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THE SYNTHESIS OF AN ATP-γ-PEPTIDYL ESTER AS A POTENTIAL PROBE OF c-AMP-DEPENDENT PROTEIN KINASES

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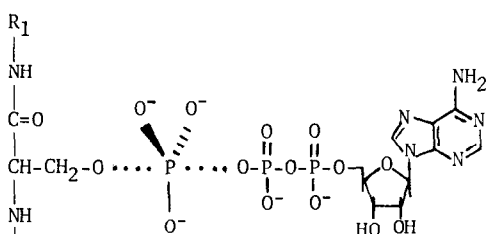
Summary: The synthesis of a γ-dipeptidyl ester of ATP has been effected by coupling a phosphopeptide (e.g. N-acetylalanyl-O-(phosphoryl)serine methyl ester) to ADP in the presence of 1,1'-carbonyldiimidazole. Compounds of this type, containing longer peptides, are proposed as potential multisubstrate adduct inhibitors of c-AMP-dependent protein kinases.

The protein kinases have been extensively studied in terms of their mechanism of action[1,2] and their role in regulation of enzyme activity.[3] Most recently, much data have been accumulated which strongly suggest that a protein kinase is the gene product responsible for initiating cell transformation by several RNA tumor viruses.[4] The cyclic-AMP-dependent protein kinases have been studied in great detail in terms of the biochemistry of hormone action, and function of the nervous system.[5] Mechanistic studies on Type II c-AMP-dependent protein kinase from beef heart have attempted to distinguish between a dissociative (S_N1-like) mechanism involving a metaphosphate intermediate, and an associative (S_N2-like) mechanism.[6] However, a definitive distinction between these two pathways using a variety of kinetic methods and special probes has been difficult. Recently, the use of chiral phosphorothioate and [^{16}O, ^{17}O, ^{18}O]-phosphate esters has allowed for the distinction between single- and double-displacement mechanisms in several kinases,[2] but still leaves unanswered the question concerning S_N1 vs. S_N2 transition states in enzyme-catalyzed phosphoryl transfer. As a means for studying this question, and at the same time providing a possible entry into a new class of drugs, we have considered the use of multisubstrate adduct inhibitors[7-9] as specific, potent inhibitors of protein kinases.

The transfer of the γ-phosphate of ATP to a protein hydroxyl group (e.g., serine) involves the transition-state shown in 1 for an associative mechanism. This mechanism suggests the synthesis of 2 as a multisubstrate adduct ("transition-state analog")[10] inhibitor of protein



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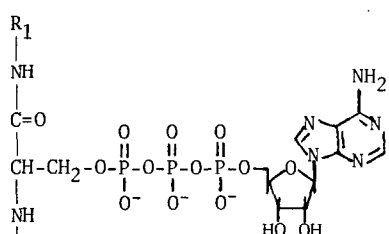


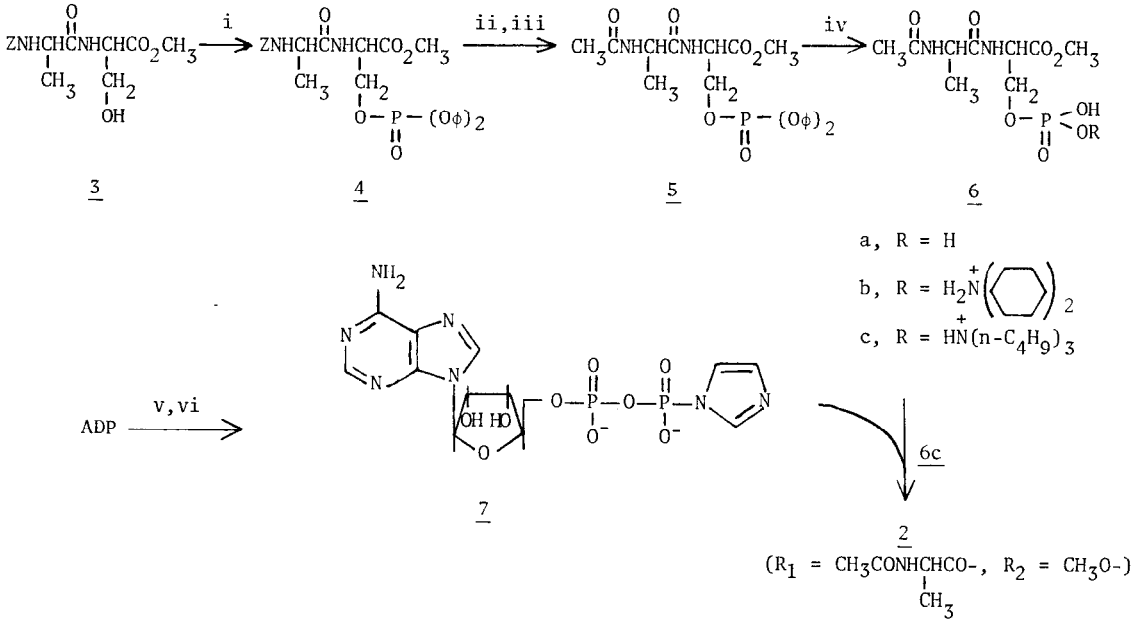
2

kinases. The discovery that small oligopeptides are effective substrates for c-AMP dependent protein kinases[11] makes the synthesis of 2 more attractive, since incorporation of an oligopeptide into 2 is a feasible synthetic undertaking. In addition, the synthesis of 2 with serine-containing peptides of varying chain length offers the possibility of probing the energetics of substrate binding to the enzyme by forming non-reactive ternary complex analogs using multisubstrate adduct inhibitors.[12] In this note, we describe the first synthesis of an ATP-γ-peptidyl ester, namely the γ-(NAcAlaSerOCH$_3$) ester of ATP (2, R_1 = $CH_3CONHCH(CH_3)CO$-, R_2 = CH_3O-).

