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PROLYL ENDOPEPTIDASE INHIBITORS[1] : *N*-ACYL DERIVATIVES OF *L*-THIOPROLINE-PYRROLIDINE

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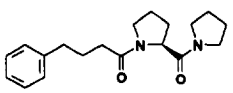
ABSTRACT : *A series of* N-*acyl derivatives of thioproline-pyrrolidine was prepared conventionally and evaluated in vitro against rat brain prolyl endopeptidase (PEP). It is argued on the basis of pharmacophore plots that an optimum chain length exists as a result of increased population of pharmacophore space and decreased solubility on increasing the alkyl chain length.* © 1997 Elsevier Science Ltd.

Prolyl endopeptidase (EC 3.4.21.26), *also known as prolyl oligopeptidase,* is widely distributed in mammals and can be purified from various organs including brain[2]. This enzyme is found both in cytosolic and membrane bound forms[3] and plays a pivotal role in the breakdown of proline-containing neuropeptides related to learning and memory functions[4, 5]. It is also believed to be involved in amyloid genesis[6]. This role, however, is still disputed. On the basis of the above observations, a novel potential mechanism arose for preventing and/or curing amnesia by disclosing the peptidergic neuronal imbalance. Therefore, PEP inhibitors are expected to have therapeutic value for treating progressive memory deficits and cognitive dysfunction related to ageing and neurodegenerative diseases of the central nervous system, such as Alzheimer's disease.

A decade ago Yoshimoto demonstrated[7] that PEP inhibitors e.g. **1** can ameliorate the scopolamine induced memory loss in rats. Several attempts have been made[8, 9] to strengthen these favourable properties. More recently JTP-4819, a specific and strong PEP inhibitor has been reported[10, 11] to potentiate neuropeptide function while enhancing learning and memory, most probably by increasing acetylcholine release.

Previous structure-activity relationship (SAR) investigations[12, 13] have revealed that large hydrophobic residues at the *N*-terminal end of the dipeptide inhibitors greatly improve their potency. Nomura[14] tentatively suggested three binding pockets for PEP. Recently Fehér et. al.[1] have proposed a three-point pharmacophore model that includes two hydrogen bond acceptor groups and a lipophilic centre. In this model, the conformation of the molecule can be described[15] as a U-shaped one. It is important that this pharmacophore criterion was fulfilled[1] not only by 4-phenylbutyryl **1**[9, 13] but also by the octanoyl derivative **2**[9].



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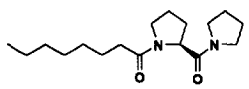

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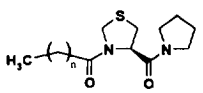

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As a consequence, the aim of present study was to examine structurally close analogues of **2** with different lengths of alkyl chain (**3**) including long chain fatty acids. **Scheme 1** outlines the procedures used to obtain the proline derivatives **3**[16] included in this study. The key intermediate **4**, an *L*-thioproline-pyrrolidine

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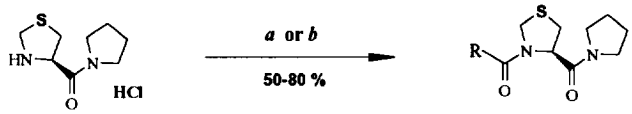
required for the synthesis of **3**, was obtained by a route previously described[13]. The standard method of synthesis involves the treatment of **4** with the appropriate acyl chlorides to produce **3**. Alternatively, several compounds were obtained by utilising the mixed anhydride method starting from the corresponding acids **6**.



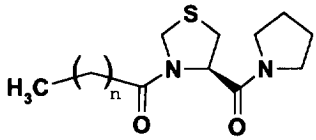
4 **3**

SCHEME 1: (a) R-COCl **5**, Et_3N, $CHCl_3$. rt (b) R-COOH **6**, pivaloyl chloride, Et_3N, $CHCl_3$, 0 °C

The compounds listed in the **Tables 1** and **2** were evaluated for their inhibitory activity against rat brain PEP. The enzyme preparation and the assay procedure[17] were performed with modifications of the method described by Atack[18].

Table 1. Effect of modifications at the alkyl chain length on PEP inhibitory activities*

Compound	n	IC_{50}[nM]		Compound	n	IC_{50}[nM]
3a	**2**	**167**		**3h**	**9**	**42**
3b	**3**	**37**		**3i**	**10**	**46**
3c	**4**	**37**		**3i**	**12**	**80**
3d	**5**	**17**		**3k**	**14**	**216**
3e	**6**	**11**		**3l**	**16**	**904**
3f	**7**	**38**		**3m**	**18**	**1157**
3g	**8**	**38**				



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* *In comparison, molecules 1 and 2 have IC_{50} values of 30 and 20 [nM] determined with the method described in ref. 17.*

On replacing a methylene group in **2** with a sulphur atom, thioproline **3e**, a slight increase in the inhibitory potency (IC_{50} = 11 nM) was observed, in accordance with literature data[19]. Subsequently we investigated the alkyl chain length dependence of the activity. Analogues having a short alkyl chain (n = 2) showed low activity against PEP. This can be explained by the fact that an insufficiently long alkyl group has reduced flexibility and, as a result, the molecule cannot assume a U-shape and the desired pharmacophore conformation at low energies. Molecules with longer alkyl chain length (n= 3-10) showed higher in vitro potency, all in the same range of IC_{50} less than 50 nM. The optimum chain length seems to be at molecule **3e** with n = 6.

