Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 4, No. 4, pp. 553-556, 1994

0960-894X/94 $6.00+0.00

0960-894X(94)E0007-2

AN ETHER-BASED PEPTIDE ANALOG AS cAMP-DEPENDENT PROTEIN KINASE INHIBITOR/SUBSTRATE

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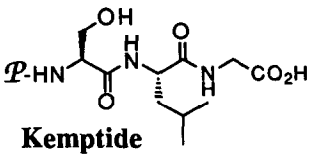
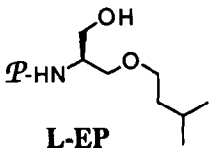
Abstract: Ether-based peptide analog L-EP contains an ether-linkage unit in place of the Ser-Leu-Gly unit of a PKA substrate, Kemptide. L-EP is a competitive inhibitor of Kemptide phosphorylation catalyzed by PKA with a K_i value of 120 μM. It also serves as a weak substrate of PKA with a K_m value 25-fold higher and a V_{max} value 3.5-fold lower than that of Kemptide.

cAMP-dependent protein kinase (**PKA**) is the most well studied member of a family of more than 200 protein kinases.[1,2] The consensus substrate sequence recognized by this enzyme has been determined to be Arg-Arg-X-Ser-Y as typified in the Kemptide sequence,[3] where X is a small residue and Y is a large hydrophobic residue.[1] The stereochemical course of phospho group transfer catalyzed by this kinase has been shown to follow an in-line displacement mechanism.[4] This prototype of protein kinase has been used as a model for designing protein kinase inhibitors based on the Kemptide sequence.[5,6,7,8] Recently, several crystal structures of the catalytic subunit[9] of **PKA** have been reported, including those complexed with a peptide inhibitor or with ATP and a peptide inhibitor. The unique peptide substrate specificity of each class of protein kinase offers the possibility of designing peptide-based, target-specific protein kinase inhibitors.[10] However, peptide ligands are not generally suitable for intracellular targets unless a delivery system can be found. Several naturally occurring polyethers are known to inhibit the related protein phosphatases, including okadaic acid which blocks phosphatases in intact cells.[11] In consideration of the dual solubility of polyethers in aqueous and lipid environments, we have been interested in designing polyether-based peptide antagonists as **PKA** inhibitors. As the first step toward this goal, we have prepared and studied Kemptide analogs **L-EP** and **D-EP**, in which the Ser-Leu-Gly unit is replaced by an ether-containing peptide surrogate unit.

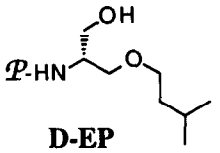


Kemptide

$\mathcal{P}$ = H_2N-**Leu-Arg-Arg-Ala**



L-EP

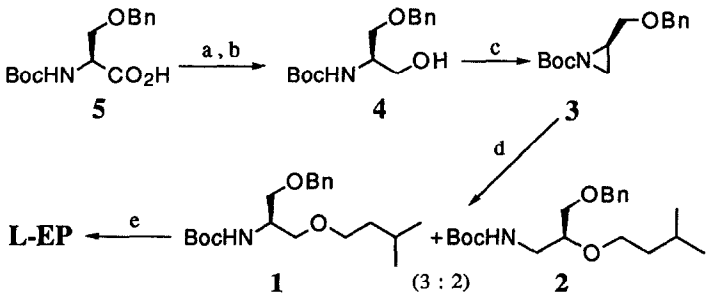


D-EP

Three synthetic methods based on Williamson's ether synthesis,[12,13,14] and a procedure using diazoacetate as the *O*-alkylating agent[15] have been reported for preparation of ether-containing peptide surrogate

units. We have recently developed a synthesis of chiral *N*-Boc-amino ethers under mild conditions via a Lewis acid-catalyzed ring-opening of *N*-acyl aziridines, which can be prepared from chiral *N*-Boc-amino alcohols.[16] As outlined in Scheme I, *N*-Boc-amino alcohol **4** was obtained from *N*-Boc-L-(*O*-benzyl)serine **5** via borohydride reduction of its active ester (98%). Ring closure of **4** to form aziridine **3** was carried out under Mitsunobu's conditions in chloroform (73%). The ring-opening of **3** was carried out in the presence of 3 equivalents of isoamyl alcohol in chloroform to give the desired amino ether **1** and its regioisomer **2** in 3:2 ratio with 0.5% borontrifluoride etherate as catalyst (49%, 32%) or with 0.2 equivalent of zinc triflate as catalyst (31%, 22%). These two isomers were readily separated from each other by flash chromatography, and chiral amino ether **1** was used for peptide synthesis by a stepwise solution phase method.[17] **D-EP**, which has the opposite configuration at the center corresponding to the α-carbon of the serine residue in Kemptide, was prepared similarly starting from the antipodal *N*-Boc-D-(*O*-benzyl)serine.

