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Synthesis and Biological Activities of N-Phosphoryl Branched Peptides

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Abstract: A series of N-phosphoryl branched peptides were synthesized by coupling of various N-phosphoryl amino acids to L-Lysine methyl ester, and their structures were confirmed by ^{31}P NMR, ^{1}H NMR, MS and elemental analysis. The results of cell biological tests indicated that compound **1d** and **1e** obviously inhibited the growth of both K562 and A2780 cells.

Keywords: N-Phosphoryl branched peptide, synthesis, biological activity, apoptosis.

Much research has shown that some N-phosphoryl peptides are endowed with important biological activities. For instance, N^{α}-(diaryloxyphosphoryl)-L-alanyl-L-prolines are moderate inhibitors of angiotensin converting enzyme and yield highly potent inhibitors when they hydrolyze under physiological condition lossing 1 mol phenol[1]; Phosphoramido (N-[(α-rhamnopyranosyloxy)hydroxyphosphinyl]-L-Leu-L-Trp) and its analogues have been shown to be inhibitors of endothelin converting enzyme, therefore, they may be used in the therapies of hypertension, acute renal failure, and coronary or cerebral vasospasm caused by overprodction of endothelin[2,3]. Dendritic branched peptides synthesized with L-lysine or its oligopolymer as matrix[4] have been widely used in the research and development of immunodiagnostic reagent[5] and synthetic peptide vaccine[6]. Furthermore, it was found that N-phosphoryl amino acids have some special properties[7]. Therefore, we synthesized a series of N-phosphoryl branched peptides by coupling of various N-phosphoryl amino acids to L-lysine methyl ester. All their structures were confirmed by ^{31}P NMR, ^{1}H NMR, MS and elemental analysis (**Table 1, 2**), and preliminary cell biological tests indicated that two of them obviously inhibited the growth of both K562 and A2780 cells.

In the literature[1,3], N-protected peptides were firstly prepared by conventional method, then phosphorylation of the deprotected products yielded N-phosphoryl peptides. In this paper, coupling of various N-phosphoryl amino acids prepared according to the method established by our laboratory[8] to L-lysine methyl ester gave N-phosphoryl branched peptides in good yields, thereby the steps of protection of amino acids and

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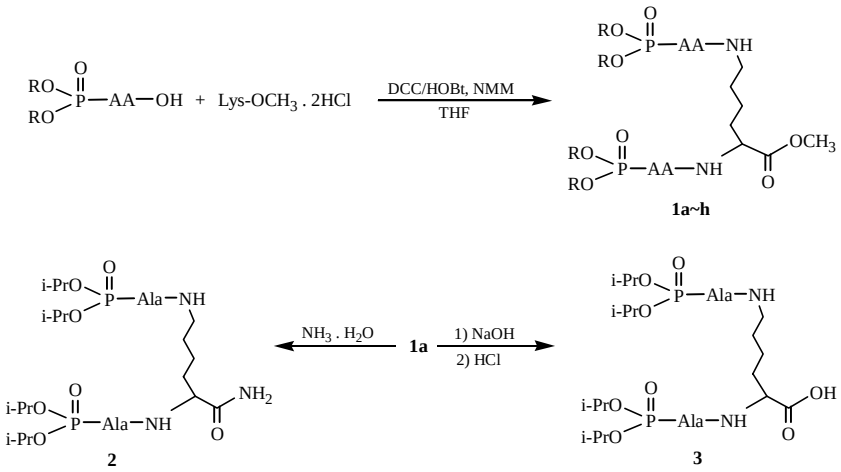
deprotection of peptides have been eliminated. Furthermore, one of the N-phosphoryl branched peptide esters has been converted into the corresponding peptide amide and peptide acid (**Scheme 1**).

Scheme 1



General procedure for the preparation of compounds **1a~h**: L-Lysine methyl ester dihydrochloride (2 mmol), N-methylmorpholine (4 mmol), N-phosphoryl amino acid (4.2 mmol) and 1-hydroxybenzotrizole (4.2 mmol) were dissolved in dry tetrahydrofuran (10 mL), the solution was stirred and cooled in an ice-water bath while dicyclohexylcarbodiimide (4.2 mmol) in THF (5 mL) was added dropwise. After stirring overnight at room temperature, the precipitate was removed by filtration and the solvent evaporated to dryness *in vacuo*. A mixture of a saturated solution of $NaHCO_3$ and $CHCl_3$ was added to the residue and the organic phase washed with saturated $NaHCO_3$, 10% citric acid, and saturated NaCl aqueous solution. The solution was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness *in vacuo*. The residue was purified by chromatography on a column of silica gel with $CHCl_3$-MeOH (9:1) as eluent, or recrystallization from THF-petroleum ether to yield compounds **1a~h**.

