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Synthesis and Separation of a Diastereomeric Pair of Phosphonopeptide Inhibitors of the Cyclic AMP-Dependent Protein Kinase Catalytic Subunit

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Abstract: In this paper we report the establishment of a novel procedure to synthesize a nonhydrolyzable phosphonopeptide dead-end inhibitor of the catalytic subunit of cAMP-dependent protein kinase. This procedure has been optimized to maximize the peptide yield and gives a diastereomeric pair of heptapeptides that can be separated on a C-18 reverse phase HPLC column. The two peptides have been characterized by NMR and the ability of these peptides to inhibit the reaction of the catalytic subunit of cAMP-dependent protein kinase. Peptide A has a dissociation constant of 9 micromolar, and is a 10-fold better inhibitor as compared to peptide B. On the basis of this 10-fold greater inhibition afforded by peptide A, this peptide is assigned the all L-form configuration. It is expected that this procedure can easily be adapted to synthesize a variety of different peptide inhibitors which involve a nonhydrolyzable phosphate on an amino acid.

INTRODUCTION

The catalytic subunit of adenosine 3',5',-monophosphate dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) phosphorylates a variety of different proteins *in vivo*, in response to elevations in the levels of cAMP, thus modulating their activity. This enzyme is most frequently assayed using peptides in place of the protein substrate. Of the peptide substrates used, Leu-Arg-Arg-Ala-Ser-Leu-Gly (SP) is the most effective[2]. A number of inhibitors have been analyzed based on the above sequence, but few have been developed that mimic the phosphorylated form of the peptide. Recently, Qamar et al.[3] have substituted Arg, Glu or α-aminobutyrate for Ser in the above sequence, and although these compete with the phosphopeptide (PSP), they do not have high affinity with K_i values in the mM range. Thus, a nonhydrolyzable phosphonopeptide [Leu-Arg-Arg-Ala-(2-amino-4-phosphonobutyrate)-Leu-Gly] was synthesized using commercially available DL-2-amino-4-phosphonobutyric acid as a starting material. Before solid phase synthesis of the peptide could be attempted using this amino acid, the amino and phosphono moieties were protected using groups that can be selectively removed at the end of the synthesis but remain intact during the synthesis. This procedure for synthesizing the phosphonopeptide yields a convenient tool that can be used to prepare a variety of different phosphonopeptides as potential inhibitors of protein kinases. The resolved diastereomeric pair of phosphonopeptides synthesized has been tested as inhibitors of the catalytic subunit.

MATERIALS AND METHODS

Chemicals. α-D-Glucose, G6PDH, HK and benzatriazolyl-N-oxy-tris (dimethylamino) phosphoniumhexafluoro phosphate (BOP) were obtained from Sigma. DL-2-Amino-4-phosphonobutyric acid was purchased from Sigma or prepared from a literature procedure[4]. $CDCl_3$, D_2O, acetone-d_6, DMSO-d_6, 1-hydroxybenzoltriazine (HOBt), N-methylmorpholine (NMM), piperidine (PID), trimethylsilyl iodide (TMSI), trimethylsilyl bromide (TMSBr), carbobenzoxy

chloride (CBo-Cl), and 9-fluorenylmethyl chloride (Fmoc-Cl) were from Aldrich. All Fmoc-amino acids, Fmoc-amino acid pentafluorophenyl esters and Fmoc-L-leucine-KA resin were from MilliGen. Dimethylformamide (DMF) was from J. T. Baker.

Synthesis of DL-2-N-carbobenzoxyamino-4-phosphonobutyric acid (1a). A suspension of 183 mg (1 mmol) of DL-2-amino-4-phosphonobutyric acid in 10 ml of water was titrated dropwise with saturated sodium carbonate to pH 9.5. The solution, stirred vigorously by sonic oscillation at 0-5 °C, was treated with carbobenzoxy chloride, 255 mg (1.5 mmol), over a period of 5-10 min. Stirring by sonic oscillation at 0-5 °C was continued for 1 hr, and a pH of 9.0-9.5 was maintained by additions of saturated sodium carbonate. The reaction mixture was then stirred an additional 6 hr at 25°C. Following ether extraction, the aqueous phase was treated with 10 mL H_2O, 5 mL conc. HCl and 15 g of crushed ice, and then extracted with ethyl acetate. After drying over magnesium sulfate, the solvent was evaporated. The resulting oil was recrystallized from ethyl acetate/hexane to yield 237 mg (75%) of white crystals which gave a single spot (R_f = 0.52) on silica gel thin layer chromatography (TLC). The solvent used was ethanol:acetic acid (1:1). IR: 1735, 1740 (C=O), 1250 (P=O). ^{1}H-NMR (acetone-d_6): 1.75 - 2.15 (4H, m, -CβH$_2$CγH$_2$-), 4.35 (1H, m, CαH), 5.05 (2H, s, PhCH_2-), 5.75 (1H, d, NH), 7.25 (5H, s, Ph-), 9.70 (3H, s, COOH and P(OH)$_2$).

