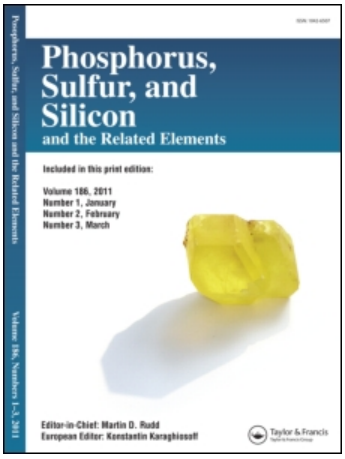

Phosphorus, Sulfur, and Silicon and the Related Elements
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713618290

SYNTHESIS OF N-PHOSPHORYL DI(OR TRI)-PEPTIDES THROUGH THE ACTIVE ESTER METHOD

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To cite this Article Xiao-Bo and Zhao, Yu-Fen(1991) 'SYNTHESIS OF N-PHOSPHORYL DI(OR TRI)-PEPTIDES THROUGH THE ACTIVE ESTER METHOD', Phosphorus, Sulfur, and Silicon and the Related Elements, 61: 1, 9 — 18
To link to this Article: DOI: 10.1080/10426509108027331
URL: http://dx.doi.org/10.1080/10426509108027331

Phosphorus, Sulfur, and Silicon, 1991, Vol. 61, pp. 9–18
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SYNTHESIS OF N-PHOSPHORYL DI(OR TRI)-PEPTIDES THROUGH THE ACTIVE ESTER METHOD

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(Received November 30, 1990; in final form January 21, 1991)

N-Phosphoryl di(or tri)-peptides were synthesized through the coupling of N-hydroxysuccinimide esters of N-phosphoryl amino acids with amino components. The active esters were prepared using ethyl dichlorophosphate (EDCP), diethyl phosphite (DEPH) or dicyclohexylcarbodiimide (DCC) as activating agents.

Key words: Ethyl dichlorophosphate; diethyl phosphite; peptides; low racemization; mixed anhydride intermediate; N-succinimidyl diethyl phosphate.

Among a wide variety of methods for the synthesis of peptides, the procedure via mixed carboxylic-phosphoric anhydride type intermediate has so far attracted a great deal of interest based on the fact that this type of compound plays an important role in the biosynthesis of proteins and peptides.[1] Regarding this procedure, some phosphorus-containing coupling agents such as alkyl or dialkyl chlorophosphites, phosphorus oxychloride and diphenyl chlorophosphate have demonstrated successful formation of peptide esters, but some racemization often resulted.[2] Some phosphates such as N-succinimidyl diphenyl phosphate (SDPP),[3] benzotriazol-1-yl diethyl phosphate (BDP)[4] and norborn-5-ene-2,3-dicarboximido diphenyl phosphate (NDP)[5] have been used for the preparation of active ester, which, in turn, could be coupled directly with amino acid salt to form racemization-free peptide acids. Limitation of this method is tediousness and difficult purification for the reagents.

In previous papers[6,7] we reported a novel method for the synthesis of dipeptides. Thus, N-protected amino acids were reacted with dialkyl phosphite and carbon tetrachloride in the presence of triethylamine to form the mixed anhydride followed by the nucleophilic substitution reaction of the amino esters to give the dipeptide ester in 70–90% yields and low racemization. The mixed anhydride could also be reacted with thiazolidine-2-thiol to afford an active amide intermediate which was treated with amino acid salt to give dipeptide acids in good yields.[8] In this paper we wish to report a new method for the preparation of an active ester of N-hydroxysuccinimide. The method is particularly convenient because the coupling

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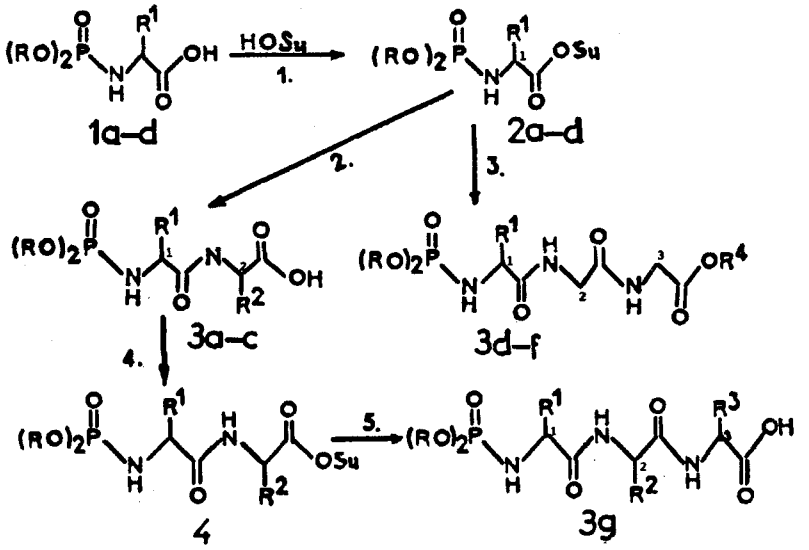
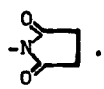
agent ethyl dichlorophosphate (EDCP) or diethyl phosphite (DEPH) and carbon tetrachloride can be added directly to a mixture of the N-protected amino acid and N-hydroxysuccinimide (HOSu) in a suitable solvent without prior formation of either the mixed anhydride or N-succinimidyl alkyl or dialkyl phosphate (Scheme I).