Table 1 Physical constants and elemental analysis data of compounds **1**, **2** and **3**

Compd.	AA	R	Yield (%)	m. p. (°C)	Formula	Elemental analysis (%, Calcd.)		
						C	H	N
1a	Ala	i-Pro	77.0	69-70	$C_{25}H_{52}N_4O_{10}P_2$	47.58(47.62)	8.39(8.25)	8.91(8.89)
1b	Ala	Et	68.7	92-94	$C_{21}H_{44}N_4O_{10}P_2 \cdot H_2O$	42.55(42.57)	7.55(7.78)	9.59(9.46)
1c	Ile	i-Pro	71.3	163-164	$C_{31}H_{64}N_4O_{10}P_2$	52.45(52.10)	9.25(8.96)	7.90(7.84)
1d	Leu	i-Pro	81.3	115-117	$C_{31}H_{64}N_4O_{10}P_2$	52.29(52.10)	9.20(8.96)	7.89(7.84)
1e	Phe	i-Pro	55.1	106-108	$C_{37}H_{60}N_4O_{10}P_2$	56.85(56.78)	7.60(7.67)	7.28(7.16)
1f	Pro	i-Pro	93.1	oil	$C_{29}H_{56}N_4O_{10}P_2 \cdot {}^3\!/_2\, H_2O$	49.01(49.08)	8.02(8.18)	8.08(7.90)
1g	Ser	i-Pro	74.0	86-88	$C_{25}H_{52}N_4O_{12}P_2$	45.07(45.32)	7.81(7.85)	8.19(8.46)
1h	Thr	i-Pro	70.3	136.5-138	$C_{27}H_{56}N_4O_{12}P_2$	46.80(46.96)	8.11(8.12)	7.85(8.12)
1i	Val	i-Pro	75.9	160-162	$C_{29}H_{60}N_4O_{10}P_2$	50.98(50.73)	8.78(8.75)	8.19(8.16)
2	Ala	i-Pro	61.2	127-128	$C_{24}H_{51}N_5O_9P_2 \cdot 1/2H_2O$	45.93(46.15)	8.09(8.33)	10.95(11.22)
3	Ala	i-Pro	71.9	55-56	$C_{24}H_{50}N_4O_{10}P_2 \cdot H_2O$	45.66(45.42)	8.16(8.20)	8.93(8.83)

