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Solid-Phase Synthesis of Adenosine Phosphopeptides as Potential Bisubstrate Inhibitors of Protein Kinases

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Abstract: A protocol has been developed for the synthesis of bisubstrate analogs of protein kinases containing adenosine 5′-phosphates (AP_n, n = 2–4) and substrate peptides attached covalently through the hydroxyl group of serine. The procedure involves the coupling of the terminal phosphate group of the phosphoadenosine to phosphorylated substrate peptide(s) after activation with 1,1′-carbonyldiimidazole. The peptide substrates were synthesized by standard Fmoc chemistry and phosphorylated at the target serine using either di-*tert*-butyl or diallyl *N,N*-diisopropylphosphoramidite, followed by oxidation with *tert*-butyl hydroperoxide. Allyl protection was preferred in the solid phase strategy since these groups could be selectively removed and the activated AP_n moieties coupled to the phosphopeptides while still bound to the resin for a yield of ≈40–80%. A less satisfactory approach was initially attempted using di-*tert*-butyl *N,N*-diisopropylphosphoramidite as the phosphite transfer reagent to yield free phosphopeptides which were subsequently coupled in a solution phase reaction (≤2–3% yield). The high efficiency of the solid phase method affords a general approach for synthesizing peptide–nucleoside analogs as well as potentially other types of covalent peptide–ligand complexes through the formation of phosphodiester linkages. In this study, a series of bisubstrate inhibitor candidates were synthesized using this new approach based on kemptide (LRRASLG), a substrate of cAMP dependent protein kinase (cAPK), and several extended analogs. The bisubstrate analogs containing ADP, ATP, and AP_4 were tested as inhibitors of the catalytic subunit of cAPK. IC_{50} values were determined and the best inhibitor in this series was the adenosine 5′-tetraphosphate peptide kemptide–AP_4 (IC_{50} = 68 μM). The efficiency of inhibition was found to decrease rapidly with shorter phosphate chains, yielding IC_{50} values of 226 and 935 μM for kemptide–ATP and kemptide–ADP, respectively. All three inhibitors in this series acted competitively with respect to ATP but not with respect to the peptide substrate, suggesting that larger peptides may be required to generate bisubstrate inhibitors for cAPK.

Introduction

Protein phosphorylation plays a critical regulatory role in numerous cellular processes. The enzymes that are responsible for protein phosphorylation, protein kinases,[1] catalyze the transfer of the γ-phosphate of ATP to the target serine, threonine, or tyrosine residues of the acceptor protein or peptide. The catalytic mechanism is rather complex and requires both MgATP and the protein as substrates. For the catalytic subunit of the cAMP dependent protein kinase (cAPK),[2] the final step in the catalytic

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(2) Symbols used for amino acids and peptides are in accordance with IUPAC-IUB Commission on Biochemical Nomenclature. Other abbreviations used: AP_4, adenosine 5′-tetraphosphate; BOC, *tert*-butyloxycarbonyl-; cAPK, cAMP dependent protein kinase; CaM kinase II, Ca^{+2}/calmodulin dependent protein kinase; CID, collision-induced dissociation; PMC, 2,2,5,7,8-pentamethylchroman-6-sulfonyl-; Fmoc, 9-fluorenylmethyloxycarbonyl-; Trt, triphenylmethyl-; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; Wang resin, (4-(hydroxymethyl)phenoxy)methyl copolymer (styrene–1% divinylbenzene); LSIMS, liquid secondary ion mass spectrometry; MS/MS, tandem mass spectrometry.

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process is the release of the phosphorylated protein and the dissociation of the enzyme–MgADP complex.[3]

The catalytic domains of different protein kinases contain a number of conserved features such as the ATP binding motif, i.e. Gly-X-Gly-X-X-Gly, as well as some less well-defined sequence similarities. On the basis of these sequence similarities, attempts have been made to separate them into a number of functional or homologous domains.[1,4] In the last two years, the crystal structure of cAPK[5,6] has been determined. Information from these three-dimensional structures should allow us to draw some conclusions regarding the kinetic mechanism across the entire kinase family. Nevertheless, even with crystallographic data, many of the mechanistic details cannot be fully understood. For example, it is still not known with certainty what amino acid is the activating base for cAPK, although at least two aspartates are considered good candidates on the basis of chemical data and their close proximity to the γ-phosphate of ATP.[5,6] The design of specific transition state and/or multisubstrate inhibitors could potentially enhance our knowledge of the catalytic mechanism. Specific inhibitors could also provide insight on the biological roles of these kinases if used under *in vivo* conditions.

Bisubstrate analogs (or multisubstrate analogs) form a special group of enzyme inhibitors. By definition, these compounds mimic both the phosphate donor (ATP) and the acceptor components (Ser-, Thr-, or Tyr-containing peptides), which need to be kept at the correct distance by an appropriate linker. From published crystallographic data on cAPK, the chemical nature of the spacer would be expected to be critical, as the Mg^{+2} metal ion complex present in the catalytic pocket makes significant contribution to the binding of the substrates. To date, several classes of inhibitors have been synthesized against tyrosine kinases,[7-10] and details of their interactions can be extrapolated to the serine-specific kinases.[11] However, none of these inhibitors takes advantage of the peptide/protein substrate specificity, and groups resembling the peptide substrate are often reduced to a single amino acid or even simpler molecules.

