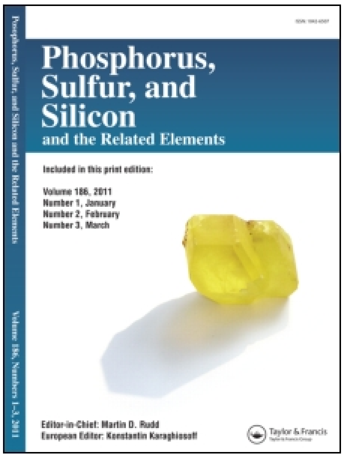

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THE STUDY OF PHOSPHORAMIDITE AS O-PHOSPHITYLATION AGENT AND ITS REACTIVITY

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THE STUDY OF PHOSPHORAMIDITE AS O-PHOSPHITYLATION AGENT AND ITS REACTIVITY

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O-phosphorylated peptide and amino acid are important in living system. In this paper, Dialkyl-N,N-dialkyl phosphoramidites (DDPA) **3** were synthesized using two methods. The reaction of DDPA with the hydroxyl group of the corresponding amino acids in the presence of tetrazole, followed by oxidation, gave O-phosphoryl amino acid methyl esters in a good yield. A systematical study of the reactivity of DDPA was presented too.

Keywords: Dialkyl-N,N-dialkyl phosphoramidites; O-phosphorylation; O-phosphoryl tyrosine methyl ester

INTRODUCTION

O-phosphorylated peptides and proteins are important molecules in the life process. It is well known that phosphorylation and dephosphorylation of protein constitute important regulation effects upon the living system[1]. Increasing interest in the biochemical process provides the impetus for the development of a general synthetic method. Phosphoramidites were used as phosphitylation agents in the synthesis of phosphoamino acid and phosphopeptide (SCHEME 1)[2].

The substituting group of used agent (R^2) were generally benzyl[3] and allyl[4] group, which were either too reactive during the reaction, or too stable to leave at the final step. Comparing to the benzyl or alkyl group,

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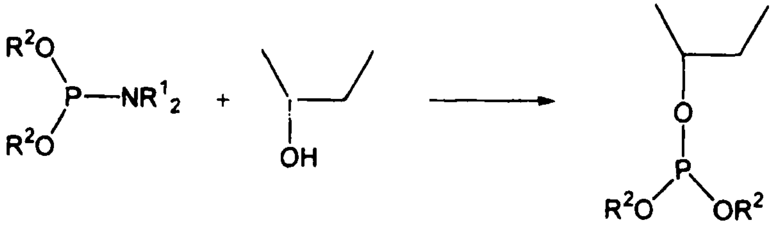
* Corresponding Author.



SCHEME 1 Phosphitylation of amino acid and peptide with phosphoramidite

alkyl substituting groups are stable enough during the reaction and easy to be moved off in the final step. In this paper, Dialkyl-N,N-dialkyl phosphoramidites (DDPA) **3** were synthesized with two methods. DDPA **3** was used as phosphitylation agent in the O-phosphitylation of tyrosine at its OH group, and O-phosphoryl tyrosine methyl ester **7** was synthesized. The reactivity of DDPA **3** was studied too.

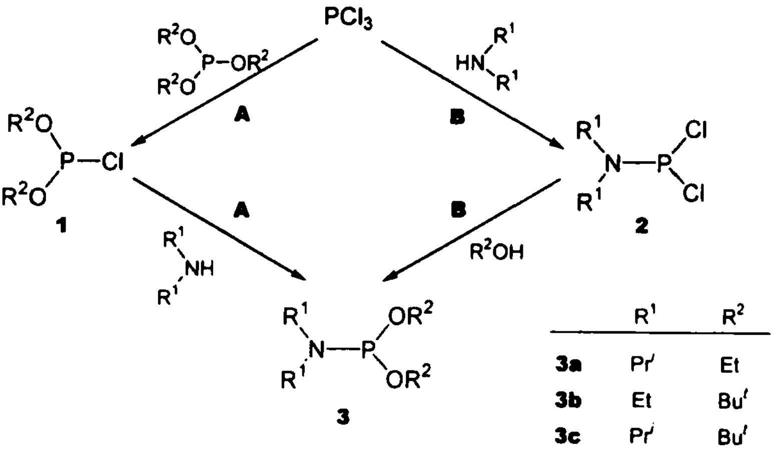
RESULT AND DISCUSSION

Synthesis of Dialkyl-N,N-dialkyl phosphoramidite

Dialkyl-N,N-dialkyl phosphoramidites were synthesized as phosphitylation agents through two methods (SCHEME 2).

In method A, dialkyl-N,N-dialkyl phosphoramidites (DDPA) **3a-c** were synthesized from chlorodialkyl phosphonates (CDPA) **1** which were monochloro derivatives of trialkyl phosphite ester, by converting the P-Cl bond into P-N bond with dialkylamine. Because CDPA **1** and phosphorus trichloride were easy to be hydrolyzed, the reaction system should be waterless. In the second step, converting the CDPA **1** into DDPA **3** with dialkylamine, excess dialkylamine will raise the conversion rate of CDPA **1**. When the mole ratio of CDPA **1** and dialkylamine was changed from 1:2 to 1:4, the conversion rate of CDPA turned from 46.5% to 69.7%.