Scheme I



[a] i-BuOCOCl, Et_3N, THF, -5°; [b] $NaBH_4$; [c] Ph_3P, DEAD; [d] isoamyl alcohol, BF_3 or $Zn(OTf)_2$; [e] Ref. 17.

Holoenzyme (R_2C_2) of bovine heart cAMP-dependent protein kinase (from Sigma) was used in the presence of cAMP to evaluate **L-EP** and **D-EP** as inhibitors against Kemptide phosphorylation.[18] Because these ether-based peptide analogs contain a hydroxyl group, they might serve as phospho group acceptors. In this case, the common protein kinase activity assay utilyzing [γ-^{32}P]-ATP might also lead to ambiguous results, so we chose an HPLC method similar to that reported by Nakanishi et al.[19] for our kinase assay.[20,21] Both **L-EP** and **D-EP** showed inhibition of Kemptide phosphorylation; however, **L-EP** was more potent than **D-EP**. **L-EP** gave IC_{50} values of 380 μM against 10 μM Kemptide and 830 μM against 25 μM Kemptide, while **D-EP** gave IC_{50} values of 620 μM and 5,000 μM, correspondingly. Further characterization of **L-EP** inhibition of **PKA**-catalyzed Kemptide phosphorylation showed an apparent competitive K_i value of 120 μM (Figure 1).[22] Our HPLC method allowed us to determine that **L-EP** was also a substrate of PKA with a K_m value 25-fold higher and a V_{max} value 3.5-fold lower than that of Kemptide. Under the same conditions no phosphorylation product of **D-EP** was detected. Although **L-EP** is a less efficient substrate than Kemptide by 80-fold according to the V_{max}/K_m values, it still binds to the enzyme with a dissociation constant similar to that of Kemptide and its analogs (Table 1).

The behavior of **L-EP** suggests that the amide bond between Ser and Leu in Kemptide is not required for recognition by the enzyme. Thomas et al.[23] had found that the depsipeptide analog of Kemptide with modification of the same amide bond is a substrate of **PKA** with a k_{cat}/K_m value 10 times lower than Kemptide, while the N-methyl analog was a poor substrate with a k_{cat}/K_m value 600 times lower than that of Kemptide. Ether-

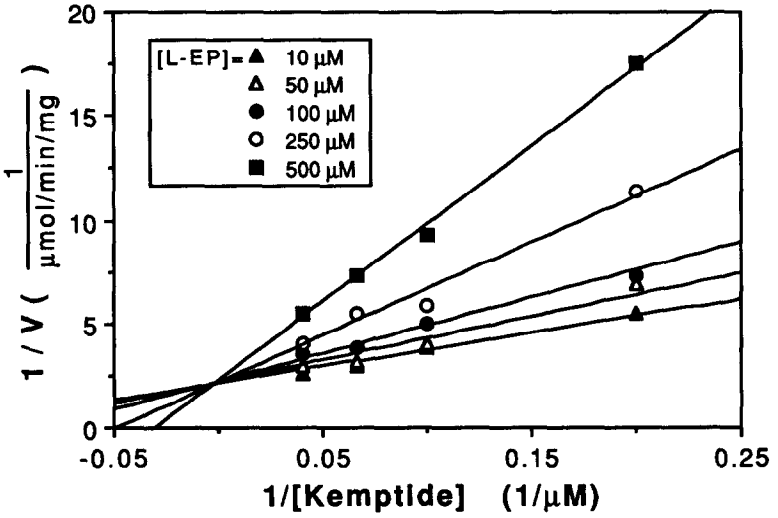
replacement implemented in **L-EP** caused no significant decrease in binding affinity, particularly in light of the fact that Kemptide has a K_d value of 250 μM and the non-subtrate peptide, Leu-Arg-Arg-Ala-Ala-Leu-Gly, has a K_i value of 490 μM.[5] The enzyme also recognizes **L-EP** with a stereospecificity similar to that of Kemptide. It might also be noted that the *C*-terminal glycine was not required for **L-EP** to achieve similar binding to **PKA** (K_i = 120 μM) as Kemptide (K_d =250 μM). This suggests that the flexibility of an ether bond has some advantage in helping peptide analogs such as **L-EP** achieve the optimal fitting conformation at the active site of the enzyme. In addition, **L-EP** is apparently specific for cAMP-dependent protein kinase (**PKA**) as evidenced by its inability to compete for histone phosphorylation catalyzed by calcium dependent protein kinase (**PKC**) at concentrations higher than 1 mM.[24] In summary, we have found a new lead structure for developing **PKA** inhibitors, the finding of this study also provides a convincing rational basis for designing other ether-based peptide antagonists of protein kinases including those with modification of other non-obligatory amide bonds in peptide substrate sequences.