Synthesis of DL-2-N-carbobenzoxyamino-4-phosphonobutyric acid triethyl ester (2a). Employing the general procedure of Schroder and Lubke[5], triethylorthoformate treatment of 316 mg of *1a* yielded 320 mg (80%) of *2a*; the product was a colorless oil and gave a single spot (R_f = 0.52) on silica gel TLC in ethyl acetate:hexane (1:3). IR: 1730, 1738 (C=O), 1250 (P=O). ^{1}H-NMR ($CDCl_3$): 1.15 (9H, m, OCH_2CH_3 for both esters), 1.70 - 2.20 (4H, m, -CβH$_2$ CγH$_2$-), 4.05 (6H, m, OCH_2CH_3 for both esters), 4.40 (1H, m, CαH), 5.08 (2H, s, PhCH_2-), 5.80 (1H, d, NH), 7.40 (5H, s, Ph-).

Synthesis of DL-2-amino-4-diethylphosphonobutyric acid (4a). Selective saponification of the carboxyl ethyl ester was accomplished by the slow addition of an equimolar amount of NaOH (1N) to an ethanolic solution of *2a* at 0°C. After 1 hr at room temperature, the ethanol was removed at reduced pressure, the remaining aqueous phase was acidified with 6N HCl at 0°C and extracted 3 times with ethyl acetate. After evaporation of the dried solvent over magnesium sulfate, a crystalline product (*3a*) was obtained which weighed 320 mg and was not purified further.

To 373 mg (1 mmol) of *3a* in 10 mL of methanol, 1 mL of water, and 4 drops of acetic acid, was added 180 mg of 10% Pd/C powder. The mixture was then stirred under H_2 gas for 4 h at room temperature. After filtration and removal of solvent at reduced pressure, a viscous oil product, *4a* (200 mg, 63% overall yield), was obtained. Paper chromatography (butanol: acetic acid: water, 2: 1: 1) gave a single spot, R_f = 0.62, that was UV negative and ninhydrin positive. IR: 1730 (C=O), 1270 (P=O). ^{1}H-NMR ($CDCl_3$): 1.21 (6H,m, P(OCH_2CH_3)$_2$), 1.65 - 2.20 (4H, m, -CβH$_2$CγH$_2$-), 4.04 (4H, m, P(OCH_2CH$_3$)$_2$), 4.35 (1H, m, CαH), 5.08 (2H, s, -NH_2), 9.25 (1H, br s., COOH).

Synthesis of DL-2-N-(9-fluorenylmethoxycarbonyl)-4-diethylphosphonobutyric acid (5a). The N-Fmoc derivative of *4a* was synthesized by established procedures[6]. From 239 mg of *4a* was obtained, after recrystallization, 300 mg of pure crystalline *5a* (60%). M.p. 141 - 142°. IR: 1735, 1770 (C=O), 1274 (P=O). ^{1}H-NMR ($CDC1_3$): 1.30 (6H, m, P(OCH_2CH_3)$_2$, 1.60 - 2.45 (4H, m, -CβH$_2$CγH$_2$-), 3.90 - 4.55 (8H, m, P(OCH_2CH$_3$)$_2$ overlapped with CαH and $\succ$CH-CH_2- of Fmoc group), 5.78 (1H, d, NH), 6.68 (1H, br s, COOH), 7.20 - 7.82 (8H, m, Ar of Fmoc group).

