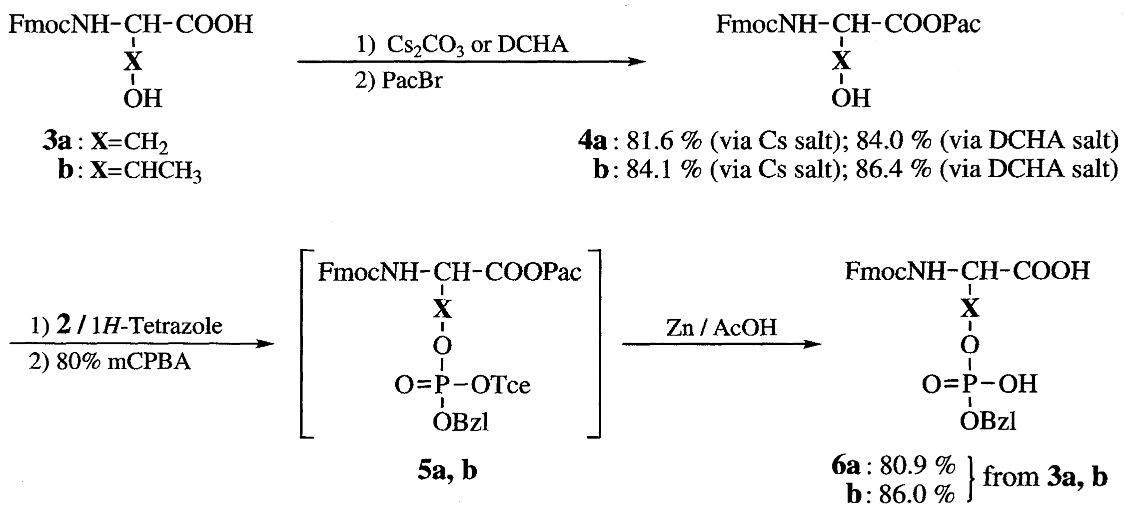
Results and Discussion

New amidite as a phosphite-forming reagent, i.e., i-$Pr_2NP(OBzl)(OTce)$[10)] (**2**), was first prepared as shown in Scheme 1.[11—14)] Fmoc–Ser (**3a**) and Fmoc–Thr (**3b**) were converted into the corresponding phenacyl (Pac) esters via Cs or DCHA salt. The formation of phosphite from Fmoc–Ser–OPac (**4a**) and Fmoc–Thr–OPac (**4b**) with amidite **2** was carried out as shown in Scheme 2. Each phosphite intermediate was then oxidized with mCPBA. The Pac and Tce groups in the phosphorylation products, **5a** and **5b**, were concurrently removed by Zn/AcOH reduction to give the desired O-[(benzyloxy)hydroxyphosphinyl] derivatives, **6a** and **6b**, respectively. (Scheme 2) Both **6a** and **6b** were obtained as crystalline compounds, which are favorable for use as builiding blocks for an automated peptide synthesis by a solid-phase method.

Of these derivatives, **6a** had already been applied to the solid-phase synthesis of phosphopeptides related to the heat-shock protein HSP27.[1e)] Although details concerning the synthetic study of phosphopeptides using **6a** and **6b** will be reported in the near future, the following important facts should be pointed out: 1) the O-phosphono group in these derivatives does not disturb the formation of the peptide bond using conventional coupling reagents; 2) β-elimination of phosphate during a standard deprotection cycle of the Fmoc group can be completely suppressed in these derivatives; and 3) the Bzl group is readily removable under conventional conditions for a final deprotection procedure using TFA and



Scheme 1.



Scheme 2.

additives.[15,16)]

Experimental

All of the melting points are uncorrected. The specific rotations were measured on a Perkin–Elmer 241 polarimeter. ^{1}H NMR and ^{31}P NMR spectra were recorded on JEOL JNM-EX 270 and JNM-GSX 400 NMR spectrometers, respectively. The chemical shifts in ^{1}H NMR (270 MHz) are given in δ values from TMS used as the internal standard, and those in ^{31}P NMR (162 MHz) from 75% aqueous phosphoric acid used as the external standard. Fast atom bombardment mass-spectra (FAB-MS) were obtained on a JEOL JMS SX-270 mass spectrometer. Silica-gel column chromatography was carried out with Merck silica gel 60 (Art. 9385, 230—400 mesh) at medium pressure (1—5 kg cm^{-2}). The compounds containing phosphorus were characterized by a color reaction with Dittmer–Lester reagent.[17)]