Using mimics which only slightly resemble the native substrates may not tell us much about the catalytic site or the mechanism of these enzymes and may not lead to inhibitors with the desired selectivity. In addition, interactions between synthetic compounds and certain amino acids at or near the catalytic site not present in the native complex may modify the structure of the transition state complex. In a previous study, we reported the enzymatic synthesis of peptides modified through the tyrosine hydroxyl group with AMP.[12] In this current study, we report the synthesis and initial testing of a series of potential bisubstrate analogs acting as inhibitors of cAPK with structures closely resembling that of the theoretical transition state complex. The inhibitor series was based on the well-known substrate peptide of cAPK, Leu-Arg-Arg-Ala-Ser-Leu-Gly (kemptide[13]). Kemptide was phosphorylated and covalently attached via the serine phosphate to the terminal phosphate groups of AMP, ADP, and ATP, yielding a series of adenosylated peptides (peptide–AP_n) with variable phosphate linkers, n = 2–4.

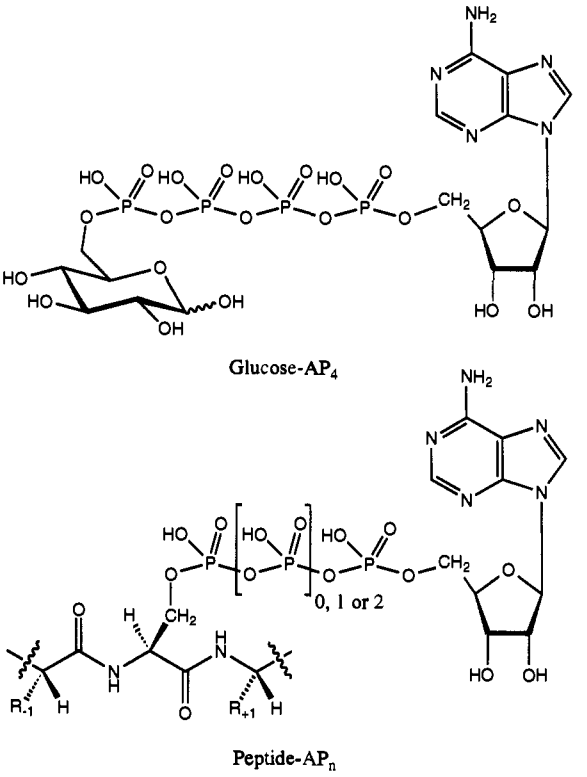

Figure 1. Structure of the glucose–AP_4 bisubstrate analog of hexokinase and the proposed peptide–AP_n analogs as potential inhibitors of serine protein kinases.

Results and Discussion

In the pioneering work of Lienhard[14] and Hampton,[15] multisubstrate analogs, such as glucose 6-phosphate coupled to various adenosine 5′-phosphates, were synthesized as potential inhibitors of enzymes including hexokinase and adenylate kinase (see Figure 1). This strategy provided a starting point in our own efforts to develop specific bisubstrate inhibitors against protein kinases (see Figure 2). However, direct application of the methods used in these syntheses, i.e. reacting nonprotected, enzymatically, or chemically phosphorylated peptides with an excess of adenosine 5′-phosphate activated with 1,1′-carbonyldiimidazole, produced the desired adenosylated peptides in only very low yields (less than 5%, as measured by HPLC). In addition, the final product needed to be separated from numerous byproducts formed from competing side reactions. The maximum yields from these reactions still remain in the range of 1–2% even when the relatively simple peptide, kemptide, was used as the peptide substrate. Therefore, it was necessary to develop a new synthetic approach based on a solid phase strategy where conditions could be found to drive the reaction to completion while also allowing for easy removal of excess reagents. Furthermore, these reaction conditions needed also to remain compatible with the chemical requirements of the appropriate side-chain protection groups in the peptide.

To achieve this goal, a phosphorylation scheme was developed where the phosphate protection could be removed selectively without affecting the linkage to the resin or removing other side-chain amino acid protection groups. Fluorenylmethyloxycarbonyl

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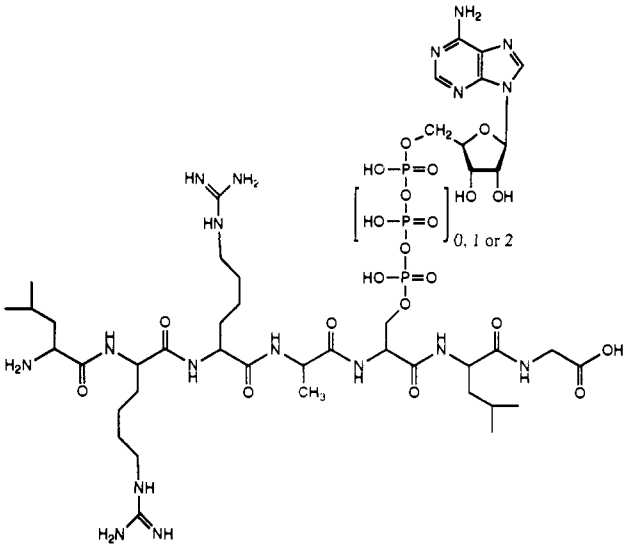

Figure 2. Structure of kemptide–adenosine 5′-phosphates (AP_n, where n = 2, 3, or 4).