Synthesis of L-Leu-L-Arg-L-Arg-L-Ala-DL-(2-amino-4-diethylphosphonobutyric acid)-L-Leu-L-Gly. Fmoc-Gly-KA resin, 1.0 g, was packaged into the reaction vessel and washed according to the manufacturer's directions. Successive peptide bond formations, initially to the glycine residue bound to the resin, were accomplished by use of Fmoc-amino acid pentafluorophenyl esters and/or by *in situ* activation using Bop/HOBt and Fmoc-amino acids. The latter method generally gave the better yields and is herein described. A 1.0 g (0.09 mmol) quantity of Fmoc-L-glycine-KA resin was washed, deblocked, and packaged into the reaction vessel according to the instrument manufacturer's instructions. The appropriate Fmoc-amino acid for each cycle, 0.36 mmol, was mixed with 159 mg (0.

mmol) of Bop and 49 mg (0.36 mmol) of HOBt. This mixture was then dissolved in a solution of 0.2 M NMM in DMF. After 5 min the solution was injected into the reaction vessel, and after the appropriate reaction time for coupling, the manufacturer's deblocking and washing procedures were followed, which prepared the resin-bound peptide for the subsequent coupling process.

Cleavage of the resin-bound heptapeptide was accomplished as follows. The resin was maintained in 10 mL of a solution of 5% phenol in TFA for 2 h. The suspension was then filtered and the resin was washed extensively with a solution of 5% phenol in TFA and then with TFA. The filtrate solution was evaporated *in vacuo* giving a brown oil. The oil was treated with ether, and the precipitated solid was removed by centrifugation. The solid was washed with ether and dried in a flow of argon gas. The peptide weighed 61 mg, and was analyzed by HPLC using a C-18 reverse phase column. The peptides were eluted by 0.1% TFA in H_2O and 0.1% TFA in acetonitrile employing a linear gradient. Two peaks (peak A and peak B) of approximately equal area and retention time were obtained, Fig. 1.

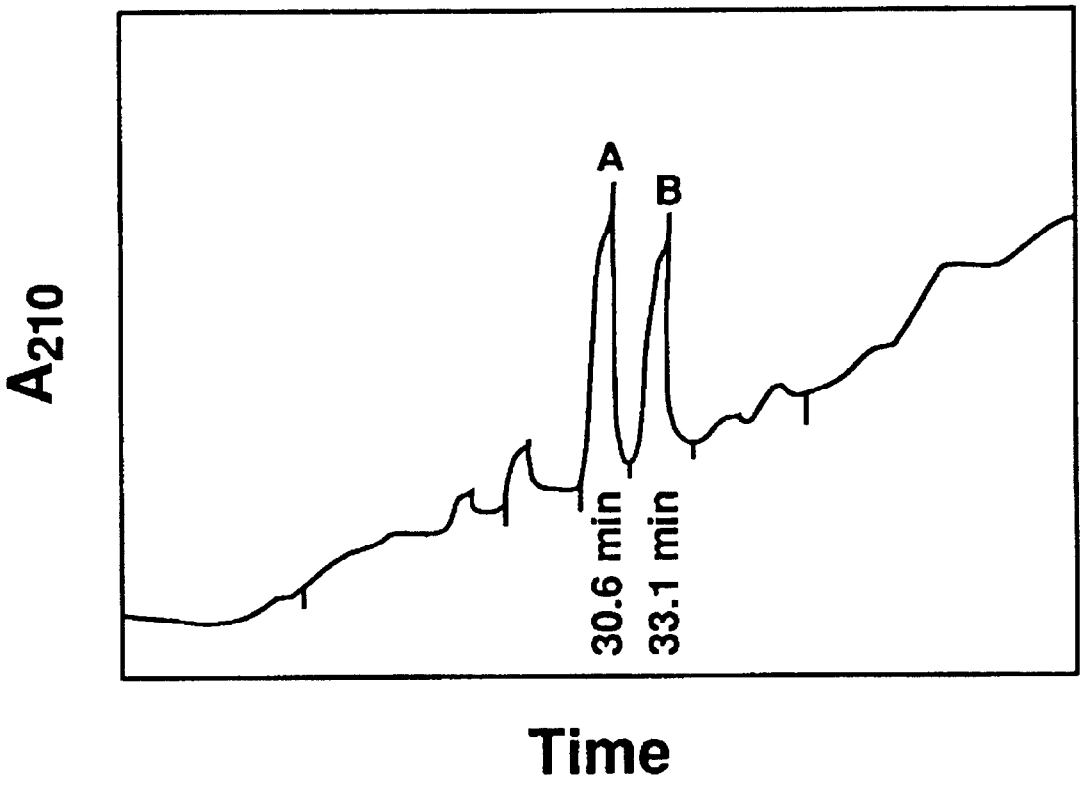

Fig. 1. HPLC separation of the diastereomeric diethylphosphonopeptide mixture according to chromatographic conditions given in the Methods section. The peptides were identified as A and B based on their elution position.