In method B[3], dichlorophosphoramidites 2 were synthesized with dialkylamine and phosphorus trichloride. Dichlorophosphoramidite **2** was converted into DDPA **3** by reacting with alcohol. In this method, it was also necessary to keep the reaction system waterless. To give the pure intermediate product **2**, the mole ratio of phosphorus trichloride and dialkylamine should be 2:1.



	R^1	R^2
3a	Pr^i	Et
3b	Et	Bu^t
3c	Pr^i	Bu^t

SCHEME 2 Synthesis of phosphoramidites with two methods

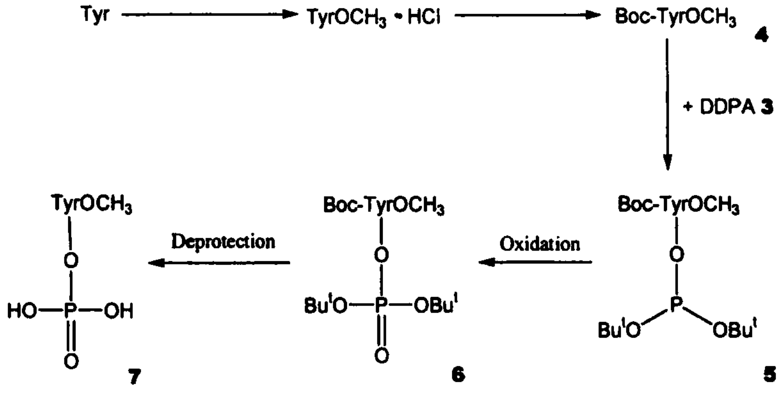
Compound **1**, **2**, **3a** and **3b** were purified by distillation. But compound **3c** could not be distilled because of its high boiling point. Compound **3a**, **3b** and **3c** were stable in alkaline solution and were hydrolyzed in acidic medium.

Synthesis of O-phosphoryl tyrosine methyl ester

To study the O-phosphorylation of tyrosine at its OH group, its amide group was protected with Boc-group[5] and its carboxyl group was protected with methyl ester[6]. The product **4** was called model compound. The model compound of serine and threonine were synthesized too.

Diethyl-N,N-diteriarybutylphosphoramidite **3b** which was used as O-phosphitylation agent was added to the solution of protected tyrosine and tetrazole in acetonitrile. From ^{31}P-NMR, it was found that after 1 minute, the peak at +134 ppm (**3b**) disappeared completely and a new peak at +142 ppm (**5a**) appeared. It means that the reaction finished completely. N-Boc-O^4-[bi-(tert-butyl) phosphino]-tyrosine methyl ester **5a** was purified with short-column chromatography.

For an evaluation of oxidation step, compound **5a** was transformed to N-Boc-O^4-[bi(tert-butyl)phosphoryl]-tyrosine methyl ester **6a** which was a phosphonic triester. In ^{31}P-NMR spectrum, the peak at +142 ppm (**5a**)



Boc=$(CH_3)_3COC(O)$-

	a	b	c
amino acid	Tyr	Ser	Thr

SCHEME 3 Synthesis of O-phosphoryl tyrosine methyl ester

disappeared completely and new peak at −15 ppm (**6a**) appeared in 15 min, which indicated that the oxidation reaction had finished. After short-column chromatography, purified compound **6a** was obtained in a yield of nearly 100%. If the water was used instead of m-chloroperbenzoic acid, compound **5a** was hydrolyzed and compound **6a** was yielded too. But the reaction could not be controlled and the yield was very low. Finally, a mixture of trifuoroacetic acid, dichloromethane and thiobenzyl ether was added to the reaction mixture, giving directly unprotected O-phosphoryl tyrosine methyl ester **7a**. In ^{31}P-NMR spectrum, the peak at −15 ppm (**6a**) disappeared completely and new peak at −2 ppm (**7a**) appeared in 1 h, which indicated that the deprotection reaction finished. Compound **7a** was white solid with little solubility in deuterated water, deuterated chloroform or deuterated benzene. It can dissolve in DMSO. Fig. 1 shows the change of the peak in ^{31}P-NMR spectrum through the reaction process.