(Fmoc) chemistry was desirable for the initial synthesis of the resin bound peptide for two reasons: (1) to establish a general method, we would need to take advantage of readily available peptide synthesis reagents, and (2) a relatively mild cleavage step would be needed for the final release of the adenosylated peptide product from the resin that would not adversely effect the adenosine moiety or the polyphosphate chain in the variable phosphoadenosine linker.

Several successful chemical methods for solid phase synthesis of phosphopeptides have been reported in the past few years. "Global phosphorylation" of peptides has been incorporated into numerous synthesis strategies using di-*tert*-butyl phosphoramidites.[16,17] Andrews et al.[18] reported the application of this method for the synthesis of peptides containing cysteine and methionine, which were previously throught to be incompatible with the oxidation step required in this procedure. Our initial attempts at using the method of Sekine et al.[19] for the selective removal of phosphate *tert*-butyl protection by trimethylchlorosilane or trimethylbromosilane,[20] however, proved to be inadequate. On the basis of an earlier report using the allyloxycarbonyl group[21] as the temporary protection for the amino terminal, the allyl group itself has been reported to be useful as a phosphate protecting group.[22] Indeed, diallyl *N,N*-diisopropylphosphoramidite was found to be an excellent reagent to introduce the phosphate group in our synthetic scheme, and the phosphoserine was easily and selectively deprotected by tetrakis(triphenylphosphine)palladium-(0) in the presence of morpholine/dimethylformamide (DMF) to form the desired resin-bound intermediate phosphopeptides.

Thus, the diallyl phosphate protection (see Figure 3) afforded an efficient means to prepare a series of intermediate phosphopeptides that were still covalently attached to the original peptide synthesis resin, including phosphokemptide, extended phosphokemptides, and modified Ca^{+2}/calmodulin dependent protein kinase (CaM Kinase II) autoinhibitory peptide (see Table 1). All the other permanent-type protection groups (Trt, OtBu, tBu, and PMC) remained intact, and no byproducts were observed due to free side chain functions. Also, acetylation of the peptide resin by acetic acid anhydride following the deprotection step did not produce any detectable amount of acetylated peptides, indicating that no acylation susceptible functional groups had been inadvertently deprotected. Methylation by diazomethane of the resin-bound peptide following the allyl cleavage and acidification resulted in monomethylated peptides where the methyl group was located on the phosphate group as indicated by the liquid secondary ion mass spectrometry (LSIMS) fragmentation, i.e. the observed β-elimination fragment gave the same m/z value as with the non-methylated phosphopeptide. In the case of the CaM kinase II autoinhibitory peptide which contained cysteine, no oxidation products were observed after the *tert*-butyl hydroperoxide oxidation step (reaction time reduced to 30 min). Details of this latter synthesis will be reported elsewhere.

Deprotection and cleavage of the adenosyl-5′-phosphopeptides proceeded smoothly using standard conditions. The intermediate carbonic acid diester formed after treatment of the ribose diol moiety with 1,1′-carbonyldiimidazole needed separate deprotection, but the carbonic acid diester could be removed easily by treatment with weak base in a water–methanol solution (see Figure 4).

During the synthesis, each step was monitored by LSIMS and the limited fragmentation observed in these spectra yielded important structural information. For example, it was noted that the thioglycerol used as part of the LSIMS matrix material formed adducts with allyl groups which later were found to be present only in samples containing aliphatic double bonds. Therefore, these artifact ions provided an excellent indicator for the presence of this structural moiety. Also, all adenosylated peptides formed extensive metal complexes in the positive-ion mode, yielding molecular ions containing Na^+, Mg^{+2}, K^+, Mn^{+2}, and Fe^{+2}.[23] Under negative-ion conditions, these metal ion adducts were largely absent and ions were seen primarily as their deprotonated species, $(M - H)^-$.

To confirm the structures of the final products, all HPLC purified compounds were subjected to high-energy collision-induced dissociation (CID) tandem mass spectrometry. This technique had been successfully used in some of our previous efforts to characterize synthetic phosphopeptides and peptides adenylated at tyrosine.[12,24,25] As an example, Figure 5 shows the spectrum obtained under negative-ion conditions for kemptide–ADP, $(M = H)^-$ at m/z 1179.6. In all these tandem spectra, characteristic fragments were identified in both the negative- and positive-ion modes that were useful in confirming that the appropriate covalent modification had been made between phoshokemptide and the phosphoadenosine moieties, AMP, ADP, and ATP (Figure 6). Details of the mass spectrometric characterization of these compounds have been presented elsewhere.[23]