Scheme I[a]

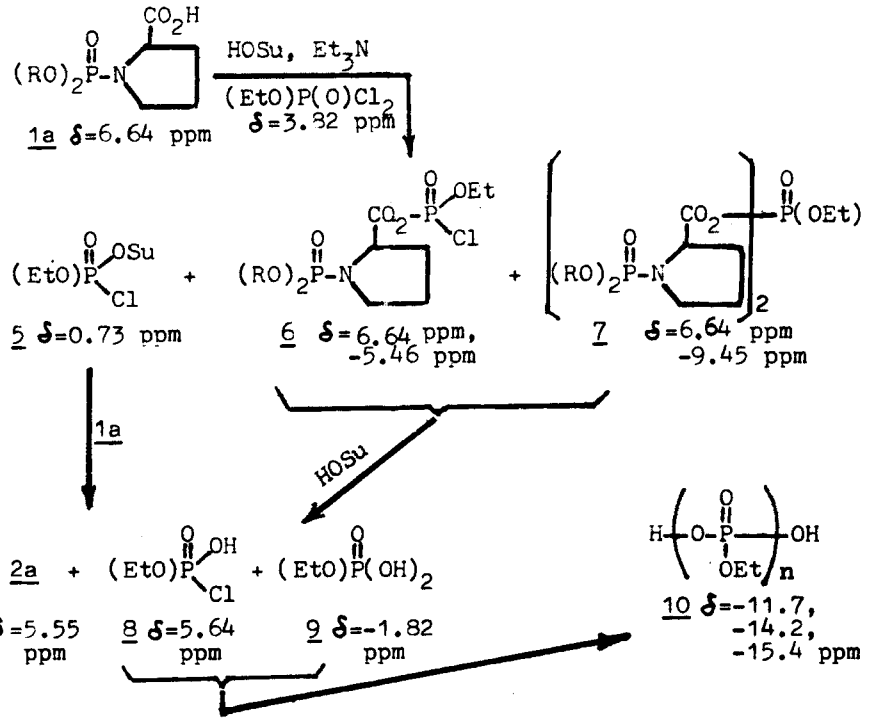


[a] R = $C_6H_2C_8H_2C_7H_2C_6H_3$, Su=  . (1) Method a: $(EtO)P(O)Cl_2$, Et_3N, AcOEt; Method b: $(EtO)_2POH$, CCl_4, Et_3N, AcOEt; Method c: DCC, Dioxane; (2) Et_3N, $R^2CH(NH_2)CO_2H$, THF/H_2O(1:1); (3) Et_3N, Gly-Gly-OR^4(R^4=H), THF/H_2O(1:1) or Et_3N, Gly-Gly-OR^4(R^4=Me), AcOEt; (4) HOSu, $(EtO)P(O)Cl_2$, Et_3N, AcOEt; (5) Et_3N, Proline, THF/H_2O(1:1).

SCHEME I

Various active esters of succinimide **2a–d** were obtained using either one of the EDCP and DEPH or dicyclohexylcarbodiimide (DCC) as activating agent (Scheme II, Table I, II). As to method (a), the activating agent (EDCP) was added to a mixture of N-protected amino acid, HOSu and triethylamine in an ice bath. In order to drive the reaction to completion, an excess of 40–50% molar EDCP and 2.5 equiv. of triethylamine should be used. When the reaction was completed, the mixture showed pH = 5. Since all by-products were water-soluble, they were readily removed by washing with aqueous sodium bicarbonate and water. After work-up, the active esters **2a–d** were pure enough to be used in the synthesis of peptide acids.

In method (b), the solution of DEPH in CCl_4 was added dropwise to a solution of **1** and HOSu slowly at -10 to -5°C. The resulting mixture was stirred at this temperature for at least 6–8 h, and subsequently at room temperature overnight. Too high a temperature and excess of DEPH lead to the formation of many by-

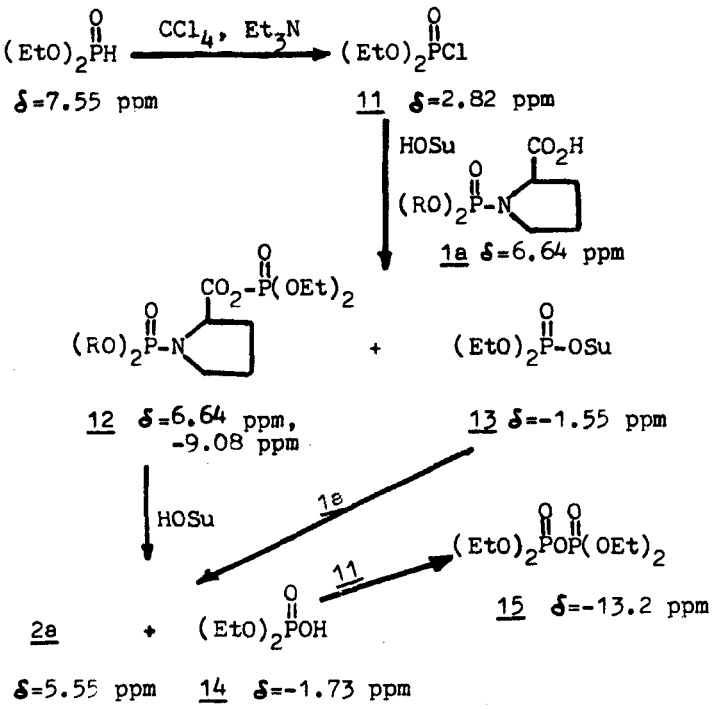


SCHEME II

products and decrease the yield of the active ester. The optimum amount of DEPH was 1.08 equiv. The phosphorus-containing by-products (Scheme III) were always difficult to remove by washing in contrast to method (a) where EDCP was used for activation.