Table 2 MS, ^{31}P NMR and ^{1}H NMR data of compounds **1**, **2** and **3**

Compd.	ESI-MS (m/z)	^{31}P NMR (δ, ppm)	^{1}H NMR (δ, ppm) **
1a	631* $[M+H]^+$	6.43	1.29-1.39(m, 24H, CH_3×8), 1.41-1.46(m, 6H, Ala CH_3×2), 1.50-1.58(m, 4H, Lys γ-CH_2 and δ-CH_2), 1.57-1.95(m, 2H, Lys β-CH_2), 3.29(t, 2H, Lys ε-CH_2), 3.75(s, 3H, OCH_3), 3.75(m, 2H, Ala α-CH×2), 4.40(m, 1H, Lys α-CH), 4.60(m, 6H, OCH×4 and Ala NH×2), 7.16(t, 1H, Lys ε-NH), 7.29(d, 1H, Lys α-NH)
1b	575.2 $[M+H]^+$	8.34	1.31(t, 12H, CH_3×4), 1.36-1.43(m, 8H, Ala CH_3×2 and Lys γ-CH_2), 1.55(m, 2H, Lys δ-CH_2), 1.81(m, 2H, Lys β-CH_2), 3.24(t, 2H, Lys ε-CH_2), 3.72(s, 3H, OCH_3), 3.79(m, 2H, Ala α-CH×2), 4.05(m, 8H, CH_2O×4), 4.53(m, 2H, Ala NH×2), 4.67(t, 1H, Lys α-CH), 7.18(m, 1H, Lys ε-NH), 7.41(m, 1H, Lys α-NH)
1c	715.4 $[M+H]^+$	6.98	0.80-1.02(m, 12H, Ile δ-CH_3×2, γ-CH_3×2), 1.05-1.20(m, 4H, Ile γ-CH_2×2), 1.28(m, 24H, CH_3×8), 1.30-1.65(m, 4H, Lys γ-CH_2, δ-CH_2), 1.70-2.00(m, 4H, Lys β-CH_2, Ile β-CH×2), 3.24(t, 2H, Lys ε-CH_2), 3.40-3.90(m, 4H, Ile α-CH×2, NH×2), 3.71(s, 3H, OCH_3), 4.30-4.70(m, 5H, CHO×4, Lys α-CH), 7.17(m, 1H, Lys ε-NH), 7.48(d, 1H, Lys α-NH)
1d	715.3 $[M+H]^+$	6.73	0.94(d, 12H, Leu CH_3×4), 1.28(m, 24H, CH_3×8), 1.40-1.95(m, 10H, Lys γ-CH_2, δ-CH_2, β-CH_2 and Leu β-CH_2×2), 2.51(m, 2H, Leu γ-CH×2), 3.24(t, 2H, Lys ε-CH_2), 3.71(m, 2H, Leu α-CH×2), 3.73(s, 3H, OCH_3), 4.09(m, 2H, Leu NH×2), 4.57(m, 5H, CHO×4, Lys α-CH), 7.28(br s, 1H, Lys ε-NH), 7.41(d, 1H, Lys α-NH)
1e	783.2 $[M+H]^+$	5.99	1.26(m, 24H, CH_3×8), 1.35-1.55(m, 4H, Lys γ-CH_2 and δ-CH_2), 1.60-1.85(m, 2H, Lys β-CH_2), 2.9-3.3(m, 6H, Phe β-CH_2×2 and Lys ε-CH_2), 3.71(s, 3H, OCH_3), 3.65-4.10(m, 4H, Phe α-CH×2, NH×2), 4.25-4.60(m, 5H, CHO×4, Lys α-CH), 7.0-7.40(m, 12H, Ph-H×10 and Lys α-NH, ε-NH)
1f	683.2 $[M+H]^+$	6.61	1.31(m, 24H, CH_3×8), 1.45(m, 2H, Lys γ-CH_2), 1.65-2.05(m, 8H, Lys δ-CH_2, β-CH_2 and Pro β-CH_2×2), 2.22(m, 4H, Pro γ-CH_2×2), 3.24(m, 6H, Lys ε-CH_2 and Pro δ-CH_2×2), 3.72(s, 3H, OCH_3), 4.14(m, 2H, Pro α-CH×2), 4.57(m, 5H, CHO×4, Lys α-CH), 7.36(t, 1H, Lys ε-NH), 7.74(d, 1H, Lys α-NH)
1g	663.3 $[M+H]^+$	7.16	1.30(m, 24H, CH_3×8), 1.42(m, 2H, Lys γ-CH_2), 1.67(m, 2H, Lys δ-CH_2), 1.88(m, 2H, Lys β-CH_2), 3.11(m, 2H, Lys ε-CH_2), 3.47(m, 2H, OH×2), 3.72(s, 3H, OCH_3), 3.65-3.80(m, 2H, Ser α-CH×2), 4.14(d, 4H, Ser CH_2×2), 4.30(m, 2H, Ser NH×2), 4.58(m, 5H, CHO×4, Lys α-CH), 7.21(t, 1H, Lys ε-NH), 7.55(d, 1H, Lys α-NH)
1h	691.2 $[M+H]^+$	7.04	1.32(m, 30H, CH_3×8 and Thr CH_3×2), 1.51(m, 2H, Lys γ-CH_2), 1.70(m, 2H, Lys δ-CH_2), 1.86(m, 2H, Lys β-CH_2), 3.07(m, 2H, OH×2), 3.54(t, 2H, Lys ε-CH_2), 3.65-3.80(m, 2H, Thr α-CH×2), 3.73(s, 3H, OCH_3), 3.85-4.17(m, 2H, Thr NH×2), 4.40(m, 2H, Thr β-CH×2), 4.62(m, 5H, CHO×4, Lys α-CH), 7.17(m, 1H, Lys ε-NH), 7.53(d, 1H, Lys α-NH)
1i	687.3 $[M+H]^+$	7.04	0.95(m, 12H, Val CH_3×4), 1.28(m, 24H, CH_3×8), 1.45-1.65(m, 4H, Lys γ-CH_2 and δ-CH_2), 1.80(m, 2H, Lys β-CH_2), 2.17(m, 2H, Val β-CH×2), 3.25(t, 2H, Lys ε-CH_2), 3.45-3.61(m, 2H, Val NH×2), 3.71(s, 3H, OCH_3), 3.81(m, 2H, Val α-CH×2), 4.56(m, 5H, CHO×4, Lys α-CH), 7.20(t, 1H, Lys ε-NH), 7.48(d, 1H, Lys α-NH)
2	616.2 $[M+H]^+$	6.29	1.33(m, 24H, CH_3×8), 1.41(m, 8H, Ala CH_3×2 and Lys γ-CH_2), 1.54(m, 2H, Lys δ-CH_2), 1.87(m, 2H, Lys β-CH_2), 2.87(br s, 1H, Ala NH), 3.26(t, 2H, Lys ε-CH_2), 3.77(m, 2H, Ala α-CH×2), 4.44(m, 1H, Ala NH), 4. 58(m, 5H, CHO×4, Lys α-CH), 6.47(d, 2H, NH_2), 7.22(m, 1H, Lys ε-NH), 7.57(d, 1H, Lys α-NH)
3	617* $[M+H]^+$	6.41	1.24-1.32(m, 24H, CH_3×8), 1.39(m, 8H, Lys γ-CH_2 and Ala CH_3×2), 1.54(m, 2H, Lys β-CH_2), 1.82(m, 2H, Lys δ-CH_2), 3.32(t, 2H, Lys ε-CH_2), 3.76(m, 2H, Ala α-CH×2), 4.08(t, 2H, Ala NH×2), 4.40(m, 1H, Lys α-CH), 4.48(m, 4H, CHO×4), 7.22(t, 1H, Lys ε-NH), 7.55(d, 1H, Lys α-NH)