As expected, fragments from the adenosine 5′-phosphate moiety dominated the spectra in negative-ion mode, yielding a series of characteristic phosphate-containing ions such as PO_3^-, $H_2PO_4^-$, $HP_2O_6^-$, $H_3P_2O_7^-$, $H_2P_3O_9^-$, etc., as well as ions containing the adenosine moiety, i.e. $(AMP - H_2O - H)^-$, $(ADP - H_2O - H)^-$, $(ATP - H_2O - H)^-$, etc. In all cases, the fragment ion containing the highest number of phosphates, i.e. 2, 3, or 4, provided conclusive evidence for the formation of the appropriate analog, AP_n. In the positive-ion mode, the dominating ions originated from the peptide itself, although a very abundant ion was always present at m/z 250 for deoxyadenosine. Also, neutral losses of adenosine and phosphoadenosines containing variable numbers of phosphates yielded a set of peptide ions containing up to four phosphates, i.e. peptide–$(HPO_3)_n$, where n = 0–4. In general, information from

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Table 1. Amino Acid Sequences of Some Synthetic Bisubstrate Analogs of cAPK and CaM Kinase II[a]

peptide	amino acid sequence
kemptide	Leu-Arg-Arg-Ala-Ser-Leu-Gly
PKI_{12-16}-kemptide	Ac-Ala-Ser-Gly-Arg-Thr-Leu-Arg-Arg-Ala-Ser-Leu-Gly
PKI_{6-16}-kemptide	Ac-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Leu-Arg-Arg-Ala-Ser-Leu-Gly
CaMPK peptide	Ac-Ser-Arg-Gln-Glu-Ala-Val-Asp-Cys-Leu-Lys-Lys-Phe-Asn-Ala-Arg-Arg-Lys-Leu-Lys-Gly-Ala

[a] The underlined serine (Ser) identifies the position of phosphorylation and adenosylation, i.e. PO_3H_2, ADP, ATP, or AP_4.

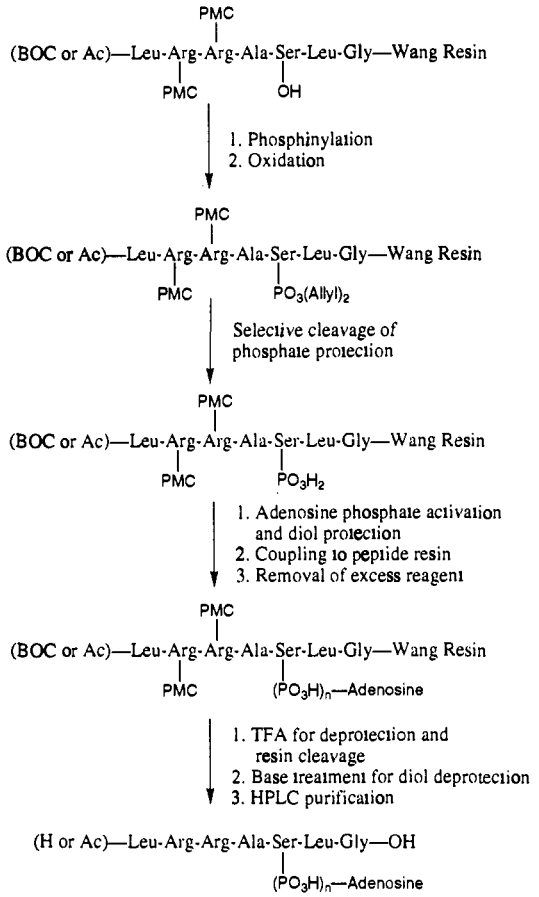

Figure 3. Solid phase synthesis scheme. The peptide was first prepared by standard Fmoc chemistry with PMC (2,2,5,7,8-pentamethylchroman-6-sulfonyl) protection of arginines and a N-terminal BOC group.

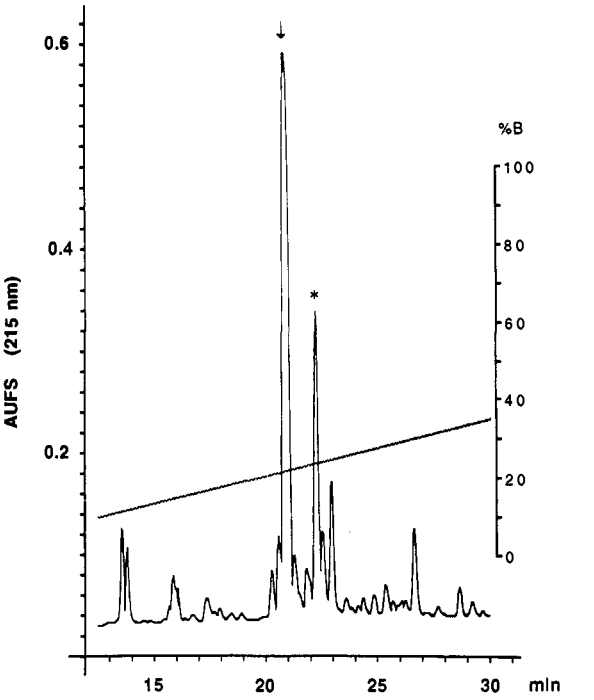

Figure 4. HPLC separation of the solid phase reaction product in one synthesis of kemptide–ADP. UV absorbance was measured at 215 nm. Arrow indicates the peak corresponding to the desired kemptide–ADP product. The major byproduct labeled with an asterisk is phosphokemptide.

both positive- and negative-ion spectra was needed to confirm absolute product identity.