In comparison with the EDCP and DEPH methods, DCC was also used as activating agent for synthesis of the active ester in 1,4-dioxane (Method c in Scheme I). It was found that the yield was high, and most of the by-product dicyclohexylurea (DCU) could be removed by filtration, but column chromatography should be used for complete removal of DCU.

To clarify the activating mechanism of EDCP and DEPH, the tracing experiments by ^{31}P NMR were performed. It was shown that the treatment of **1a** and HOSu with EDCP (0.75 equiv.) in the presence of Et_3N (2.5 equiv.) gave the major peaks at 5.55 ppm, 0.73 ppm and −1.82 ppm which corresponded to **2a**, **5** and **9** respectively (Figure 1a and Scheme II). One hour later, the intermediate **5** was transformed to products completely. But a great deal of **1a** still existed (Figure 1b). In order to complete the reaction, more Et_3N (2.5 equiv.) and EDCP (2.5 equiv.) were successively added to the reaction mixture at 0°C. At this time the ^{31}P NMR spectra became more complicated (Figure 1c). **1a** was almost consumed completely, and most of the products **8** and **9** were transformed to product **10**. It is noteworthy that the intermediates **6** and **7** were observed since the reaction proceeded more mildly, and they disappeared after standing for 2 h. These facts suggested that **2a** was actually obtained through two paths. Thus, either by esterification of the intermediates **6** and **7** with HOSu or esterification of **1a** by inter-



SCHEME III

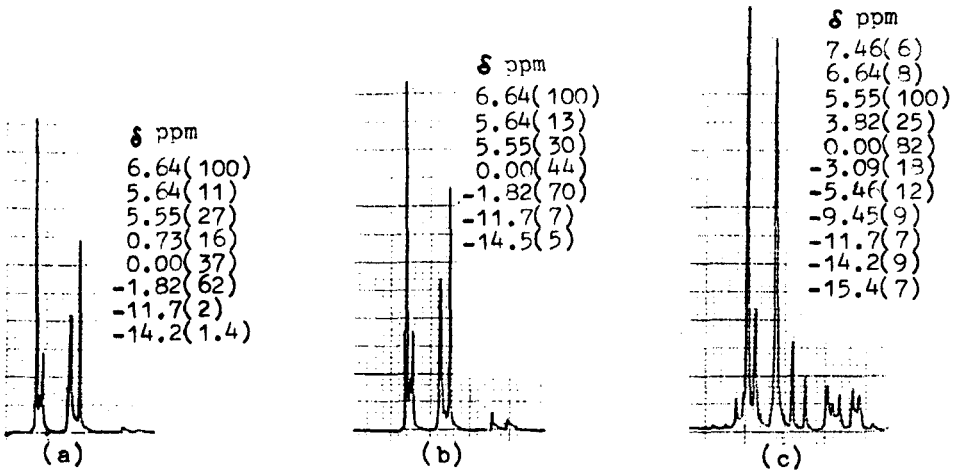


FIGURE 1 ^{31}P NMR tracing experiment for synthesis of **2a** activating by EDCP. (a) 6 min. later (b) 58 min. later (c) 2 min. after more EDCP and Et_3N were added.
* The data in parentheses are relative intensities.

mediate **5**. The reaction proceeded mainly via intermediate **7** when the amount of EDCP was less than 1.0 equiv., but **5** and **6** were major intermediates when more than 3.0 equiv. of EDCP were used.

As to method (b), the tracing experiments by ^{31}P NMR were performed as following. DEPH and carbon tetrachloride were added to a solution of HOSu in

AcOEt in the absence of DBP-Pro-OH **1a**. After storing in an ice bath for 10 minutes, ^{31}P NMR spectra show two new signals. The one at 2.82 ppm which disappeared after standing for more than one hour could be diethyl phosphorochloridate intermediate **11**. The other at -1.55 ppm was ascribed to succinimidyl diethyl phosphate which was isolated from the reaction mixture by column chromatography and verified by ^{13}C, ^{1}H, ^{31}P NMR, IR and FABMS. On the other hand, when DEPH and carbon tetrachloride were added to a solution of HOSu and DBP-Pro-OH **1a** in AcOEt at 0°C, the signals at 2.82 ppm and -1.55 ppm were also observed in ^{31}P NMR (Figure 2a and Scheme III). In addition, the signals at -9.08 ppm, -1.73 ppm and -13.18 ppm were assigned to compounds **12, 14** and **15**, respectively (Figure 2a–b). The signal at -9.08 ppm vanished after standing at 0°C for 40 minutes. Upon heating at 50°C for 5 minutes, the signal at -1.55 ppm weakened and the one at -1.73 ppm strengthened (Figure 2c). It was shown by further experiment that compound **13** ($\delta = -1.55$ ppm) could react with DBP-Pro-OH **1a** at 40–50°C to afford **2a** similar to N-succinimidyl diphenyl phosphate.[3] It seems that DEPH was first transformed into diethyl phosphorochloridate **11**, which could phosphorylate **1a** and HOSu to form intermediates **12** and **13** respectively, followed by esterification of **12** with HOSu and **1a** by **13** to give products **2a** and **14**, as it is shown in Scheme III.