Separation of the diethylphosphonopeptide diastereoisomers. The diastereoisomers separated by HPLC as described above were collected, dried, hydrolyzed and analyzed for amino acid composition. The resultant data for each product were consistent with the anticipated amino acid compositions. ^{31}P NMR analyses were conducted on the peptide mixture. Peptide A gives a δ of 34.2 ppm, while peptide B has a δ of 34.5 ppm using HOD as the locking signal. The resonances corresponding to each of the individual peptides was determined from the isolated peptide.

Removal of the protective ethyl ester groups from the phosphonopeptides. The ester protecting groups were successfully removed by use of 42% HBr in glacial acetic acid (AcOH). The time required for complete diester hydrolysis was determined in trial runs by following the disappearance of ^{1}H NMR peaks associated with the heptapeptide diethyl substituents. Diethylpeptides A and B (20 mg of each) were dissolved in 0.5 mL HBr/acetic acid and maintained at 25 °C with stirring for 20 h. After solvent removal *in vacuo*, two mL of benzene was added, the mixture was stirred rapidly, and the benzene was removed *in vacuo*. The latter process was repeated several times. The final deprotected phosphonopeptides were then purified on HPLC using a TSK SP-5PW cation exchange column (7.5 x 57 mm) with isocratic elution (1 mL/min) using 5% acetonitrile in 10 mM triethylamine formate, pH 2.8. The effluent was monitored at 210 nm. Diethylphosphonopeptides treated as described above gave one major peak in the chromatogram. Peak fractions were combined after removing the solvent *in vacuo* and yielded 15 mg of each of the deprotected phosphonopeptides termed A and B. The resulting phosphonopeptides, showing no ethyl peaks in the ^{1}H NMR spectra, were analyzed by ^{31}P NMR; a single peak was observed for each deprotected phosphonopeptide.