The highest conversion yield (≈80%) was obtained from the reaction of phosphopeptide with ADP, and yields were somewhat lower with the other two adenosine 5′-phosphates, AMP and ATP (≈40–50%). We suspect that in the case of AMP the steric effects of the bulky arginine protection (PMC) and the closeness of the adenine ring may have been a limiting factor. Extended reaction times up to 48 h and repeated addition of fresh activated AMP did not increase the yield significantly. For the reaction of phosphokemptide with ATP, the reduced stability of the tetraphosphate chain could have been the limiting factor and the reaction might have proceeded to near completion had not trifluoroacetic acid (TFA) cleavage destroyed part of the desired product. The identification of such decomposition products after separation of the final product mixture by HPLC supports this hypothesis.

To evaluate the inhibitory efficacy of these bisubstrate analogs, all three adenosine 5′-phosphopeptides were tested in a coupled enzyme assay[3] against the catalytic subunit of cAPK. The phosphorylation activity of the enzyme was monitored directly by measuring the absorbance change at 340 nm characteristic of NADH oxidation. The measured K_m values for kemptide and ATP using this assay were 15.3 and 9.6 μM, respectively, and were in good agreement with the earlier published results.[13,26]

The synthetic adenosine 5′-phosphopeptides were found to be effective inhibitors of cAPK; IC_{50} values of 68, 226, and 935 μM were established for tetra-, tri-, and diphosphate compounds, respectively. The inhibition mechanism was found to be competitive with respect to ATP ([ATP] = 10 μM) but not with respect to the peptide substrate ([kemptide] = 16 μM). Inhibitor concentrations up to 10 times higher than that of the peptide substrate had little or no effect on the phosphorylation rate. Although contribution to the inhibitor binding can be expected from both the peptide and the AP_n moiety in the bisubstrate analog molecule, our experiments indicated that these compounds acted primarily as ATP analogs under these assay conditions. It is likely that the phosphoadenosine groups on the target serine of kemptide resulted in some conformational or steric perturbations in these bisubstrate analogs and may have diminished the binding of the peptide moiety. However, in earlier studies of bisubstrate analogs of protein kinases,[7] variations in the length of the linker did not effect inhibition. From our earlier experiments, it was also clearly established that the phosphopeptides do not inhibit the phosphorylation reaction, i.e. the reaction went to completion. In a separate experiment, dimethylphosphokemptide was also tested but no inhibition was observed even at concentrations up to 100 times that of the peptide substrate. This suggests that ionic interaction between the introduced phosphate group and the basic peptide itself is not primarily responsible for the release of products from the enzyme.

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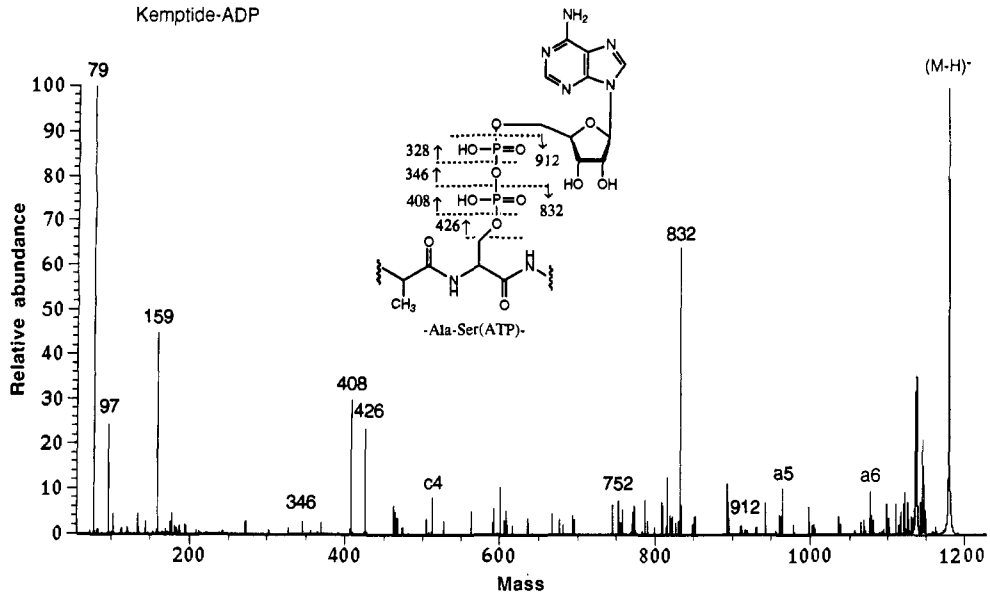

Figure 5. Tandem MS/MS spectrum of kemptide–ADP in the negative-ion mode. All ions are listed as their nominal masses. Peptide backbone fragmentation is labeled according to standard nomenclature, i.e. a_n, b_n, etc.[31]