From the results described above, it is demonstrated that both EDCP and DEPH can be used for the synthesis of active ester **2a** directly by mixing it with **1a** and HOSu without the necessity of isolating the intermediates. These methods were also applied successfully for the synthesis of active esters **2b–d** (Table I, II).

Similar to the N-hydroxysuccinimide ester of benzyloxycarbonyl-amino acids,[9] the N-hydroxysuccinimide ester of N-(dibutyloxyphosphonyl) amino acids **2** could also be reacted with amino acid, peptide acid or their esters in the presence of

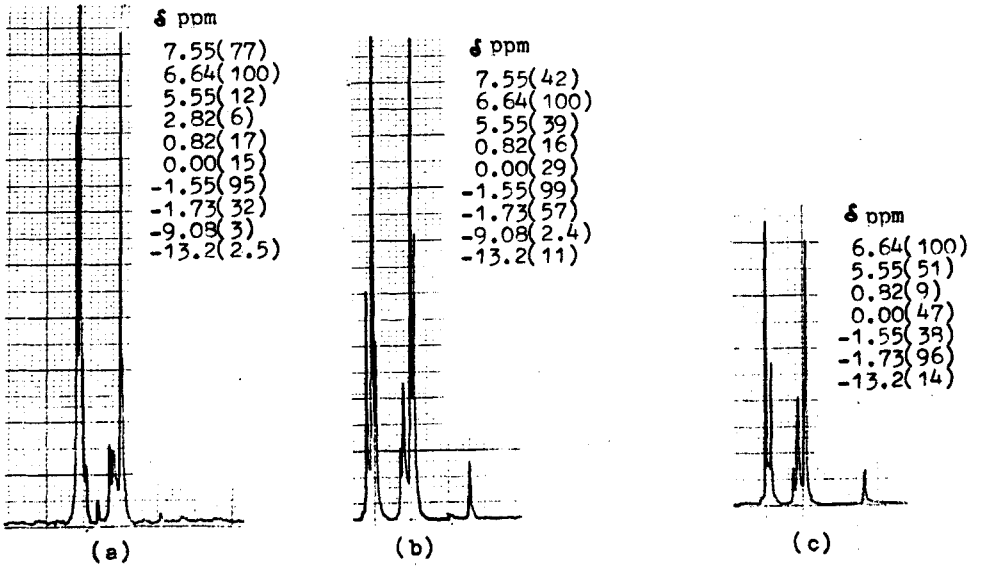


FIGURE 2 ^{31}P NMR tracing experiment for synthesis of **2a** activating by DEPH. (a) 5 min. later (b) 20 min. later (c) then at 50°C for 5 min.

TABLE I

Yields and physical properties of compounds **2a–d**, **3a–g** and **16**

Entry	Compound	Active agent	Yield[b] (%)	$[\alpha]_D$(c,AcOEt) 30 °C	mp (°C)	FAB-MS $(M+H)^+$	ir(cm^{-1})
2a	DBP-L-Pro-OSu	EDCP	84.9	-38.7(0.80)			1740
		DEPH	67.2	-40.0(0.80)	Oil	405	1790
		DCC	87.0	-36.3(0.80)			1820
2b	DBP-L-Ala-OSu	EDCP	66.7	-46.7(0.90)			1740
		DEPH	58.5	-43.3(0.90)	Oil	379	1785
		DCC	80.2	-47.8(0.90)			1825
2c	DBP-L-Leu-OSu	EDCP	75.0	+33.8(0.80)			1735
		DEPH	71.0	+33.8(0.80)	51-52	421	1770
		DCC	85.8	+30.0(0.80)			1815
2d	DBP-L-Met-OSu	EDCP	61.9	-61.3(0.80)			1746
		DEPH	46.5	-57.5(0.80)	66-67	439	1780
		DCC	77.0	-61.3(0.80)			1820
3a	DBP-L-Pro-L-Asn-OH	EDCP	70.2	+38.1(0.92,$CHCl_3$)	58-59	422	1730,1670
3b	DBP-L-Ala-L-Tyr-OH	EDCP	85.1	-57.3(0.90)	75-77	445	1770,1700
3c	DBP-L-Pro-Gly-Gly-OH	EDCP	66.8	-76.1(0.96,EtOH)	34-35	422	1725,1662
3d	DBP-L-Leu-Gly-Gly-OH	EDCP	66.4	-35.0(0.80,EtOH)	85-86	438	1730,1640
3e	DBP-L-Pro-Gly-Gly-OMe	EDCP	74.7	-65.0(0.57,EtOH)	Oil	436	1744,1665
3f	DBP-L-Pro-L-Pro-OH[a]	EDCP	83.1	-67.1(0.66)	Oil	---	---- ----
3g	DBP-L-Pro-L-Pro-L-Pro-OH	EDCP	77.3	-43.5(0.74)	Oil	502	1728,1656
16	Bz-L-Leu-Gly-OEt	EDCP	67.2	-30.6(3.1,EtOH)[c]	153-155	---	---- ----
		DEPH	54.5	-32.2(3.1,EtOH)			

[a] Compound **3f** has been known.[7]

[b] Yields for compounds **3a–g** and **5** are from the aminolysis of the active ester with amino acid or the ester.