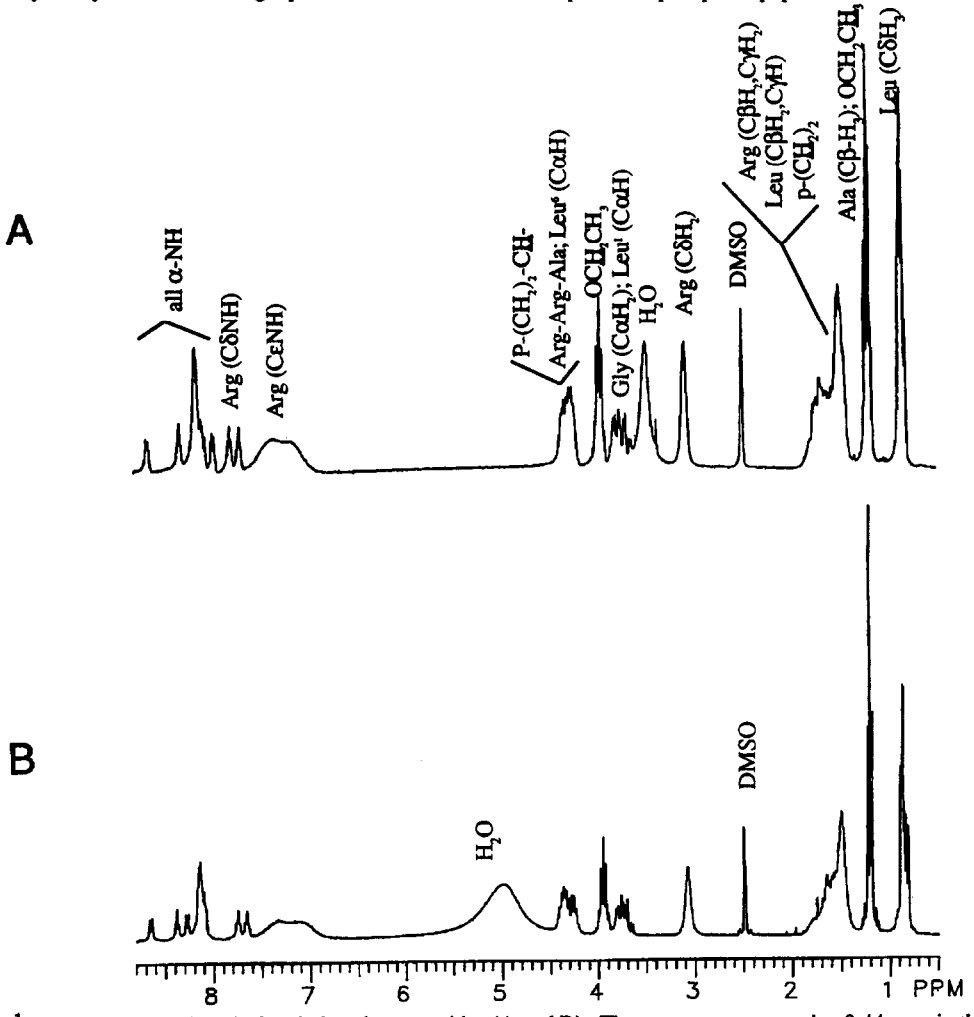

Fig. 2. ^{1}H NMR spectra of the isolated phosphonopeptides (A and B). The resonance centered at 3.44 ppm in the A spectrum is from a small amount of water in the sample. In the B spectrum, the resonance is shifted to 4.96 ppm because of the presence of trifluoroacetic acid. In both spectra, the resonance at 2.5 ppm reflects DMSO-d_5.

NMR spectroscopy. For the ^1H NMR, Fig. 2, and spectra of the various phosphonoamino acid derivatives, a Varian Gemini-200 Spectrometer operating at 200 MHz was employed; samples were dissolved in chloroform-d_3 or acetone-d_6. Chemical shifts are expressed in ppm downfield from a tetramethylsilane internal standard. The ^1H NMR spectral parameters were as follows: sweep width, 3,000.3 Hz; data points, 11,968 zero-filled to 16 K; acquisition time, 1.994 s; acquisition delay, 0 s. For the ^{31}P NMR spectra, a Varian VXR-300 Spectrometer operating at 121.4 MHz was used. Samples were dissolved in deuterium oxide, and 85% H_3PO_4 was the external standard. The ^{31}P NMR spectral parameters were as follows: sweep width, 50,000 Hz; data points, 30,016 zero filled to 32 K; acquisition time, 0.3 s; acquisition delay, 0 s. A Varian XL-300 NMR Spectrometer operating at 299.9 MHz for ^1H and 75.4 MHz for ^{13}C was used to acquire spectral data for the final phosphonopeptide products. Samples were prepared as dilute solutions in DMSO-d_6. Proton spectra were referenced to the center line of residual DMSO-d_5 at 2.49 ppm (δ), and ^{13}C spectra were referenced to the center line of the CD_3 group of DMSO-d_6 at 39.5 ppm (δ). Proton NMR spectral parameters were as follows: sweep width, 2810.6 Hz; data points, 10,500 zero-filled to 16 K; acquisition time, 1.87 s; acquisition delay, 2 s; acquisitions, 32. Carbon NMR spectral parameters were as follows: sweep width, 17,094 Hz; data points, 32 K; acquisition time, 0.96 s; acquisition delay, 2 s; acquisitions, 18,000 in double precision mode.

Carbon spectral lines. The ^{13}C NMR spectrum of heptapeptide A exhibited the following resonances: δ 172.1, 172.0, 170.9, 170.8, 170.7, 170.2, 168.8, 156.8, 61.1, 61.0, 52.5, 52.2, 52.1, 50.7, 50.6, 48.0, 29.1, 29.0, 25.1, 25.0, 24.9, 24.8, 24.0, 23.4, 22.9, 22.5, 21.8, 21.5, 19.9, 17.8, 16.2, and 16.1 ppm. The ^{13}C NMR spectrum of heptapeptide B exhibited the following absorptions: δ 172.4, 172.2, 171.1, 170.8, 170.3, 168.9, 156.9, 156.8, 61.2, 61.1, 52.4, 52.3, 52.1, 50.7, 50.6, 48.3, 29.3, 29.0, 25.9, 25.0, 24.2, 23.5, 23.2, 22.6, 21.9, 21.1, 20.1, 18.5, 16.3, and 16.2 ppm.