Study of the reactivity of diethyl-N,N-diteriarybutylphosphoramidite 3b with different amino acid

It was found that different amino acids had different reactivity with phosphoramidite. Fig. 2 shows the ^{31}P-NMR spectrum of the O-phosphitylat-

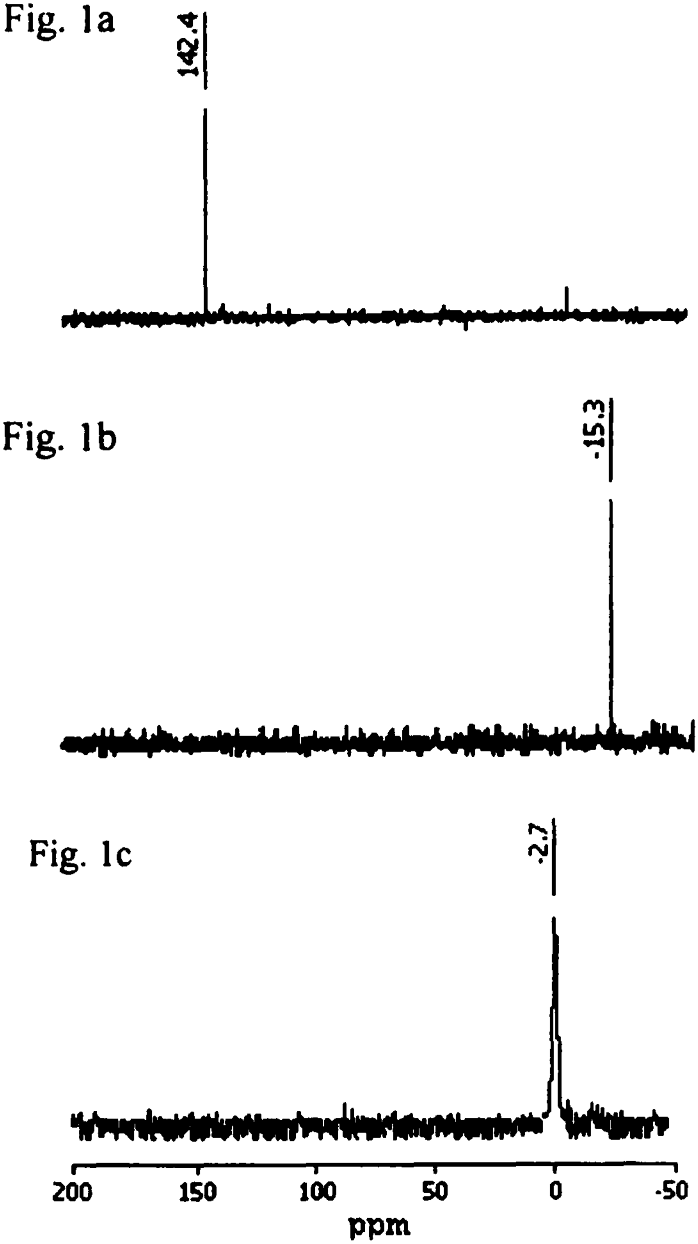
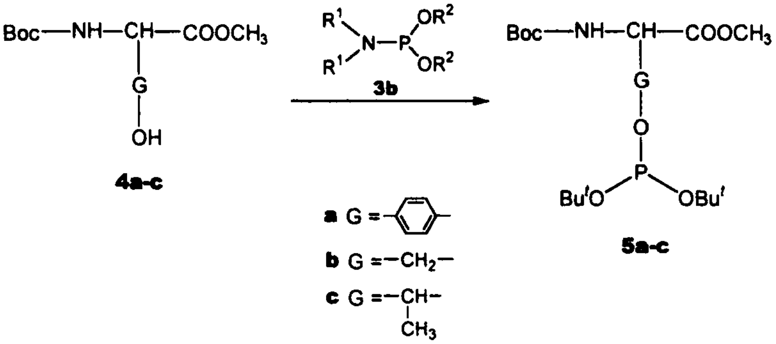


FIGURE 1 ^{31}P-NMR spectrum of the O-phosphorylation process of tyrosine ing reaction of different model compounds, in which the O-phosphitylating agent was DDPA **3b** (SCHEME 4). In Fig. 2, the peak near +140 ppm is triester phosphite **5**, and the peaks between −5 ppm and 10 ppm are the hydrolysis products of DDPA **3b**.



SCHEME 4 O-phosphitylation of different model compounds

In Fig. 2a, in which model compound was Boc-Tyr-OCH_3, the height of the peak at +142 ppm (**5a**) is greater than that of the peak at −2 ppm. That means that only a small part of DDPA **3b** was hydrolyzed. The conversion of DDPA **3b** in the reaction was greater than 90%. In Fig. 2b, in which the model compound was Boc-Ser-OCH_3, the height of the peak at +133 ppm (**5b**) is almost the same with that of the peak near −2 ppm. That means that about half of DDPA **3b** was hydrolyzed. The conversion of DDPA **3b** in the reaction was only about 50%. Fig. 2c shows the reaction of Boc-Thr-OCH_3 with DDPA, in which the height of the peaks near −2 ppm is much greater than the peak at +139 ppm (**5c**). Thus, the conversion of DDPA **3b** in Fig. 2c was much less than that in Fig. 2a and Fig. 2b. in this reaction.