[c] Specific rotation of compound **16** was measured at 20°C.

triethylamine (Scheme I, Table I, II). Note should be made that, partial hydrolysis always occurred to give the original acid which was difficult to remove from the product when the active ester was stirred with aqueous amino acid salt at room temperature. This hydrolysis could partly be suppressed when the aqueous solution of amino acid salt was added dropwise to the solution of N-protected amino acid in THF. Further studies on finding better active esters for synthesis of peptide acids are in progress in our laboratory.

In order to examine the extent of racemization for this procedure to synthesize peptides, one of the standard racemization tests, the Young test, was used. The model compound N-benzoyl-L-leucyl-glycine ethyl ester was synthesized via the above active ester method, i.e., EDCP or DEPH was reacted with N-benzoyl-L-leucine and N-hydroxysuccinimide to form the active ester followed by the coupling reaction of glycine ethyl ester to give rise to the model compound. It was shown that the optical purity $(C_L/C_L + C_{DL})\%$ of the model compound was in the range 90.0–94.7% when EDCP or DEPH was used as activating agent.

In conclusion, the presented method provides an alternative efficient synthetic route to active N-hydroxysuccinimide ester and peptides. The advantages are: simplicity of the procedure, moderate yield and good optical purity, possible use of an amino component as amino acid salt, and EDCP or DEPH being inexpensive and available in large scale.

TABLE II

^{31}P and ^{13}C NMR chemical shifts (ppm) and coupling constants (Hertz in parentheses) of compounds **2a–d**, **3a–e** and **3g**

Compd	^{31}P NMR	^{13}C NMR								
		C-α	C-β	C-γ	C-δ	C_1, C_2, C_3	C=O	R^1	R^2, R^3 or S_{Si}	
2a	5.81	66.4(5.9)	47.1(4.4)	18.6	13.4	58.1(5.9)	168.6	32.2 31.8	32.0 31.5	169.1
2b	6.47	66.2(5.9)	47.9	18.2	13.1	31.8(5.8)	169.1(5.8)	13.3	31.5 31.6	173.6
2c	6.66	68.8(5.9)	42.6(5.8)	17.9	13.3	66.5(5.8)	168.9(4.4)	24.8 23.4 12.7	31.3 31.5	169.8
2d	7.08	65.2(5.8)	47.3	17.9	12.9	33.0(4.4)	168.0	39.4 38.6 28.9	31.4 31.7	169.0
3a	7.69	66.4(4.3)	32.0(2.9)	18.4	13.2	49.0 47.2	173.0(4.4) 175.1	25.1(5.8) 24.8	31.0	173.8
3b	7.45	66.9(2.9)	32.2(5.9)	18.6	13.4	53.6 51.1	173.5(5.9) 155.7	17.9	36.7 126.8	130.3 115.5
3c	7.08	66.4(2.9)	42.3(4.4)	18.9	13.5	65.4(5.9) 61.5(4.4)	170.6(3.0) 174.4 175.4	42.7(4.4) 32.8 31.1		
3d	7.63	66.3	41.2(3.0)	18.3	13.1	43.0(2.9) 54.0 53.2	171.6(4.6) 169.7 174.5	32.0 31.3 24.1		
3e	8.11	65.1(5.9)	46.4(3.0)	17.5	12.2	60.2(4.4) 59.1 58.8	172.2(2.9) 173.7 168.8	45.8(4.4) 39.8 31.3		
3g	5.38	65.8(4.5)	45.5(3.1)	19.4	13.2	57.8(5.8) 60.8 59.1	171.6(5.1) 169.7 173.4	58.9 31.2 29.6	59.0 28.6 23.1	30.9 22.2 26.5

EXPERIMENTAL

^1H, ^{13}C and ^{31}P NMR spectra were taken on a JEOL FX-100 spectrometer. ^{31}P NMR spectra were recorded by the broadband decoupling program using 85% phosphoric acid as external reference. ^{13}C NMR spectra used chloroform-d as the internal reference at 76.9 ppm. TMS was used as the internal standard for the ^1H NMR spectra. Positive-ion FAB-MS data were obtained on a KYKY Zhp-5 double-focusing mass spectrometer from Scientific Instrument Factory (Beijing, China) equipped with a standard KYKY fast atom gun. FAB high resolution mass spectral (FABHR-MS) data were obtained on a VG ZAB-HS mass spectrometer. Infrared spectra were measured as KBr plates or film on NaCl on a Shimadzu 430 spectrometer. Column chromatography was performed on 10–40 μm silica gel under 0.8 atm of nitrogen (N_2). Elemental analyses were performed by the Analytical Laboratory, Institute of Chemistry, Academia Sinica, Beijing, China. The optical rotation were measured with WXG-4 polarimeter meter made by Daqing Optical Instrument Factory (Shanghai, China).