Benzyl Tetraisopropylphosphorodiamidite [(i-Pr$_2$N)$_2$P(OBzl)] (1).[14)] **Method A:** To a solution of diisopropylamine (DIPA) (144 g, 1.42 mol) in anhydrous hexane (600 ml) was added dropwise a solution of trichlorophosphine (48.8 g, 355 mmol) in anhydrous hexane (60 ml) over 40 min with stirring at 0 °C in an atmosphere of N_2; as far as we examined, it is not necessary to use argon instead of N_2. The mixture was stirred for 3 h at r.t., and then heated under reflux for 19 h.[11)] To the reaction mixture neutralized with triethylamine (TEA) was added a solution of benzyl alcohol (BzlOH) (38.4 g, 355 mmol) and TEA (36.3 g, 355 mmol) in anhydrous diethyl ether (300 ml) over 1 h with stirring at 0 °C in an atmosphere of N_2. After stirring for 30 min at r.t., the precipitated salt was filtered off, and the filtrate was concentrated in vacuo. The residue was dissolved in hexane (800 ml), and the hexane solution was washed with acetonitrile (80 ml×4), followed by evaporation in vacuo to obtain (i-Pr$_2$N)$_2$P(OBzl) as an oily substance. Yield 78.6 g (65.5%). The thus-obtained crude material was submitted to the following reaction without further purification.

Method B: Diisopropylphosphoramidous dichloride[12,13)] (14.9 g, 73.7 mmol) and DIPA (9.66 ml, 73.7 mmol) were dissolved in anhydrous hexane (210 ml); the solution was then refluxed for 3 d in an atmosphere of N_2. To the reaction mixture cooled at 0 °C was added TEA (10.3 ml, 73.7 mmol), and then BzlOH (7.70 ml, 73.7 mmol) over 5 min with stirring; the mixture was stirred for 2.5 h at r.t. in an atmosphere of N_2. After TEA (4 ml) was added to the reaction mixture, insoluble material was filtered off, and the filtrate was concentrated in vacuo. The residue dissolved in hexane (200 ml) was washed with CH_3CN (30 ml×3), and then hexane layer was concentrated in vacuo to obtain (i-Pr$_2$N)$_2$P(OBzl) (19.9 g, 79.9%).

Benzyl 2,2,2-Trichloroethyl Diisopropylphosphoramidite [(i-Pr$_2$N)P(OBzl)(OTce)] (2). To a solution of **1** (78.6 g, 232 mmol) and 1H-tetrazole (16.3 g, 232 mmol) in anhydrous CH_2Cl_2 (500 ml) was added dropwise 2,2,2-trichloroethanol (TceOH) (22.3 ml, 232 mmol); the solution was stirred for 2 h at r.t. in an atmosphere of N_2. The reaction mixture was washed with saturated aqueous $NaHCO_3$ (100 ml×2), dried over anhydrous $MgSO_4$ in an atmosphere of N_2, and concentrated in vacuo. An oily residue was purified by medium-pressure column chromatography [Merck silica gel 60, Art. 9385, 500 g; silica gel was sufficiently prewashed with hexane–TEA (10 : 1)] and pure i-Pr$_2$NP(OBzl)(OTce) was obtained by elution with hexane–TEA (100 : 1) as a colorless oil. Yield 65.5 g (73.0%); FAB-MS: m/z 386.1 [(M+H)$^+$] (calcd 386.1). ^{1}H NMR (DMSO-d_6) $\delta = 1.16$, 1.20 (each 6H, d, $J=7.0$ Hz, –CH(C$\underline{H}_3$)$_2$×2), 3.58—3.77 (2H, m, –C$\underline{H}$(CH$_3$)$_2$×2), 4.24 and 4.29 (each 1H, dd, $J=6.1$ and 11.8 Hz, CH$_2$CCl$_3$), 4.72 and 4.79 (each 1H, dd, $J=8.6$ and 12.8 Hz, C$\underline{H}_2$Ph), 7.24—7.42 (5H, m, CH$_2\underline{\text{Ph}}$). ^{31}P NMR (DMSO-d_6) $\delta = 151.52$ (s).