Enzyme assay and inhibition studies. Initial velocities measured for the catalytic subunit of cAMP-dependent protein kinase made use of the assay described by Qamar et al.[3]. The production of MgATP is coupled to the hexokinase and glucose-6-phosphate dehydrogenase reactions and the appearance of NADPH at 340 nm is continuously monitored. A background rate was obtained in the absence of the catalytic subunit and was subtracted from the rate obtained upon the addition of the catalytic subunit to obtain the corrected initial velocity. All data were collected using a Gilford 260 spectrophotometer connected to a strip chart recorder. The inhibition constants for both the phosphonopeptides, A and B, were obtained from Dixon plots of initial velocity vs. inhibitior[7] in which MgADP was maintained saturating and PSP was maintained equal to its K_m.

RESULTS AND DISCUSSION

The 2-amino-4-phosphonobutyric acid (phosphonoamino acid), which was used to synthesize the phosphonopeptides is an isosteric phosphonic acid analog of phosphoserine, contained in the PSP product of the reaction of the catalytic subunit. The carbon-phosphorus bond of the phosphonopeptide is incapable of being hydrolyzed by the catalytic subunit, thus yielding a dead-end inhibitor of the enzyme.

It was decided at the outset that commercially available, racemic 4-phosphono-2-aminobutyric acid, appropriately protected, would be employed in the synthesis of the heptapeptide inhibitor. The reasoning was that the separation of the two diasteriomeric forms of the final heptapeptide could be effected without difficulty. The latter proved to be the case. However, the assignment of the D and L forms for the substituent phosphonoamino acid residue proved to be difficult. Therefore, the assignment was made on the basis of the assumption that the all L-form of the heptapeptide should exhibit the higher inhibitory activity towards the catalytic subunit of cAMP-dependent protein kinase.

Several synthetic schemes were attempted for protection of the amino and phosphonate groups of the phosphonoamino acid. The method described in this paper proved to be the best of those attempted (Fig. 3).

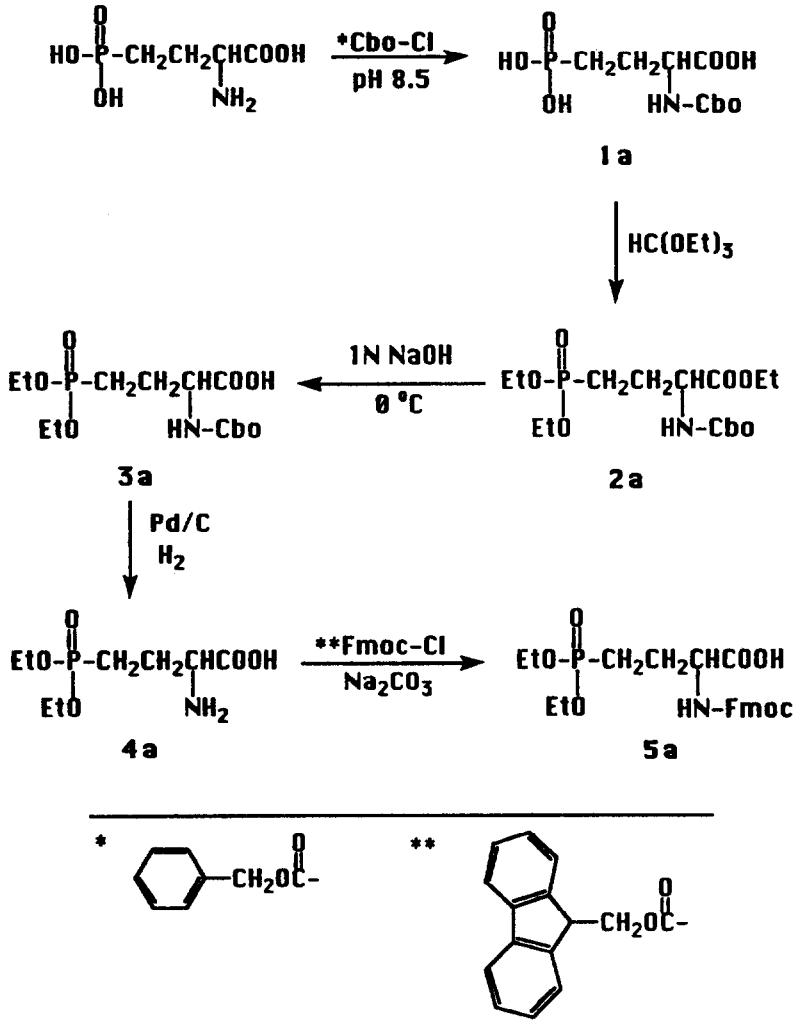

Fig. 3. Scheme for the synthesis of protected DL-2-amino-4-phosphonobutyric acid.