General Procedure for the Preparation of N-Hydroxysuccinimide Ester of N-(dibutyloxyphosphoryl) Amino Acids **2a–d**. Each of compounds **2a–d** was prepared by methods (a), (b) and (c) as described below.

(a) To a solution of N-(dibutyloxyphosphoryl) amino acid (4.0 mmol) and N-hydroxysuccinimide (4.0 mmol) in dry AcOEt (15 ml) cooled to an ice bath, was added triethylamine (10 mmol). After stirring for 20 minutes, ethyl dichlorophosphate (5.6 mmol) was added dropwise. The mixture was stirred at 0°C for 3 h, and subsequently at room temperature for 2 h. At this time the reaction mixture showed pH = 5. After washing with aqueous sodium bicarbonate (4 × 10 ml) and water (3 × 10 ml), drying over anhydrous magnesium sulfate and evaporating in vacuo the residue was purified by silica gel column chromatography eluted with AcOEt and petroleum ether (2:1).

(b) To a solution of N-(dibutyloxyphosphoryl) amino acid (6.5 mmol) and N-hydroxysuccinimide (6.5 mmol) in AcOEt (20 ml) cooled at below −5°C was added dropwise in turn triethylamine (13 mmol) and diethyl phosphite (7.0 mmol) in carbon tetrachloride (7.0 mmol). The mixture was stirred below −5°C for 6–8 h, and then at room temperature overnight. Work-up similar to (a) afforded **2**.

(c) To a stirred solution of N-(dibutyloxyphosphoryl) amino acid (5.0 mmol) and N-hydroxysuccinimide (5.0 mmol) in 5 ml of dioxane at 0°C was added dropwise a solution of N,N-dicyclohexylcarbodiimide (5.5 mmol) in dioxane (5 ml). The mixture was stirred at 0°C for 4 h, and then allowed to stand in the refrigerator overnight. The formed dicyclohexylurea (DCU) was filtered off and washed with ethyl acetate. The filtrate was concentrated in vacuo to dryness. The residue was again dissolved in 25 ml of ethyl acetate and washed with aqueous sodium bicarbonate and water (filtered again if more DCU precipitated). After drying over anhydrous magnesium sulfate and evaporation, the residue was purified by column chromatography as described above.

N-Hydroxysuccinimide Ester of N-(dibutyloxyphosphoryl)-L-proline, **2a**. ^{1}H NMR: 0.9 (t, 6H, $2CH_3$), 1.2–2.4 (m, 12H, $3CH_2CH_2$), 2.8 (s, 4H, CH_2CH_2), 3.2–3.5 (m, 3H, NCH, NCH_2), 3.8–4.2 (m, 4H, $2CH_2O$, $^3J_{HP}$ = 7.4 Hz). Anal. Calcd for $C_{17}H_{29}N_2O_7P$: C, 50.50; H, 7.18; N, 6.93. Found: C, 50.20; H, 7.07; N, 6.63.

N-Hydroxysuccinimide Ester of N-(Dibutyloxyphosphoryl)-L-alanine, **2b**. ^{1}H NMR: 1.0 (t, 9H, $3CH_3$), 1.2–1.9 (m, 8H, $2CH_2CH_2$), 2.7–2.8 (m, 1H, NCH), 2.8 (s, 4H, CH_2CH_2), 3.7–4.4 (m, 5H, $2CH_2O$, NH, $^3J_{HP}$ = 7.6 Hz). Anal. Calcd for $C_{15}H_{27}N_2O_7P$: C, 47.62; H, 7.14; N, 7.41. Found: C, 47.25; H, 7.10; N, 7.32.

N-Hydroxysuccinimide Ester of N-(Dibutyloxyphosphoryl)-L-leucine, **2c**. ^{1}H NMR: 1.0 (t, 12H, $4CH_3$), 1.2–1.9 (m, 11H, $2CH_2CH_2$, CH_2, CH), 2.7–2.8 (m, 1H, NCH), 2.9 (s, 4H, CH_2CH_2), 3.8–4.3 (m, 5H, $2CH_2O$, NH, $^3J_{HP}$ = 7.9 Hz). Anal. Calcd for $C_{18}H_{33}N_2O_7P$: C, 51.43; H, 7.86; N, 6.67. Found: C, 51.25; H, 7.73; N, 6.54.

N-Hydroxysuccinimide Ester of N-(Dibutyloxyphosphoryl)-L-methionine, **2d**. ^{1}H NMR: 0.9 (t, 6H, $2CH_3$), 1.2–1.8 (m, 12H, $3CH_2CH_2$), 2.1 (s, 3H, CH_3S), 2.8 (s, 4H, CH_2CH_2), 3.2–3.5 (m, 1H, NCH), 3.8–4.1 (m, 5H, $2CH_2O$, NH, $^3J_{HP}$ = 7.7 Hz). FABHRMS, $(M + H)^+/z$ 439.1627 ($C_{17}H_{32}N_2O_7PS$ requires 439.1627).