From the result above, it can be seen that the reactivity of different amino acids protected with DDPA **3b** is:

$$\text{Tyr} > \text{Ser} > \text{Thr}$$

There may be two reasons for the different reactivity of these three protected amino acids: One is the difference of OH group. In the O-phosphitylation reaction, tetrazole acting as proton donor attacked DDPA **3b** first. Then, tetrazolylphophite **8**, which is the intermediate product, was

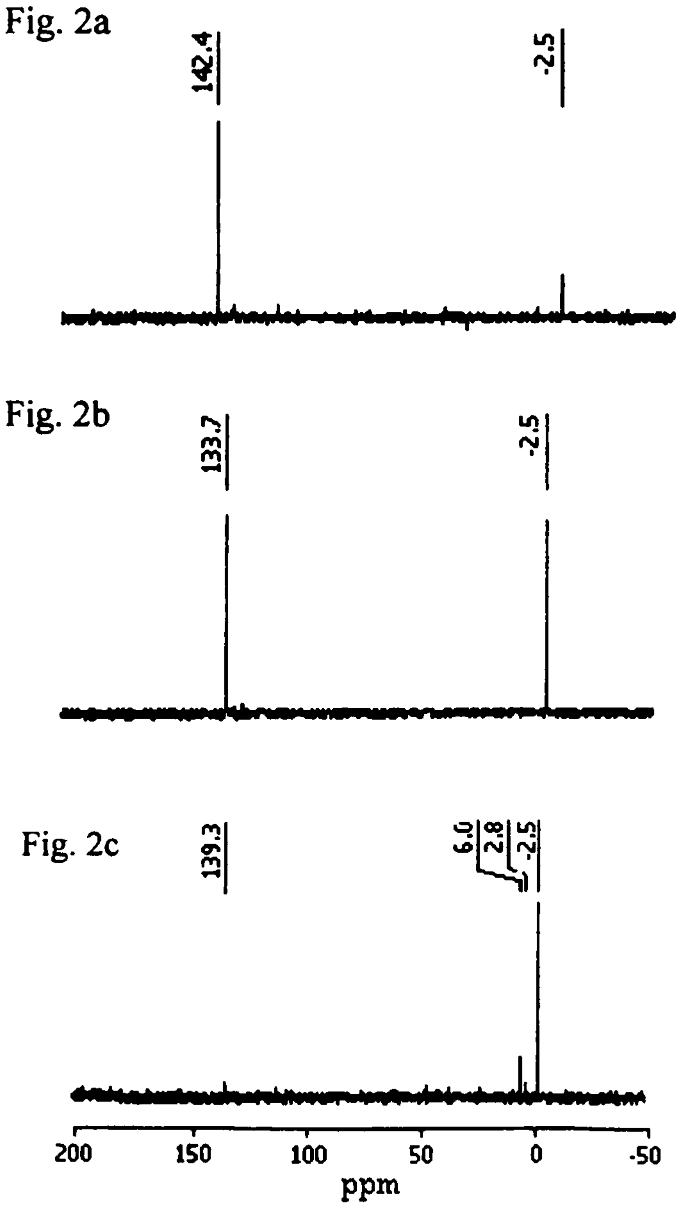
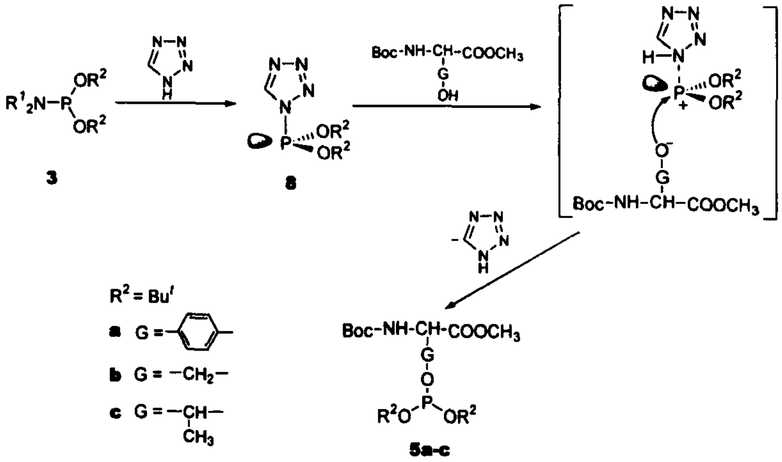


FIGURE 2 ^{31}P-NMR spectrum of the O-phosphitylating reaction of DDPA with different amino acid obtained. **8** is more reactive than DDPA **3**[7]. Then the hydrogen atom migrated from OH group to the nitrogen atom of tetrazolylphophite **8**. The phosphorus atom of tetrazolylphophite **8** which had positive charge is easy to be attacked by nucleophile. Then the oxygen atom with lonepair electrons would attack it as nucleophile (SCHEME 5). So the easilier the hydrogen atom of OH group leaved, the easier the reaction between DDPA and amino acid was. The OH group of tyrosine is a phenolic hydroxyl group. Because of conjugative effect, it is a weak acid and its hydrogen atom is easy to leave. So its oxygen atom is easy to attack phosphorus atom as nucleophile. Thus, the reaction of DDPA with protected tyrosine is easier than the reaction of DDPA with the other two model compounds.