The N-carbobenzoxy derivative of the phosphonoamino acid (*1a*) was prepared as a first step in order to increase the solubility of the starting material such that the triethylation reaction would occur. Several attempts to synthesize the triester (*2a*) from the underivatized phosphonoamino acid were unsuccessful, due to the very limited solubility of the compound in triethylorthoformate. The N-Fmoc protecting group was not synthesized initially, since subsequent reactions required the hydrolysis of the ethyl ester of the carboxyl group of compound *2a* under alkaline conditions; the lability of the N-Fmoc group to such conditions would have negated its initial usage. Saponification of the labile ethyl ester of the carboxyl group proceeded smoothly in 1 N NaOH to yield *3a*. After hydrogenolytic removal of the carbobenzoxy group from *3a*, the resulting product was treated with Fmoc-Cl to give the appropriately protected product, *5a*, which was used in the synthesis of the heptapeptide.

Selective removal of the ethyl ester protecting groups from the phosphonoamino acid residue of the heptapeptide proved somewhat difficult. In addition to acidolysis with HBr/AcOH[8-10], trimethylsilyl iodide deblocking[11], and phosphodiesterase hydrolysis[12] were studied. Trimethylsilyl iodide caused peptide bond cleavage after only 15 min in the presence of the diethylated peptide. Phosphodiesterase gave only slight hydrolysis after long incubation periods. Successful removal of the ethyl groups with virtually no detectable peptide bond hydrolysis (HPLC analysis), was achieved by 20 h exposure to 42% HBr in glacial acetic acid. Shorter exposures (e.g., 16 h) gave significant quantities of the monoethylphosphonate product, while longer exposures (e.g., >22 h) produced some peptide bond hydrolysis.

NMR characterization of protected heptapeptides A and B. Although it was not possible to fully analyze and assign NMR resonances, each heptapeptide exhibited spectral characteristics consistent with structures dictated by the synthetic methodology (Fig. 2). Isomer A (Fig. 2A) showed high field proton NMR absorptions for 39 hydrogens in the region of 0.75 - 1.85 ppm. These included a 12-proton multiplet centered at 0.85 ppm for four methyl groups on the two Leu units, a 9-proton absorption centered at 1.20 ppm for the two methyl groups of the ester and the methyl group of the Ala unit, and a complex 18-proton absorption between 1.4 and 1.8 ppm containing overlapping methylene and methine protons from two Leu units (6H, β and γ), two Arg units (8H, β and γ), and the phosphonobutyrate unit (4H, β and γ). The high field proton region of isomer B was virtually identical (Fig. 2B). With the exception of the ester methyl group absorption, these portions of both spectra in Fig. 2 are strikingly similar to the published ^{1}H NMR spectrum of SP.[13]

The central region of the proton NMR spectrum from 3.0 to 4.4 ppm for isomer A contained all eight α-hydrogens (3H, 3.60 - 3.85 ppm and 5H, 4.15 - 4.40 ppm), a 4-proton multiplet centered at 3.95 ppm for two methylene groups of the ester, and a broad triplet at 3.08 ppm for four protons for the two δ-CH_2 groups of the Arg units. The corresponding hydrogens in the proton spectrum of isomer B appeared as similar but slightly better resolved absorptions in the region of 2.95 to 4.45 ppm. In both spectra, the five α-hydrogens of the internal residues were unresolved in the region 4.15-4.50 ppm. At higher field (3.60-3.85 ppm), the α-proton resonances of the two terminal residues appeared in excellent agreement with previous NMR studies of SP[13] and related peptides.[14]

All N-H absorptions occurred between 6.8 and 8.8 ppm in both isomers. The broad signals (6.8-7.6 ppm) are from the C(ε)-$[N(2)H_2]_2$ of the Arg moieties. Only three other peaks, all triplets, could be assigned definitively: Gly-NH (8.33 ppm in isomer A; 8.38 ppm in isomer B), and Arg-C(δ)NH (7.70 and 7.81 ppm for isomer A; 7.65 and 7.75 ppm for isomer B).