Tracing Experiments by ^{31}P NMR. (a) To an ice-salt-cold tube for NMR measurement was added a solution of DBP-Pro-OH **1a** (1.0 mmol), HOSu (1.0 mmol) and Et_3N (2.5 mmol) in AcOEt (2.0 ml) followed by EDCP (0.75 mmol). ^{31}P NMR spectra were first recorded four times at 2 minutes' intervals, and then the fifth time after standing at 0°C for 50 minutes. Finally again four times at 2 minutes' intervals after more Et_3N (2.5 mmol) and EDCP (2.5 mmol) were successively added to the reaction mixture at 0°C. (b) To an ice-salt-cold tube for NMR measurement was added a solution of HOSu (1.63 mmol) and Et_3N (3.26 mmol) in AcOEt (2.0 ml) followed by DEPH (1.76 mmol) in CCl_4 (1.76 mmol). ^{31}P NMR spectra were recorded six times at 10 minutes' intervals, and then three times at room temperature at one hour's intervals. (c) To an ice-salt-cold tube for NMR measurement was added a solution of DBP-Pro-OH **1a** (1.63 mmol), HOSu (1.63 mmol) and Et_3N (3.26 mmol) in AcOEt (2.0 ml) followed by DEPH (1.76 mmol) in CCl_4 (1.76 mmol). ^{31}P NMR spectra were first recorded four times at 0°C at 5 minutes' intervals, and then twice at 5 minutes' intervals upon heating at 50°C.

Phosphorylation of N-Hydroxysuccinimide by DEPH. A mixture of HOSu (2.0 g, 17.4 mmol) and Et_3N (4.8 ml, 38.4 mmol) in AcOEt (40 ml) was cooled to 0°C and treated dropwise with a solution of DEPH (3.60 g, 26.1 mmol) in CCl_4 (2.5 ml, 26.1 mmol). The mixture was stirred for 6–8 h. After filtering the filtrate was concentrated in vacuo, and the residue was purified by silica gel column chromatography. Elution with 2:1 AcOEt-petroleum ether afforded 2.8 g (64.2%) of succinimidyl diethyl phosphate as a liquid. ^{1}H NMR: 1.4 (t, 6H, $2CH_3$), 3.0 (s, 4H, $2CH_2$), 4.0–4.4 (m, 4H, $2CH_2O$). ^{13}C NMR: 169.0 (C=O, $^3J_{CP}$ = 5.7 Hz), 66.2 (CH_2O, $^2J_{CP}$ = 4.4 Hz), 25.0 (CH_2CH_2), 15.6 (CH_3, $^3J_{CP}$ = 5.9 Hz). ^{31}P NMR: δ = −1.545 ppm. FABMS: $(M + H)^+/z$ 252.

General Procedure for the Preparation of N-(Dibutyloxyphosphoryl)di- or tripeptide Acids **3a–e**.

To a solution of N-hydroxysuccinimide ester of N-(dibutyloxyphosphoryl) amino acid (10.0 mmol) in THF (12 ml) was added dropwise a mixture of amino acid or glycyl-glycine (12.0 mmol), water (10 ml) and triethylamine (12.0 mmol). The mixture was stirred at room temperature for 5–8 h before the organic solvent was removed in vacuo. After washing with ethyl acetate (2 × 10 ml), the aqueous solution was acidified with 2N HCl to pH = 3 in an ice bath, and then extracted with ethyl acetate (4 × 10 ml). The organic layer was washed with aqueous citric acid (3 × 10 ml) and water (3 × 10 ml), dried over anhydrous magnesium sulfate, and then evaporated in vacuo to give an oil which was purified by column chromatography eluted by methanol and chloroform to give **3a–e**.

N-(Dibutyloxyphosphoryl)-L-prolyl-L-asparagine, **3a**. ^{1}H NMR: 1.0 (t, 6H, $2CH_3$), 1.1–2.4 (m, 14H, CH_2, $3CH_2CH_2$), 2.9–3.3 (m, 4H, 2CHN, CH_2N), 4.0–4.2 (m, 4H, $2CH_2O$), 6.9, 7.4, 7.9 (3a, brd, 3H, NH, NH_2), 10.1 (s, brd, 1H, CO_2H). FABHRMS: $(M + H)^+/z$ 422.2037 ($C_{17}H_{33}N_3O_7P$ requires

422.2073). Anal. Calcd for $C_{17}H_{32}N_3O_7P \cdot H_2O$: C, 46.47; H, 7.74; N, 9.57. Found: C, 46.63; H, 7.88; N, 9.61.

N-(Dibutyloxyphosphoryl)-L-alanyl-L-tyrosine, **3b**. ^{1}H NMR: 0.8–1.0 (m, 6H, $2CH_3$), 1.0–1.8 (m, 13H, CH_3, CH_2, $2CH_2CH_2$), 2.8–3.2 (m, 2H, 2CHN), 3.5–4.3 (m, 4H, $2CH_2O$, $^3J_{HP}$ = 7.1 Hz), 4.7 (s, brd, 1H, OH), 6.6–7.1 (m, $4H_{arom}$), 8.4 (s, brd, 3H, 2NH, CO_2H). Anal. Calcd for $C_{20}H_{33}N_2O_7P \cdot H_2O$: C, 51.95; H, 7.58; N, 6.06. Found: C, 52.43; H, 7.13; N, 6.06.