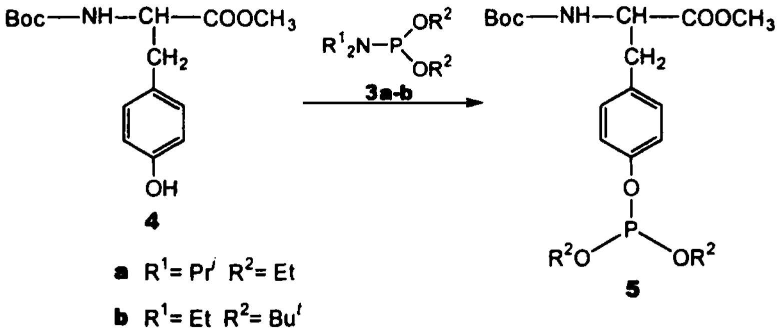


SCHEME 5 Mechanism of O-phosphitylating reaction of DDPA with model

The other reason is the steric effect. The steric hindrance of OH group of different amino acids is: Tyr < Ser < Thr. So the reactivity is the same with the result above.

Study of the reactivity of Protected tyrosine with different DDPA 3

In our study, it was found that different DDPA **3** has different reactivity with model product **4**.



SCHEME 6 O-phosphitylating reaction of Boc-Tyr-OCH_3 with different DDPA

Fig. 3 shows the ^{31}P-NMR spectrum of the O-phosphitylating reaction of Boc-Tyr-OCH_3 with DDPA **3**. Fig. 3a shows the ^{31}P-NMR spectrum of the O-phosphitylating reaction of Boc-Tyr-OCH_3 with DDPA **3a**. The peak at +140 ppm is triester phosphite **5**, and the peaks near −2 ppm are the hydrolysis product of DDPA **3a**. The height of the peak at +140 ppm is almost equal with that of the peak at −2 ppm. That means that nearly half of DDPA **3a** was hydrolyzed. Fig. 3b shows the ^{31}P-NMR spectrum of the O-phosphitylating reaction of Boc-Tyr-OCH_3 with DDPA **3b**, the peak at +142 ppm is triester phosphite **5**, and the peak near −2 ppm is the hydrolysis product of DDPA **3b**. The height of peak at +140 ppm is greater than that of the peak near −2 ppm. That means that a large part of DDPA **3b** was transformed into triester phosphite **5**. So DDPA **3b** has higher reactivity with model product. It is a good O-phosphitylating agent in the O-phosphitylating reaction of the OH group of protected amino acid.

CONCLUSION

It is found that dialkyl-N,N-dialkyl phosphoramidites (DDPA) **3** are good O-phosphitylation agents. Among them, diethyl-N,N-diteriarybutylphosphroamide **3b** is the best one. Through the process of O-phosphitylation, oxidation and deprotection, O-phosphoryl tyrosine methyl ester was yielded. Different amino acids have different reactivity with DDPA. Tyrosine has the highest reactivity, Serine's reactivity is less, and Threonine reacts most difficultly with DDPA.