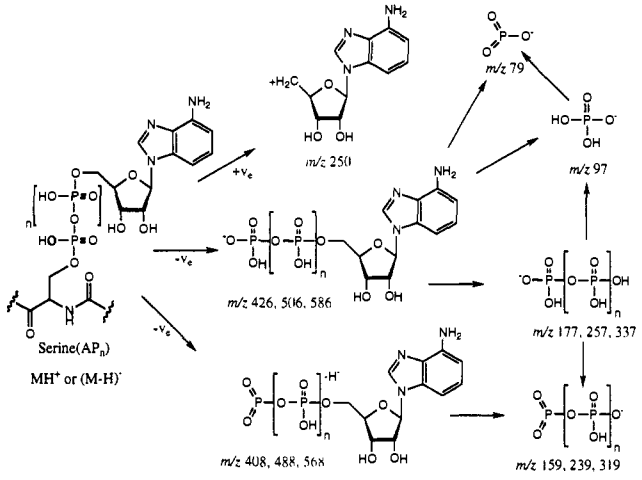

Figure 6. Characteristic fragments observed under CID conditions for all bisubstrate analogs.

Conclusion

A new synthetic protocol has been developed on the basis of solid phase peptide synthesis strategies that provides an efficient means to synthesize bisubstrate analogs of protein kinases based on phosphoadenosines (AMP, ADP, and ATP) coupled to phosphopeptides. These bisubstrate analogs have close structural similarity to the transition state complex and could potentially act as specific inhibitors of a number of protein kinases. In addition, these analogs could be used in X-ray crystallographic experiments by cocrystallization with various protein kinases and provide important tools to investigate the precise molecular interactions of the substrates at the catalytic site. The strategy also opens up new synthetic pathways for the design of small peptide–nucleotide compounds and potentially other peptide analogs containing secondary ligands attached through phosphodiester linkages.

Successful inhibitors have now been developed in this work by covalently coupling two substrates of cAPK (both kemptide and AP_n) in a single compound, yielding bisubstrate analogs where the peptide portion has now been extended to include other residues beside the phosphorylated amino acid. Previously, Kruse et al.[9,10] synthesized a series of multisubstrate analogs that contained tyrosine mimics linked through phosphodiester bonds to nucleoside analogs. It is our long term goal to construct peptide-based inhibitors that better exploit the differences in protein/peptide substrate recognition among kinases by constructing bisubstrate analogs which extend beyond the target amino acid and contain peptide substrates.

In the adenosine phosphopeptides analogs synthesized and tested in this study, the effect of the length of the phosphate chain on inhibition was found to be significant, and the best inhibitor in the group was kemptide–AP_4 with an IC_{50} value of 68 μM toward ATP. However, this initial set of kemptide–AP_n analogs exhibited no competition between the peptide portions of these bisubstrate mimics, suggesting that the substitution on the target serine had significantly reduced the binding between the peptide and the enzyme. It is likely that changes will be required in the peptide component of these bisubstrate analogs if significant inhibition ($IC_{50} \leq$ nmol) is to be achieved. To achieve this goal, we are in the process of completing the synthesis of a complete series of adenosylated peptides (AP_n, $n = 2$–4) containing extended sequences based on the heat stable peptide inhibitor PKI (see Table 1), which has much more extensive interactions with cAPK[27] and should take advantage of additional interactions outside the catalytic site. However, our results do not rule out the possibility that peptides of the same size as kemptide, or even smaller, can be found that will act as more potent bisubstrate inhibitors.

Experimental Section

General Procedure. Solid phase peptide synthesis was carried out in 0.1–0.2 mmolar scale on a Rainin Instruments PS-3 peptide synthesizer using standard Fmoc chemistry and HBTU as the coupling agent. General conditions of the synthesis were those recommended by the manufacturer. Protected amino acids, Wang resin coupled amino acids, and other peptide synthesis chemicals were purchased from either Rainin or Novabiochem USA. DMF and all HPLC solvents were obtained from Fisher Scientific. All the other chemicals were purchased from either Aldrich or Sigma. The phosphinylation reagent, diallyl *N,N*-diisopropylphosphoramidite, was synthesized in-house by the method of Bannwarth et al.[22]

All HPLC separations were carried out on a Rainin Instruments gradient HPLC system using either a Vydac C_{18} wide-pore analytical (4.6 mm × 25 cm, 300 Å) or semipreparative column (9.8 mm × 25 cm, 300 Å). Eluting products were detected by UV at 215 nm for peptides and phosphopeptides and at 215 and 260 nm for AP_n-peptides using an Applied Biosystems 1000S diode array detector. Solvent A was 0.1% TFA (v/v) in water, and solvent B was 0.08% TFA (v/v) in 70%

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acetonitrile–water. A flow rate of 1 mL/min was used for all analytical separations and a 4.0 mL/min flow rate for semipreparative runs. In either case, a linear gradient program was carried out starting from 100% solvent A and reaching 35% solvent B (30% acetonitrile) for 25 min.

TLC was performed on cellulose plates with a fluorescent indicator (Eastman Kodak) using the solvent system 2-propanol–0.25 M NH_4HCO_3 (65:35).