N^α-(9-Fluorenylmethoxycarbonyl) β-Hydroxy α-Amino Acid Phenacyl Ester (4). Fmoc–Ser–OPac (4a)—Method A (as

a general procedure via Cs salt): To a solution of Fmoc–Ser–OH (**3a**) (22.0 g, 67.2 mmol) in dioxane (130 ml) was added Cs_2CO_3 (10.9 g, 33.6 mmol) in H_2O (50 ml) at 0 °C, and the solution was lyophilized. To a solution of the residue in DMF (200 ml) were added phenacyl bromide (PacBr) (14.5 g, 67.2 mmol) and Cs_2CO_3 (1.09 g, 3.36 mmol) at 0 °C. After stirring for 5 min at 0 °C and for 3 h at r.t., the reaction mixture was neutralized with citric acid, and concentrated in vacuo. To the residue were added AcOEt (200 ml) and 10% citric acid (50 ml). AcOEt layer separated was washed successively with 10% citric acid (30 ml×2), saturated aqueous $NaHCO_3$ (30 ml×3), and brine (30 ml×3). The organic layer was dried over anhydrous $MgSO_4$, and concentrated in vacuo. The thus-obtained solid residue was triturated with hexane, and collected by filtration. The crude product (28.6 g) was recrystallized from AcOEt. Yield 24.4 g, (81.6%), mp 110—113 °C, $[\alpha]_D^{22}$ −24.3° (*c* 1.00, $CHCl_3$). Found: C, 69.40; H, 5.26; N, 3.11%. Calcd for $C_{26}H_{23}NO_6$: C, 69.53; H, 5.11; N, 3.19%. ^1H NMR (DMSO-d_6) $\delta = 3.76$—3.86 (2H, m, CH_2/Ser), 4.21—4.38 (4H, m, CH/Fmoc, CH_2/Fmoc, and CH/Ser), 4.98 (1H, bs, OH), 5.50 and 5.57 (each 1H, d, $J = 17.0$ Hz, CH_2/Pac), 7.30—7.98 (14H, m, NH, aromatic/Fmoc, and Ph/Pac).

Method B (as a general procedure via DCHA salt): To a solution of Fmoc–Ser–OH (9.00 g, 27.5 mmol) in AcOEt was added DCHA (5.48 ml, 27.5 mmol). After allowing to stand for 2 h, precipitated DCHA salt was filtered; yield 14.0 g (quant).

A part of the thus-obtained Fmoc–Ser–OH·DCHA (5.00 g, 9.89 mmol) and PacBr (2.15 g, 10.8 mmol) were dissolved in 50 ml of DMF. To the solution was added TEA (138 μl, 0.989 mmol); the mixture was then stirred for 3.5 h at r.t. Precipitated DCHA·HBr was filtered off, and the filtrate was concentrated in vacuo. The residue was worked up in a similar manner as described in method A: Yield 3.70 g (84.0%).

Fmoc–Thr–OPac (**4b**) was also prepared similarly by both methods A and B: Yield 84.1% (method A) and 86.4% (method B); mp 133.5—136.5 °C [83—88 °C (sintering)]; $[\alpha]_D^{22}$ −45.8° (*c* 1.02, $CHCl_3$). Found: C, 70.25; H, 5.41; N, 3.09%. Calcd for $C_{27}H_{25}NO_6$: C, 70.30; H, 5.50; N, 3.04%. ^1H NMR (DMSO-d_6) $\delta = 1.20$ (3H, d, $J = 6.3$ Hz, CH_3/Thr), 4.20—4.34 (5H, m, CH/Fmoc, CH_2/Fmoc, α- and β-CH/Thr), 4.79 (1H, bs, OH), 5.53 (2H, s, CH_2/Pac), and 7.30—7.98 (14H, m, NH, aromatic/Fmoc, and Ph/Pac).

***N*$^\alpha$-(9-Fluorenylmethoxycarbonyl)-β-[[(benzyloxy)(2,2,2-trichloroethoxy)phosphinyl]oxy] α-Amino Acid Phenacyl Ester (5). Fmoc–Ser[PO(OBzl)(OTce)]–OPac (5a) (as a general procedure):** To a solution of **4a** (5.00 g, 11.2 mmol) in anhydrous THF (25 ml) were added amidite **2** (6.08 g, 15.7 mmol) and 1*H*-tetrazole (2.36 g, 33.6 mmol) in an atmosphere of N_2. After stirring for 1.5 h at r.t. in an atmosphere of N_2, to the reaction mixture was added 80% *m*CPBA (6.05 g, 28.0 mmol) at 0 °C, followed by stirring for 10 min at 0 °C and for 30 min at room temperature; the oxidation was quenched by the addition of $NaHSO_3$ (2.93 g, 28.0 mmol) in H_2O (10 ml) at 0 °C. The mixture was stirred for 5 min at 0 °C,and concentrated in vacuo. To the residue was added AcOEt (50 ml); the insoluble material was then filtered off. The filtrate was washed successively with aqueous 10% citric acid (20 ml×3), brine (20 ml×1), saturated aqueous $NaHCO_3$ (20 ml×6), and brine (20 ml×3). The organic layer was dried over anhydrous $MgSO_4$, and concentrated in vacuo. The oily residue was subjected to the following cleavage reaction of the Pac and Tce groups without further purification.[18)]