* FAB-MS. ** $CDCl_3$ as solvent.

The preparation of compound **2:** Compound **1a** 0.5 g (0.8 mmol) was dissolved in methanol (5 mL), and 5 mL of 25% ammonia solution was added. The mixture was stirred at room temperature for 4 hours, then evaporated *in vacuo* to remove methanol. 5 mL of water was added to the residue and extracted with $CHCl_3$ 3 times. The $CHCl_3$

extracts were washed with saturated NaCl solution, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The residue was recrystallized from THF to give compounds **2** (white solid, 0.3 g).

The preparation of compound **3:** A solution of compound **1a** 0.88 g (1.4 mmol) in methanol (6 mL) was stirred and cooled in an ice-water bath. An aqueous solution of 1 mol/L NaOH (1.7 mL) was added and stirring was continued for 2 hours at room temperature. After removing methanol by evaporation *in vacuo*, 5 mL of water was added and the solution was acidified to pH = 3 with 1 mol/L HCl. A small amount of undissolved substance was filtered off and the filtrate extracted with ethyl acetate 3 times. The combined ethyl acetate extracts was washed with saturated NaCl solution, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The residue was recrystallized from ethyl acetate-petroleum ether to yield compound **3** (white solid, 0.62 g).

Tumor growth inhibition of synthesized compounds on solid or non-solid human tumor cell lines has been determined by MTT (3-(4, 5-dimethylthiozol-2-yl)-2, 5-diphenyltetrazolium bromide) tests. The preliminary results showed that percentage of growth inhibition on K562 and A2780 cells of compounds **1d** and **1e** are more than 50% at the concentration of 100 μg/mL, while that of other compounds are less than 50%. Further MTT tests of compounds **1d** and **1e** at the concentration of 1, 10, 20, 40, 80 and 100 μg/mL showed that IC_{50} of compound **1d** on K562 and A2780 cells are 21.37 μg/mL and 90.41 μg/mL; and IC_{50} of compound **1e** on K562 and A2780 cells are 17.44 μg/mL and 37.82 μg/mL, respectively. It was observed under microscope that the cell shape exhibited typical characteristic of apoptosis, so it is presumed that the tumor growth inhibition of these two compounds is effected by inducing cell apoptosis. The mechanism and structure-activity relationship remain to be further studied.

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