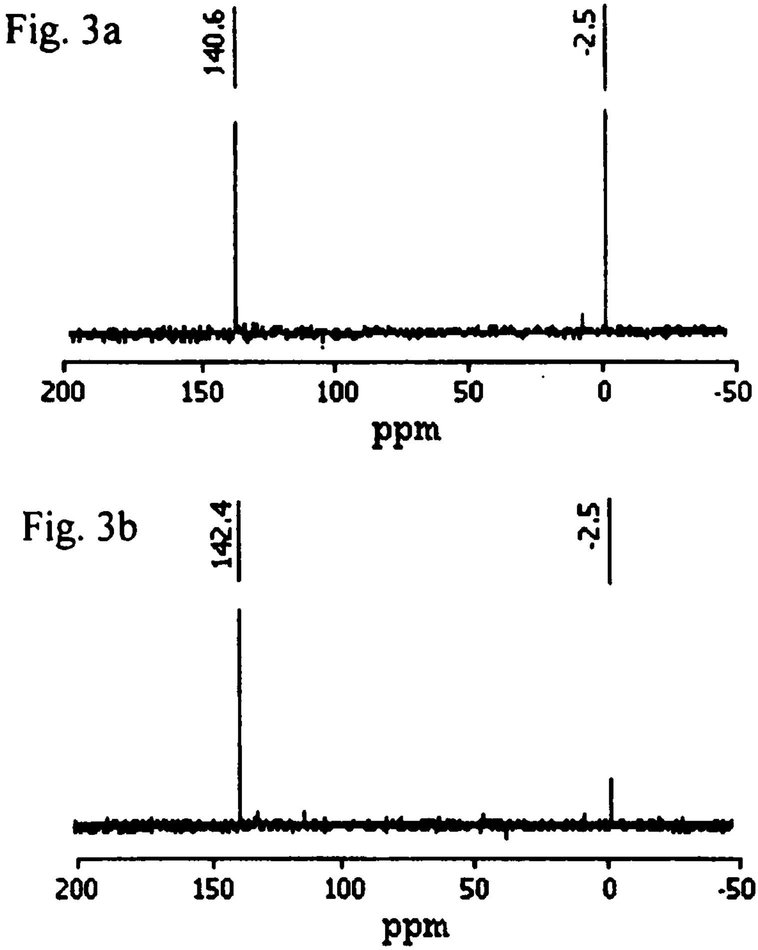


FIGURE 3 ^{31}P-NMR spectrum of the O-phosphitylating reaction of Boc-Tyr-OCH$_3$ with different DDPA

EXPERIMENTAL

Methods and Apparatus

The ^{31}P-NMR, ^{1}H-NMR, ^{13}C-NMR spectra were taken on Bruker AC-200 spectrometer and Bruker AC-300 spectrometer. The ^{31}P-NMR shifts used 85% phosphoric acid as the external reference. The ^{31}P-NMR spectra were recorded by the broadband decoupling program. The ^{13}C-NMR spectra used chloroform-d as the internal reference at 76.9ppm. TMS was used as the internal standard for the ^{1}H-NMR spectra.

Synthesis ofphosphoramidite 3

Take the synthesis of diethyl-N,N-diisopropylphosphoramidite **3a** for example.

Method A

Chlorodiethyl phosphonate $ClP(OEt)_2$ 1a To 0.05mol PCl_3(4.7ml), 0.1mol $P(OEt)_3$ (17ml) was added dropwise under N_2. The mixture was stirred and kept slightly boiling for 1 hour. Then the mixture was stirred at 140°C for 1 hour. The residue was distilled under reduced pressure(55°C/25mmHg) and chlorodiethyl phosphonate $ClP(OEt)_2$ **1a** was obtained. ^{31}P-NMR: +166.0 ppm.

Diethyl-N,N-diisopropylphosphoramidite $(EtO)_2PN(Pr^i)_2$ 3a

To a solution of 37.5mol **1a** (5.88g) in 100ml dry ethyl ether, a solution of 7.5mmol triethylamine (7.59g) in 50 ml dry ethyl ether was added dropwise within 60 minutes under N_2 with ice-salt bath. After a removing of ice-bath, the mixture was stirred overnight at r.t.. The precipitate was removed by filtration and washed twice with 20ml dry ethyl ether. After evaporating of Et_2O, the residue was distilled under reduced pressure (95°C/2mmHg). Diethyl-N,N-diisopropylphosphoramidite $(EtO)_2PN(Pr^i)_2$ **3a** was obtained as colorless oil. ^{31}P-NMR: +146.5 ppm.

Method B

N,N-Diisopropylphosphroamide dichloridite $Cl_2PN(Pr^i)_2$ 2a To a solution of 0.1mol PCl_3 (9.2ml) in 50ml dry Et_2O, a solution of 0.2mol $HN(Pr^i)_2$in 25ml dry Et_2O was added dropwise within 1 hour by stirring under N_2in ice-salt bath. The resulting mixture was stirred at room temperature for 3 hours more, then filtered under N_2. The precipitate $[(Pr^i)_2NH \cdot HCl]$ was washed with 2 x 20ml dry Et_2O. After evaporation of Et_2O, N,N-Diisopropylphosphroamide dichloridite $Cl_2PN(Pr^i)_2$ **2a** was obtained as colorless oil. ^{31}P-NMR: +168 ppm.

Diethyl-N,N-Diisopropylphosphroamide $(EtO)_2PN(Pr^i)_2$ 3a

To a solution of 25mmol **2a** (5.038g) in 50ml dry Et_2O, a solution of 50mmol dry EtOH (2.308g) and 0.1mol $N(Et)_3$ in 50ml dry Et_2O was added dropwise within 40 minutes by stirring under N_2. The resulting white slurry was stirred at room temperature for 3 hours more, then filtered. The precipitate $[Et_3N \cdot HCl]$ was washed with 2 × 20ml dry Et_2O. After evaporating of Et_2O, the oil was poured into 10ml 5% $NaHCO_3$, which was then extracted 3 times with CH_2Cl_2. The combined org. layers were dried with Na_2SO_4 and then evaporated. Diethyl-N,N-Diisopropylphosphroamide $(EtO)_2PN(Pr^i)_2$ **3a** was obtained as colorless oil. ^{31}P-NMR: +146.5 ppm ^{1}H-NMR($CDCl_3$): 1.18 (d, 6H, $CH(C\underline{H}_3)_2$), 1.26 (t, 6H, $CH_2C\underline{H}_3$), 3.66(q, 4H, $C\underline{H}_2CH_3$), 3.67 (m, 2H, $C\underline{H}(CH_3)_2$).