The ^{13}C spectra for these isomers which were complicated by overlaps and couplings of carbon with phosphorus, provided salient diagnostic absorptions in each case. As expected, the ^{13}C NMR spectrum of isomer A contained seven carbonyl carbon lines at 172.1, 172.0, 170.9, 170.8, 170.7, 170.2, and 168.8 ppm. The two guanidino carbons of the Arg units overlapped at 156.8 ppm. For isomer B, the carbonyl absorptions occurred at 172.4, 172.2, 171.1, 170.8 (2 carbons), 170.3, and 168.9 ppm while the two guanidino carbons of the Arg units appeared at 156.9 and 156.8 ppm.

Attempts to characterize further these two heptapeptides using 2-dimensional NMR techniques were not successful due to limited sample size (the 1-D C-13 spectrum required 18,000 acquisitions), signal overlaps, and other complications. It is of interest to note, however, that the phase-sensitive NOESY spectra of the two isomers were virtually identical, indicating that the solution structures of the two compounds are likely very similar.

Inhibition studies. The concentrations of the peptides were obtained by chromatographing both peptides on a C-18 reverse phase column against a standard of a known concentration of SP, and comparing the peak areas of the unknown phosphonopeptide to that of a known SP concentration.

The inhibition constants for both phosphonopeptides A and B, competitive vs. phospho-SP, were obtained from Dixon plots obtained using the enzyme assay for the catalytic subunit described above, and corrected for the presence of the two substrates as described above. Peptide A has a K_i of about 9 μM, while peptide B has a K_i of about 90 μM. Peptide A is about 10-fold better as an inhibitor of the catalytic subunit of cAMP-dependent protein kinase as compared to peptide B and is likely the L diastereomer. Peptide A is also a good inhibitor when compared to other dead-end analogs. The substrate PSP has a K_m of 2 mM. When the serine of SP is replaced by α-aminobutyrate, Glu and Asp, the K_i values of 1.3, 2, and 3 mM are obtained. Thus, the presence of the diionic phosphate is important for optimum binding. In

addition, the difference in C-C-P and C-O-P bond angles likely also contributes to the more effective binding of the phosphonopeptide.

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REFERENCES

1. Present address is the Department of Biological Sciences, University of North Texas, Denton, TX 76023.
2. Bramson, H. N.; Mildvan, A. S.; Kaiser, E. T. *CRC Crit. Rev. Biochem.* **1984**, 15, 93-124.
3. Qamar, R.; Yoon, M.-Y.; Cook, P. F. *Biochemistry* **1992**, 31, 9986-9992.
4. Wasielweki, C.; Antczak, K. *Synth. Commun.* **1981**, 6, 540-541.
5. Schroder, E.; Lubke, K. *The Peptides,* Vol. l; Academic Press: New York, 1965; p. 22.
6. Meienhofer, J.; Waki, M.; Heimer, E. P.; Lambros, T. J.; Makofske, R. C.; Chang, C.-D. *Int. J. Pept. Prot. Res.* **1979**, 13, 35-42.
7. The apparent K_i for the phosphonopeptides A and B, obtained from the Dixon plots were corrected for the concentrations of MgADP and phosphorylated serpeptide present in the assay mixture to obtain the true K_i, by using the following eq. and using known values for the kinetic parameters.[3]

$$v = VAB/[(K_{ia}K_b + K_bA)(1 + I/K_i) + K_aB + AB]$$

8. Kafarski, P.; Lejczak, B.; Mastalerz, P.; Dus, D.; Radzikowski, C. *J. Med. Chem.* **1985**, 28, 1555-1558.
9. Valerio, R.; Perich, J. W.; Kitas, E. A.; Alewood, P. F.; Johns, R. B. *Aust. J. Chem.* **1989**, 42, 1519-1525.
10. Valerio, R.; Alewood, P. F.; Johns, R. B.; Kemp, B. E. *Int. J. Peptide Protein Res.* **1989**, 33, 428-438.
11. Blackburn, G. M.; Inglson, D. *J. Chem. Soc. Perkin Trans 1* **1980**, 1150-1153.
12. Razzell, W. E.; Khorana, H. G. *J. Biol. Chem.* **1959**, 234, 2105-2113.
13. Granot, J.; Mildvan, A. S.; Bramson, H. N.; Thomas, N.; Kaiser, E. T. *Biochemistry* **1981**, 20, 602-610.
14. Rosevear, P. R.; Fry, D. C.; Mildvan, A. S.; Doughty, M.; O'Brian, C.; Kaiser, E. T. *Biochemistry* **1984**, 23, 3161-3173.

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