Fmoc–Thr[PO(OBzl)(OTce)]–OPac (**5b**) was prepared by the same method as described above, and the oily product was subjected to the following reaction.

***N*$^\alpha$-(9-Fluorenylmethoxycarbonyl)-β-[[(benzyloxy)hydroxyphosphinyl]oxy] α-Amino Acid (6). Fmoc–Ser[PO(OBzl)(OH)]–OH (6a) (as a general procedure):** To a solution of Fmoc–Ser[PO(OBzl)(OTce)]–OPac (**5a**) (11.2 mmol) in 90% acetic acid (110 ml) was added zinc dust (11.0 g, 168 mmol) in several portions. After stirring for 1.5 h, insoluble materials were filtered off. The insoluble materials were treated with a mixture of AcOEt (50 ml) and 1 M HCl (20 ml, $M = mol\ dm^{-3}$) saturated with NaCl; a part of Fmoc–Ser[PO(OBzl)(OH)]–OH, which precipitated as Zn salt, can be recovered by this treatment. The filtrate was concentrated in vacuo, and the residue was triturated with diethyl ether. A powdery product collected by filtration was dissolved in a mixture of AcOEt (100 ml) and 1 M HCl (30 ml) saturated with NaCl, and aqueous layer separated was extracted again with AcOEt (20 ml×2). All AcOEt extracts were combined, washed with brine (30 ml×4), and dried over $MgSO_4$.[19)] After stirring for 5—10 min, $MgSO_4$ was filtered off, and the filtrate was concentrated in vacuo to give an oily residue, which could be crystallized by trituration with AcOEt–diethyl ether–hexane. The thus-obtained material was sufficiently pure to be used for peptide synthesis. Yield 4.50 g, (80.9%). A part of the product was recrystallized from AcOEt–diethyl ether–hexane to obtain an analytical sample: Mp 110—111 °C, $[\alpha]_D^{24}$ +6.3° (*c* 1.1, EtOH). Found: C, 59.57; H, 4.93; N, 2.92%. Calcd for $C_{25}H_{24}NO_8P \cdot 0.5H_2O$: C, 59.29; H, 4.98; N, 2.77%. ^1H NMR (DMSO-d_6) $\delta = 4.08$—4.36 (6H, m, CH/Fmoc, CH_2/Fmoc, CH/Ser, and CH_2/Ser), 4.93 (2H, d, $J = 7.0$ Hz, $\underline{CH_2}Ph$), 7.28—7.89 (14H, m, NH, aromatic/Fmoc, and $CH_2\underline{Ph}$). ^{31}P NMR (DMSO-d_6) $\delta = -0.89$ (s).

Fmoc–Thr[PO(OBzl)(OH)]–OH (**6b**) was prepared by the same method as described above. Yield 86.0%, mp 154.5—156.5 °C, $[\alpha]_D^{26}$ +5.5° (*c* 1.0, EtOH). Found: C, 60.95; H, 5.13; N, 2.84%. Calcd for $C_{26}H_{26}NO_8P$: C, 61.06; H, 5.12; N, 2.74%. ^1H NMR (DMSO-d_6) $\delta = 1.30$ (3H, d, $J = 6.3$ Hz, CH_3/Thr), 4.15—4.28 (4H, m, CH/Fmoc, CH_2/Fmoc, and α-CH/Thr), 4.75—4.81 (1H, m, β-CH/Thr), 4.92 (2H, d, $J = 6.9$ Hz, $\underline{CH_2}Ph$), 7.23—7.94 (14H, m, NH, aromatic/Fmoc, and $CH_2\underline{Ph}$). ^{31}P NMR (DMSO-d_6) $\delta = -1.48$ (s).

This work was partly supported by Grands-in-Aid for Scientific Research Nos. 04640526 and 05680503 from the Ministry of Education, Science and Culture, and SUNBOR GRANT from Suntory Institute for Bioorganic Research.