For LSIMS mass spectrometric analysis, a Kratos MS50S double-focusing mass spectrometer equipped with a high-field magnet and cesium ion source was used.[28] Samples were dissolved in a water–acetonitrile mixture at 400–600 pmol/μL, and ≈1 μL was applied to the probe tip mixed with 1 μL of thioglycerol–glycerol (1:1) (v/v) matrix containing 1% TFA for positive-ion mode operations. Mass assignments were made by manual calibration using an Ultramark 1621 as an external reference to an accuracy of better than ±0.3 Da. The high-energy CID analyses were performed using a Kratos Concept IIHH four-sector tandem mass spectrometer also equipped with a cesium ion source.[29]

Evaluations of synthetic bisubstrate analogs were performed using a cAPK catalytic subunit in a coupled enzyme assay[3] on a Hewlett-Packard 8452A UV/vis diode array spectrophotometer using the supplied kinetic analysis software package. All the enzymes and other biochemicals were from Sigma.

Activation of Adenosine 5′-Phosphate. Typically, 1 mmol of the free acid[30] of adenosine 5′-phosphate was converted to its tri-*n*-butylammonium salt by first dissolving the acid in 10 mL of water followed by the addition of 4 mmol (0.76 mL) of tri-*n*-butylamine and then ethanol to obtain a clear solution. The volatile components were evaporated in vacuo, and the oily residue was dried by repeated evaporations of ethanol. The precipitate was further dried in vacuo over P_2O_5 overnight. The residue was then dissolved in 6 mL of dry DMF, 5 mmol (810 mg) of 1,1′-carbonyldiimidazole was added, and the mixture was left standing at room temperature (≈20–22 °C). Activation of the phosphoadenosines were monitored by TLC and was complete for ADP in 3 h but required overnight (15 h) activation for AMP and ATP. The reaction mixture was treated with methanol (260 μL) for 30 min to destroy excess, 1,1′-carbonyldiimidazole and was added to the peptide resin in a 10-fold excess.

Synthesis of Phosphopeptide Resin. BOC-Leu-Arg(PMC)-Arg-(PMC)-Ala-Ser-Leu-Gly-Wang resin (0.1 mmol) was prepared as described in the General Procedure. The N-terminal amino acid (Leu) was introduced with BOC protection. Serine was introduced without side chain protection. For Fmoc peptide coupling, ≈0.4 mmol of activated amino acid was used in a 4-fold excess. After the synthesis was complete, 5 mmol (350 mg) of 1*H*-tetrazole in 5 mL of DMF and 1 mmol (245 mg) of diallyl *N,N*-diisopropylphosphoramidite were added to the swollen peptide resin. The transfer of diallyl phosphite to the hydroxyl group on serine was continued for 60 min with periodic agitation by nitrogen bubbling. The resin was then washed on the machine in the reaction vessel with DMF (9×) to remove excess reagent followed by the delivery of 1 mL of 2 M *tert*-butyl hydroperoxide in water–DMF. After 40 min, excess reagent was removed by through washings with DMF (12×). For analytical purposes, ≈1 μmol of resin (1/100) was removed, washed with methanol, dried, and treated with 0.5 mL of TFA containing 15 μL of water and 15 μL of thioanisole (solvent X) for 60 min at room temperature. Volatile reagents were removed by evaporation, and the residue was washed with diethyl ether and dried in vacuo. The cleaved peptide was then dissolved in a water–acetonitrile mixture (10–20% acetonitrile) and the resin removed via filtration. The final product was then separated from the nonvolatile contaminants and reactants by HPLC. In each case, the major components were subjected to LSIMS for product identification. Both diallyl and monoallyl compounds were detected along with a very small amount of unprotected phosphopeptide, indicating the resistance of allyl groups toward mild acid cleavage.

Selective cleavage of the phosphate protecting groups was achieved by adding 1 mmol (87 mg) of morpholine in 1 mL of DMF and 0.5 mmol (578 mg) of tetrakis(triphenylphosphine)palladium(0) in 5 mL of DMF to the wet resin and mixing with periodic nitrogen bubbling for 60 min at room temperature. The catalyst was removed by repeated washes with DMF, and 1 mmol (0.24 mL) of tri-*n*-butylamine was added to form the tributylammonium salt. Excess base was removed by washing the resin with DMF and methanol and drying over P_2O_5 overnight. An aliquot of the resin was cleaved for analytical purposes as described earlier.

Synthesis of Adenosine 5′-Phosphopeptides (Adenosylated Peptides). Coupling was carried out by resolvating the resin in DMF and adding the activated adenosine 5′-phosphate (1 mmol) to the peptide resin at 50 °C. The reaction mixture was stirred for 15 h in the case of ADP and was continued up to 24 h with AMP and ATP. Reaction was terminated by filtering and washing the resin with DMF and methanol on a sintered glass filter funnel. The resin can be stored dry in a desiccator for months.