3b was prepared with method A and method B, and purified with distilling under reduced pressure (75°C/5mmHg). ^{31}P-NMR: +134.1ppm

3c was prepared with method B. The final product was not distilled under reduced pressure but washed with 5% $NaHCO_3$. ^{31}P-NMR: +131.1ppm

Synthesis of protected amino acid

Esterification of amino acid

The preparation of amino acid methyl ester was carried out according to the literature[6]. All physical constants and spectroscopic data of the products synthesized agreed with the literature.

Protection of the amino-group of amino acid ester with Boc-group

To a solution of 8mmol $SerOCH_3 \cdot HCl$ (1.244g) in 31ml pyridine, 1.1mmol $N(Et)_3$(1.08ml) was added dropwise by stirring. 15 minutes later, the white precipitate was filtered under reduced pressure and 8mmol $(Boc)_2O$ (1.746g) was added to the solution. The mixture was stirred at room temperature overnight. After evaporating of pyridine, the residue was dissolved in acetic ether. The solution was washed with 0.1mol/L HCl, 5% $NaHCO_3$, saturated NaCl solution respectively. The combined org. layer were dried with Na_2SO_4 and then evaporated. The solid residue was purified with column chromatography (silica gel) with petroleum ether:acetic ether=2:1. 1.356g Boc-Ser-OCH_3 **4b** was obtained with yield 78.4% as white solid (TLC: R*f*=0.41, petroleum ether: acetic ether=2:1).

^{1}H-NMR(CDCl$_3$): 1.37(s, 9H, $C(C\underline{H}_3)_3$), 3.68(m, 1H, CH), 3.69(s, 3H, $OC\underline{H}_3$), 3.78 (m, 2H, $C\underline{H}_2$), 4.25(br, 1H, OH), 5.71(d, 1H, NH)

4a was prepared in the same way as **4b**, but purified with column chromatography (silica gel) with petroleum ether: acetic ether=4:1. 1.376g Boc-Tyr-OCH$_3$ **4a**(TLC: R*f*=0.47, petroleum ether: acetic ether=2:1) was obtained with yield 77.7% as white solid. ^{1}H-NMR(CDCl$_3$): 1.37(s, 27H, $C(C\underline{H}_3)_3$), 2.56(m, 1H, CH) 2.94(d, 2H, CH$_2$), 3.65(s, 3H, OCH$_3$), 4.46(br, 1H, OH), 4.92(d, 1H, NH), 6.64~6.92(q, 4H, Ar-H). ^{13}C-NMR(CDCl$_3$): 28.0 ($-OC(\underline{C}H_3)_3$, 37.3 ($-Ph-\underline{C}H_2-$), 51.9 (-OCH$_3$), 54.5 (-NH-$\underline{C}$-CO-), 80.8($-O\underline{C}(CH_3)_3$), 120.9, 121.0, 126.7, 130.0 (Ar-C), 155.3 (-NH-COO-), 172.5 (-$\underline{C}$OOCH3)

4c was prepared in the same way as **4b**, but purified with column chromatography of silica gel with petroleum ether:acetic ether=4:1. 0.966g Boc-Thr-OCH$_3$ **4c** (TLC: R*f*=0.48, petroleum ether: acetic ether=2:1) was obtained with yield 68.3% as white solid. ^{1}H-NMR(CDCl$_3$): 1.37(s, 3H, $CHC\underline{H}_3$), 1.38(s, 9H, $C(C\underline{H}_3)_3$), 3.69(s, 3H, $OC\underline{H}_3$), 4.09 (m, 1H, $NHC\underline{H}$), 4.15(br, 1H, OH), 4.63(m, 1H, $C\underline{H}CH_3$), 5.35(d, 1H, NH)

Synthesis of O-phosphoryl tyrosine methyl ester

N-Boc-O^4-[bi(tert-butyl) phosphino]-tyrosine methyl Ester (Boc-Tyr(P(OBu^i)$_2$)-OMe) 5a

To a solution of 1.1mmol Boc-TyrOCH$_3$(0.33g) and 2.2mmol tetrazole(0.154g) in 22ml MeCN, 1.1 mmol **3b**(0.243g) was added with stirring under N$_2$at r.t.. Stirring was continued for 3 hours more, and the mixture was poured into 5% NaHCO$_3$ solution. After extracted with (3 × 150ml) CH$_2$Cl$_2$, the combined org. layers were dried with Na$_2$SO$_4$and evaporated. 0.437g solid was obtained. This solid was then purified with short-column chromatography (silica gel). The column was eluted with the following solvent mixture: Et$_2$O:petroleum ether=2:3, Et$_2$O:petroleum ether=1:1, Et$_2$O:petroleum ether=2:1. The pure fraction were collected (TLC: R*f*=0.55, Et$_2$O/petroleum ether=2:1) and concentrated. **5a** was obtained as colorless oil. ^{31}P-NMR: +142 ppm ^{1}H-NMR(CDCl$_3$): 1.45(s, 27H, P-O-C(CH$_3$)$_3$), 1.55(s,9H,C-O-C(CH$_3$)$_3$), 3.00(d, 2H, CH$_2$), 3.69(s, 3H, OCH$_3$), 4.60 (m, 1H, CH), 4.94 (d, 1H, NH), 6.64~6.92(m, 4H, Ar-H) ^{13}C-NMR (CDCl$_3$): 27.4($-P-OC(\underline{C}H_3)_3$), 28.0 ($-COOC(\underline{C}H_3)_3$), 37.6 ($-Ph-\underline{C}H_2-$), 52.1 (-OCH$_3$), 54.4 ($-NH-\underline{C}H-CH_2-$), 77.6($-COO\underline{C}(CH_3)_3$), 80.8 (-Ph-OC($CH_3$)$_3$), 115.3 120.7, 126.7, 130.0(Ar-C), 155.3 (-NH-COO-), 172.5 (-COOCH3).