References

1) A part of this work was presented in: a) K. Saruta, T. Wakamiya, J. Yasuoka, and S. Kusumoto, "the 67th National Meeting of the Chemical Society of Japan," Tokyo, March 1994, Abstr., No 2E16; b) T. Wakamiya, K. Saruta, Y. Yasuoka, and S. Kusumoto, *Chem. Lett.*, **1994**, 1099; c) T. Wakamiya, K. Saruta, J. Yasuoka, and S. Kusumoto, "the 23rd European Peptide Symposium," Braga, September 1994, Abstr., No P364; e) T. Wakamiya, K. Saruta, J. Yasuoka, and S. Kusumoto, "the 32nd Japanese Peptide Symposium," Fukuoka, October 1994, Abstr., No. O2, and published in "Peptide Chemistry 1994," ed by M. Ohno, Protein Research Foundation, Osaka (1995), p. 5.

2) T. Wakamiya, M. Kawahara, S. Kusumoto, S. Imajoh-Ohmi, and S. Kanegasaki, "Peptide Chemistry 1991," ed by A. Suzuki, Protein Research Foundation, Osaka (1992), p. 383.

3) T. Wakamiya, *Chem. Express*, **7**, 577 (1992).

4) T. Wakamiya, K. Saruta, S. Kusumoto, K. Nakajima, K. Yoshizawa-Kumagaye, S. Imajoh-Ohmi, and S. Kanegasaki, *Chem. Lett.*, **1993**, 1401.

5) T. Wakamiya, K. Saruta, S. Kusumoto, S. Aimoto, K. Yoshizawa-Kumagaye, and K. Nakajima, "Peptide Chemistry 1993," ed by Y. Okada, Protein Research Foundation, Osaka (1994), p. 17.

6) K. Sato, K. Ishiguro, A. Omori, J. I. Kim, T. Uchida, K. Saruta, and T. Wakamiya, "Peptide Chemistry 1993," ed by Y. Okada, Protein Research Foundation, Osaka (1994), p. 109.

7) T. Wakamiya, K. Saruta, J. Yasuoka, and S. Kusumoto, *Bull. Chem. Soc. Jpn.*, **68**, 2699 (1995).

8) J. -M. Lacombe, F. Andriamanampisoa, and A. A. Pavia, *Int. J. Pept. Protein Res.*, **36**, 275 (1990).

9) This methodology is applicable to the synthesis of phosphotyrosine-containing peptides, since tyrosine is not β-hydroxy α-amino acid.

10) Abbreviations-AcOEt: ethyl acetate; Bzl: benzyl; Boc: *t*-butoxycarbonyl; *m*CPBA: *m*-chloroperbenzoic acid; DCHA: dicyclohexylamine; DMF: *N*,*N*-dimethylformamide; Fmoc: 9-fluorenylmethoxycarbonyl; *i*-Pr: isopropyl; Pac: phenacyl; Ser: serine; Thr: threonine; Tce: 2,2,2-trichloroethyl; TFA: trifluoroacetic acid; THF: tetrahydrofuran.

11) R. B. King and P. M. Sundaram, *J. Org. Chem.*, **49**, 1784 (1984).

12) T. Shimidzu, K. Yamana, and S. Maikuma, *Tetrahedron Lett.*, **25**, 4237 (1984).

13) T. Tanaka, S. Tamatsukuri, and M. Ikehara, *Tetrahedron Lett.*, **27**, 199 (1986).

14) C. E. Dreef, C. J. J. Elie, P. Hoogerhout, G. A. van der Marel, and J. H. van Boom, *Tetrahedron Lett.*, **29**, 6513 (1988).

15) C. G. Fields and G. B. Fields, *Int. J. Pept. Protein Res.*, **35**, 161 (1990).

16) D. S. King, C. G. Fields, and G. B. Fields, *Int. J. Pept. Protein Res.*, **36**, 255 (1990).

17) J. D. Dittmer and R. L. Lester, *J. Lipid Res.*, **5**, 126 (1964).

18) When the oily residue was triturated with AcOEt–diethyl ether–hexane, **5a** can be obtained as a crystalline compound; mp 124—125 °C and $[\alpha]_{\mathrm{D}}^{22}$ +4.9° (c 1.0, $CHCl_3$) after recrystallization from AcOEt and hexane. However, the isolation of **5a** results in a lowering of the total yield from **4a** to **6a**.

19) Hydrogenphosphate moiety of phospho amino acid ester tends to form Na salt during dryness over Na_2SO_4. The use of $MgSO_4$ is recommended to suppress such salt formation.