Cleavage from the resin was carried out similar to that described for the analytical samples. To about 200 mg of resin was added 10 mL of solvent X, and the mixture was stirred for 60 min at room temperature. The resin was filtered out and washed with a small amount of TFA, combined filtrates were evaporated in vacuo, the residue was triturated with diethyl ether, and the precipitated peptide was filtered out on a sintered glass funnel. The product was then dissolved in 5 mL of water and 2 mL of methanol. To remove the carbonic acid diester from the ribose moiety, the pH was adjusted to between 10.5 and 11.0 with TEA. After 30 min, this solution was neutralized with acetic acid and lyophilized. The crude product was purified by gel filtration on a Biogel P-2 column in 5% acetic acid (2.5 × 75 cm) and the eluate monitored at 260 nm. UV absorbing fractions at the exclusion limit were pooled and lyophilized again. Preparative anion exchange chromatography on a MonoQ and/or RP-HPLC using Vydac C_{18} columns was used to obtain sufficiently pure compounds for enzyme assays.

Analytical Section

Amino Acid Analysis. Amino acid composition was determined after standard 24 h hydrolysis in 6 N HCl and reported in relative molar amounts. The expected molar ratios are shown in parentheses to the right. Kemptide–ADP: Leu, 1.71 (2); Arg, 1.68 (2); Ala, 0.94 (1); Ser, 1.16 (1); Gly, 1.51 (1). Kemptide–ATP: Leu, 2.02 (2); Arg, 1.98 (2); Ala, 1.10 (1); Ser, 1.37 (1); Gly, 1.78 (1). Kemptide–AP_4: Leu, 1.80 (2); Arg, 1.79 (2); Ala, 0.95 (1); Ser, 1.00 (1); Gly, 1.46 (1).

LSIMS Data. Molecular ions for the individual kemptide–AP_n analogs were determined to an accuracy of ±0.3 Da in both the positive- and negative-ion modes and reported against their expected mass values listed in parentheses to the right. Kemptide: $(M + H)^+$ at m/z 772.6 (772.5) and $(M - H)^-$ at m/z 770.7 (770.5). Kemptide–Ser(P): $(M + H)^+$ at m/z 852.6 (852.5) and $(M - H)^-$ at m/z 850.6 (850.4). Kemptide–ADP: $(M + H)^+$ at m/z 1181.5 (1181.5) and $(M - H)^-$ at m/z 1179.6 (1179.5). Kemptide–ATP: $(M + H)^+$ at m/z 1261.5 (1261.5) and $(M - H)^-$ at m/z 1259.7 (1259.5). Kemptide–AP_4: $(M + H)^+$ at m/z 1341.5 (1341.4) and $(M - H)^-$ at m/z 1339.7 (1339.4).

Characteristic Fragment Ions from MS/MS Analysis. Detailed interpretation of the data has been published elsewhere.[23] Kemptide–ADP, positive-ion mode: 250.1 (deoxyadenosine); 754.5 (β-elimination of ADP from peptide, i.e. dehydroalanine-containing peptide + H); 852.4 (peptide–PO_3H + H); 932.4 (peptide–$(PO_3H)_2$ + H); 965.3 (a_5); 1046.4 (peptide–$(PO_3H)_2$ – ribose + H); 1124.4 (Leu side chain loss). Kemptide–ADP, negative-ion mode: 78.8 (PO_3^-); 158.8 ($HP_2O_6^-$); 408.0 (ADP – H_2O – H); 426.0 (ADP – H). Kemptide–ATP, positive-ion mode: 250.1 (deoxyadenosine); 754.6 (β-elimination of ATP + H); 834.4 (peptide–PO_3H – H_2O + H); 852.3 (peptide–PO_3H + H); 932.4 (peptide–$(PO_3H)_2$ + H); 1012.3 (peptide–$(PO_3H)_3$ + H); 1045.5 (a_5); 1204.5 (Leu side chain loss). Kemptide–ATP, negative-ion mode: 78.8 (PO_3^-); 158.8 ($HP_2O_6^-$); 408.0 (ADP – H_2O – H); 426.0 (ADP – H); 488.1 (ATP – H_2O – H); 506.1 (ATP – H); 912.8 (peptide–$(PO_3H)_2$ – H_2O – H); 1202.7 (Leu side chain loss). Kemptide–AP_4, positive-ion mode: 250.1 (deoxyadenosine); 754.6 (β-elimination of AP_4 + H); 852.5 (peptide–PO_3H + H); 932.3 (peptide–$(PO_3H)_2$ + H); 1012.6

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(30) If the free acid was not available; the appropriate adenosine 5′-phosphate salt was dissolved in water (3 mL) and the solution percolated through a column of Dowex 50×2-200 resin (5 mL, pyridinium form), detection at 260 nm. Washings were performed with water. Eluting UV absorbing fractions were combined and concentrated in vacuo to 5 mL. This solution was used in the following steps.

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(peptide–$(PO_3H)_3$ + H); 1092.6 (peptide–$(PO_3H)_4$ + H); 1284.5 (Leu side chain loss). Kemptide–AP_4, negative-ion mode: 78.8 (PO_3^-); 158.8 ($HP_2O_6^-$); 238.8 ($H_2P_3O_9^-$); 318.7 ($H_3P_4O_{12}^-$); 408.1 (ADP – H_2O – H); 488.1 (ATP – H_2O – H); 506.0 (ATP – H); 586.2 (AP_4 – H); 992.7 (peptide–$(PO_3H)_3$ – H_2O – H).

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