Oxidation of 5a to N-Boc-O^4-[bi(tert-butyl) phosphoryl]-tyrosine methyl Ester (Boc-Tyr(P(OBu^i)$_2$)-OMe) 6a

To a solution of 0.27mmol **5a** (0.129g) in 15ml CH_2Cl_2, m-chloroperbenzoic acid was added by stirring at r.t. Stirring was continued for 2 hours more. After diluting with another 15ml CH_2Cl_2, the mixture was washed twice with 10% $NaHSO_3$. After extracting with CH_2Cl_2 (3 × 20ml), the combined org. layers were dried with Na_2SO_4 and then concentrated. 0.559g of **6a** was obtained as white solid. This rude product was then purified with short-column chromatography (silica gel). The column was eluted with Et_2O:petroleum ether=2:1. The pure fraction were collected (TLC: R*f*=0.22, Et_2O:petroleum ether=2:1) and concentrated. 6a was obtained as white sold with yield 98.9%. ^{31}P-NMR: −15 ppm ^{1}H-NMR($CDCl_3$): 1.35 (s, 27H, P-O-C(CH_3)$_3$), 1.44(s, 9H, C-O-C(CH_3)$_3$), 2.93(d, 2H, CH_2), 3.64(s, 3H, OCH_3), 4.45(m, 1H, CH), 4.92(d, 1H, NH), 6.66~6.90 (m, 4H, Ar-H) ^{13}C-NMR ($CDCl_3$): 28.2(-P-OC(CH_3)$_3$), 30.3(-COOC(CH_3)$_3$), 37.4(-Ph-CH_2-), 52.1(-OCH_3), 54.6(-NH-C-CH_2-), 77.6(-COOC(CH_3)$_3$), 80.1(-Ph-OC(CH_3)$_3$), 115.6, 126.9, 130.2, 155.3 (Ar-C), 155.6 (-NH-COO-), 172.5 (-$COOCH_3$)

Deprotection of N-Boc-O^4-[bi(tert-butyl) phosphoryl]-tyrosine methyl Ester 6a

To 0.27mmol **6a** (0.129g), a solution of 0.8ml CH_2Cl_2, 1.5ml CF_3COOH and 0.3ml $PhSCH_3$ was added by stirring at r.t. Stirring was continued for 2 hours more. After removing of CH_2Cl_2, **7a** was obtained as colorless oil. Dissolving the oil in CH_2Cl_2 and then removing the solvent. Repeat this procedure many times until white solid was obtained. ^{31}P-NMR: −2 ppm ^{1}H-NMR(D_2O): 2.18(s, 2H, -CH_2-), 3.65(s, 3H, OCH_3), 5.30(s, 1H, CH), 7.06~7.2 (m, 4H, Ar-H) ^{13}C-NMR (DMSO-d): 38.4(-Ph-CH_2-), 46.3(-OCH_3), 71.8(-NH-CH-CO_2-), 118.7, 118.8, 120.0, 121.7 (Ar-C), 170.5 (-$COOCH_3$)

Study of the reactivity of Diethyl-N,N-diteriarybutylphosphoramidite 3b with different amino acid

To a solution of 1.1mmol Boc-TyrOCH_3 (0.33g) and 2.2mmol tetrazole(0.154g) in 22ml MeCN (a solution of 2.2 mmol Boc-SerOCH_3

(0.487g) and 4.4mmol tetrazole(0.310g) in 44ml MeCN or a solution of 2.3mmol Boc-ThrOCH$_3$ (0.551g) and 4.6mmol tetrazole (0.322g) in 44ml MeCN), 1.1mmol **3b** was added with stirring under N$_2$ at r.t.. 1 minute later, the reaction system was traced by ^{31}P-NMR.

Study of the reactivity of Protected tyrosine with different DDPA 3

To a solution of 1.1mmol Boc-TyrOCH$_3$ (0.33g) and 2.2 mmol tetrazole (0.154g) in 22ml MeCN, 1.1mmol **3a** (**3b**) was added with stirring under N$_2$at r.t.. 1 minute later, the reaction mixture was traced by ^{31}P-NMR.

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