Table 1. Peptide-based ligands of PKA.

Peptide ligand	K_d / K_i (μM)	K_m[a] (μM)	V_{max}[a] (μmol/min/mg)
LRRA—**Ser-Leu-Gly** (Kemptide)	250[b]	4.6 ± 0.5	0.380 ± 0.030
LRRA—**SerΨ[CH$_2$O]isoamyl** (L-EP)	120	108 ± 14	0.107 ± 0.015
LRRA—**Ala-Leu-Gly**	490[b]		
LRRA—**Cys(Npys)-Leu-Gly**	40[c]		
LRRA—**Phe(*p*-Bz)-Leu-Gly**	110[d]		

[a] Values from this study are the average of three experiments. [b] Ref. 5. [c] Ref. 6. [d] Ref. 8.

Figure 1. Lineweaver-Burk plots of L-EP inhibition.



Acknowledgment: We are grateful to the American Heart Association, New York State Affiliate for support of this research, to Dr. Kentaro Murakami for performing the PKC assay and to Dr. Brenda Wilson for helpful comments.

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17. We have applied the frequently used strategy in solid phase synthesis, mainly excess of acylating agents (symmetrical Boc-amino acid anhydride or Boc-amino acid-OBt active ester) and short coupling time (less than 1 hr), in our solution phase method. Starting from **1**, deprotection was carried out in TFA on ice for 30 min, the solvent was removed by rotary evaporation. The amount of excess TFA was further reduced by evaporating with hexane or EtOAc or by gel filtration on an LH-20 column in MeOH. The TFA salt of the deprotected amine was dissolved in DMF and treated with an excess amount of triethylamine (5 to 10 eq.) on ice. This was immediately followed by addition of the acylating reagent (2 to 3 eq.). TLC analysis indicated the coupling was usually completed in less than 30 minutes. Product obtained after each coupling was purified by flash chromatography on silica gel with MeOH/EtOAc as the mobile phase. Desired peptides were obtained with yields of 63%, 91%, 86%, 89% for four consecutive coupling steps. The final deprotection was carried out by hydrogenolysis in HOAc with Pd-C as catalyst (60% after subsequent HPLC purification on a Vydax C_4-column). 300 MHz ^1H NMR spectrum of **L-EP** was consistent with that anticipated for the desired peptide.
18. The phospho group transfer was carried out in 50 mM MOPS buffer at pH 7.0, containing 2 mM ATP, 150 μM cAMP, 2 mg/mL BSA, 200 μM DTT, 10 mM $MgCl_2$, 150 mM KCl, and various amount of Kemptide and/or inhibitor at 30°C.
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20. The extent of phospho group transfer was determined by the peak area ratio between phospho-peptide and substrate peptide recorded at 210 nm. The peak area ratio was calculated from the values of H x $W_{1/2}$, where H is the peak height and $W_{1/2}$ is the width at half peak height.
21. Phospho derivative of **L-EP** was purified by HPLC and its authenticity was confirmed by Plasma Desorption Ionization Mass Spectrometry: M/Z (M+H) = 658.4 for **L-EP** and M/Z (M+H) = 739.2 for phospho **L-EP**.
22. The K_i value was determined by solving $v = V_{max}/(1+(1+[I]/K_i)[S]/K_m)$ using a least square method.
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(*Received in USA* 16 *November* 1993; *accepted* 17 *December* 1993)