Initially, we planned to synthesize 2 (R_1 = NAcAla-, R_2 = CH_3O-) via a dicyclohexyl carbodiimide(DCC)-mediated coupling of N-acetylalanylserine methyl ester to ATP. Although this type of reaction has precedent in the synthesis of the γ-methyl ester of ATP,[13,14] and γ-phosphoramides,[14] we were able to obtain the desired γ-methyl ester in only low yield after purification on DEAE-cellulose. The major product (67%) in the DCC-mediated coupling reaction was eluted first on DEAE-cellulose (0.07 M NH_4HCO_3), and between AMP and ADP on anion exchange HPLC. The second peak on DEAE-cellulose (0.115 M NH_4HCO_3) proved to be the desired ATP-γ-methyl ester (γ-CH_3ATP). We abandoned attempts to improve on this synthetic route when it became apparent that poor nucleophiles such as simple alcohols and serine containing peptides, could not be used in stoichiometric amounts, but were needed in large excess (i.e., as solvent), in order to obtain even the low yield of the γ-ester described above. In contrast, stronger nucleophiles such as amines, when added in stoichiometric amounts, readily couple to ATP, in the DCC-mediated reaction.[14] The procedure of Hoard and Ott[15] in which the coupling of $ROPO_3H_2$ and ADP is effected with the aid of carbonyldiimidazole, via an intermediate ADP-β-imidazolidate, was then investigated. Using this procedure, γ-CH_3ATP was obtained in good yield from methyl phosphate and ADP.

Our attention then turned to the synthesis of the appropriate peptidyl phosphate required for analogous coupling to ADP to yield 2. The base lability of phosphopeptides has been known for many years.[16] Therefore, amine and phosphate protecting groups were employed which would be removed under acidic or neutral conditions. Diphenylphosphochloridate is known to phosphorylate serine-containing peptides, and removal of the phenyl esters is accomplished using H_2/PtO_2.[16] Thus, reaction of N-(benzyloxycarbonyl)alanylserine methyl ester, 3, with diphenyl phosphochloridate gave the phosphorylated peptide, 4, as an oil, in quantitative yield. This material was subjected to the action of HBr/HOAc in order to remove the benzyloxycarbonyl blocking group, and the resulting amine salt was acetylated with acetic anhydride to give N-acetylalanyl-O-(diphenylphosphoryl)serine methyl ester, 5, in overall yield from 4 of 46%. This material was slightly contaminated with an unidentified impurity, which was indicated by nmr spectroscopy. However, it was sufficiently pure for use in the synthesis of the free phosphoric acid, 6a, prepared in 93% yield by hydrogenation of the diphenyl ester using PtO_2 as catalyst. The free acid, 6a, was obtained as a light yellow, oil and this was readily converted to a crystalline dicyclohexylamine (DCHA) salt, 6b, in 70% yield (m.p. 179-181°) for complete characterization. Coupling of the phosphodipeptide, as its tri(n-butyl)ammonium salt[17], 6c, to ADP was effected using the carbonyldiimidazole procedure of Hoard and Ott.[15] Ion-exchange chromatography of the crude product on DEAE-cellulose resulted in isolation of the desired γ-peptidyl-ATP, 2 (R_1 = NAcAla-, R_2 = CH_3O-), as the major product in 60% yield. It should be noted that, although ADP and

γ-CH_3ATP were not separated by chromatography on DEAE-cellulose, the separation of the desired product from unreacted ADP was readily accomplished by this procedure. The purified product was shown to be pure by anion-exchange HPLC (t_R = 24.9 min)[18], phosphate:adenosine ratio,[19,20] and amino acid analysis. It was completely characterized by both UV and NMR (^1H, ^{31}P) spectroscopy,[21] and found to have spectral properties completely consistent with the assigned structure. The synthetic route used to obtain 2 (R_1 = NAcAla-, R_2 = CH_3O-) is shown in Scheme 1.



Scheme 1[a]

[a]Reagents: (i) $(\phi O)_2P$-Cl, pyridine; (ii) HBr/HOAc; (iii) Ac_2O; (iv) H_2/PtO_2; (v) $(n\text{-}C_4H_9)_3N$; (vi) carbonyldiimidazole.

The synthesis reported here is the first described for a γ-peptidyl ester of ATP. Esters containing longer oligopeptides (i.e., 2) should allow for a study of the energetics of enzyme binding associated with a stepwise addition of specific amino acid residues to the multisubstrate adduct analog of the ternary complex. The synthesis of these larger γ-peptidyl-ATP's is currently in progress in our laboratory.

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18. HPLC analysis was performed on a Partisil-SAX (Whatman) column using a linear phosphate (pH 3.3) gradient (0.001-0.05 M) over a period of 30 min.; phosphate concentration was then increased to 0.1 M over a 5 min. period, and held at that concentration for an additional 5 min. In this HPLC system, operating at a flow rate of 1 ml min^{-1}, AMP was eluted at t_r = 22 min., and ATP at t_r = 39 min.
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21. High-field ^{1}H-nmr spectra were recorded on a Bruker HX-270 superconducting nmr spectrometer operating at 270 MHz. ^{31}P-nmr spectra were obtained at 32 MHz using a modified Varian CFT-20 spectrometer.

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