N-(Dibutyloxyphosphoryl)-L-propyl-glycyl-glycine, **3c**. ^{1}H NMR: 0.8–1.1 (m, 6H, $2CH_3$), 1.1–2.3 (m, 12H, $3CH_2CH_2$), 3.1–3.4 (m, 2H, CH_2N), 3.5–4.4 (m, 9H, CHN, $2CH_2N$, $2CH_2O$), 8.1 (s, brd, 2H, 2NH), 10.1 (s, brd, 1H, CO_2H). Anal. Calcd for $C_{17}H_{32}N_3O_7P$: C, 48.46; H, 7.60; N, 9.98. Found: C, 48.17; H, 7.51; N, 9.69.

N-(Dibutyloxyphosphoryl)-L-leucyl-glycyl-glycine, **3d**. ^{1}H NMR: 1.0 (t, 12H, $4CH_3$), 1.2–2.0 (m, 11H, CH_2, CH, $2CH_2CH_2$), 3.4–4.5 (m, 9H, $2CH_2O$, CHN, $2CH_2N$), 2.8 (s, brd, 3H, 3NH), 11.2 (s, brd, 1H, CO_2H). FABHRMS: $(M + H)^+/z$ 438.2379 ($C_{18}H_{37}N_3O_7P$ requires 438.2379).

General Procedure for Preparation of N-Protected Di- or Tripeptide Esters **3e** *and* **16** *Using EDCP or DEPH as Activating Agents.*

(a) A solution of N-protected amino acid (4.3 mmol), HOSu (4.3 mmol) and Et_3N (9.8 mmol) in AcOEt (10 ml) was stirred at 0°C for 10 minutes. To the solution was added EDCP (6.0 mmol). The resulted mixture waas stirred at 0°C for 4 h, and then at room temperature for 2 h.

(b) A solution of N-protected amino acid (4.3 mmol), HOSu (4.3 mmol) and Et_3N (5.6 mmol) in AcOEt (10 ml) was cooled to −5°C. To the solution was added dropwise DEPH (4.7 mmol) and CCl_4 (6.4 mmol). The mixture was stirred at −5°C for 5 h, and then at room temperature for 2 h.

To a solution of amino acid or peptide ester hydrochloride salt (4.3 mmol) in methanol (5 ml) was added Et_3N (11 mmol) with shaking. The mixture was evaporated in vacuo to dryness. The residue was added to the reaction mixture described in (a) or (b), and stirring was continued at 0°C or −5°C for an additional 4 h. After being washed sequentially with aqueous sodium bicarbonate (3 × 10 ml), water (10 ml), citric acid (4 × 10 ml) and water (3 × 10 ml), the organic layer was dried over anhydrous magnesium sulfate and evaporated in vacuo. The residue was purified by recrystallization from AcOEt and light petroleum (solid) or column chromatography (liquid).

Benzoyl-L-leucyl-glycine Ethyl Ester, **16**. ^{1}NMR: 0.8–1.1 (m, 6H, 2 CH_3), 1.2 (t, 3H, CH_3), 1.5–1.9 (m, 3H, CH_2, CH), 2.8 (t, 1H, CHN), 4.0 (d, 2H, CH_2N), 4.15 (t, 2H, CH_2O), 4.8 (s, brd, 1H, NH), 6.9 (s, brd, 1H, NH), 7.3–7.9 (m, $5H_{arom}$).

N-(Dibutyloxyphosphoryl)-L-prolyl-glycine Methyl Ester, **3e**. ^{1}H NMR: 0.9 (t, 6H, $2CH_3$), 1.2–2.4 (m, 12H, $3CH_2CH_2$), 3.1–3.4 (m, 2H, CH_2N), 3.7 (s, 3H, CH_3O), 3.9–4.4 (m, 9H, $2CH_2O$, CHN, $2CH_2NH$), 7.54 (s, brd, 2H, 2NH). FABHRMS, $(M + H)^+/z$ 436.2219 ($C_{18}H_{35}N_3O_7P$ requires 436.2222).

N-(Dibutyloxyphosphoryl)-L-prolyl-L-prolyl-L-proline, **3g**. N-Hydroxysuccinimide ester of N-(dibutyloxyphosphonyl)-L-prolyl-L-proline was prepared similar to the preparation of DBP-L-Pro-OSu. After simple washing with aqueous sodium bicarbonate and water, it was directly treated with aqueous proline and triethylamine as described above. Usual work-up afforded an oil. ^{1}H NMR: 1.2 (t, 6H, 2 CH_3), 1.3–2.3 (m, 2OH, $5CH_2CH_2$), 3.2–3.7 (m, 9H, 3CHN, $3CH_2N$), 4.0–4.7 (m, 4H, $2CH_2O$, $^3J_{HP}$ = 7.7 Hz), 11.0 (s, brd, 1H, CO_2H). FABHRMS, $(M + H)^+/z$ 502.2717 ($C_{23}H_{41}N_3O_7P$ requires 502.2713).

N-Hydroxysuccinimide, ethyl dichlorophosphate and N-(dibutyloxyphosphonyl) amino acid were prepared according to References 9, 11 and 12 respectively.

ACKNOWLEDGEMENTS

We are grateful to Associate Professor Shi-kang Xi and the referee for helpful advice and comments. Thanks are also expressed to the financial support of the National Science Foundation for Fund for YFZ, 2890068, 2860227 and the National Science and Technology Committee for a Grant (89)-157 for YFZ.

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