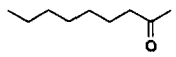
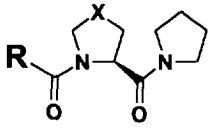
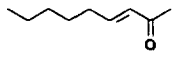
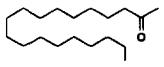
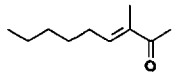
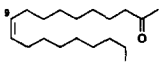
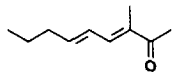
Further increase in the length of the side chain leads to a dramatic reduction of potency. It seems that there are two major factors that may account for our findings. On the one hand, molecules with alkyl chains of different lengths might be unable to adopt the conformation necessary for effective inhibition of PEP. On the other hand, the decreased solubility on increasing the length of the alkyl chain may lead to diminished activity. This may arise when the sample is not fully dissolved in the test mixture, in which case the observed Hill plot is shifted towards lower concentrations, i.e. a lower apparent activity is observed. In addition, it was thought

that the steric bulkiness of long straight alkyl chains might simply create an unfavourable steric clash, preventing the inhibitor molecule to fit into the proposed hydrophobic pocket of the enzyme.

In order to study the effect of differently sized alkyl chains on the pharmacophore geometries that can be adopted by the molecule, the method of pharmacophore plots was applied[20]. In this approach the possible conformations of the molecules are generated and the distances of pharmacophore centres are monitored. It was found that no region of pharmacophore space exists that is only populated by molecules with shorter chains. This proves that on the basis of the pharmacophore hypothesis alone, molecules with bulkier side chains should have increased inhibitory activity. In general, increased conformational flexibility could lead to low population of the conformers that may represent the pharmacophores. This, however, is not the case here, as the spatially less defined nature of the liphophilic centre implies that many conformers of the flexible molecules can fit the required pharmacophore.

In order to investigate the importance of folded conformations, we introduced unsaturated bond(s) into the alkyl chain (see **Table 2.**) These more rigid analogues[21] **7a, 7b** were substantially less active than the corresponding parent compound **2** and the trend is well pronounced. Making the side chain more rigid (as in **7c**) leads to a further decrease in activity. Moreover when a *cis* double-bond (**3n**) was introduced into the side chain of the weak inhibitor **3l** the activity increased dramatically.

Table 2. Conformational side chain flexibility and the PEP inhibitory activities.

N°	RCO; X=CH_2	IC_{50}[nM]		N°	RCO; X=S	IC_{50}[nM]
2		20				
7a		34		3l		904
7b		73				
			7 [21]	3n		104
7c		207				

In conclusion, there appear to be two major factors affecting the activity of alkyl-substituted (thio)prolyl pyrrolidines with side chains of different lengths. An increase in activity as a result of a greater proportion of the molecules being able to assume the required folded pharmacophore conformation and a decrease on account of higher lipophilicity. As a result an optimum chain length for activity is expected and has indeed been found (n = 6). It is anticipated that on exceeding a certain size, the alkyl chain would sterically fit less and less into the binding pocket of the enzyme. This effect, however, is parallel to the possible decrease in observed activity as a result of poor solubility and cannot be distinguished from that.

This study failed to reveal any outstanding candidate for development as a prolyl endopeptidase inhibitor. Nonetheless, it provided some insights regarding the effects of structure modifications on the activity of PEP inhibitors that may help to design more potent analogues[22].

References and Notes

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16. Satisfactory data from ^{1}H-NMR, MS and elemental analysis were obtained for all new compounds using chromatographically homogeneous samples.
17. After removal of the cerebellum whole brain of male rats (Sprague Dawley,180-200g) were homogenised in 0.1 M Tris-HCl, 1 mM EDTA buffer, pH = 7.5. The homogenate was centrifuged and the 40000xg supernatant was used at 300 times final dilution in the reaction mixture. Enzyme reaction was performed at room temperature for 15 minutes in the presence of 62.5 μM *Z*-glycyl-prolyl-7-amino-4-methyl-coumarin as a highly specific synthetic substrate of PEP. The inhibitory effects of the compounds was tested at 100 to 0.001 nM inhibitor concentration. The formation of 7-amino-4-methyl-coumarin was detected spectrofluorometrically at 370 nm exitation and 440 nm emission wavelength. The concentrations of the compounds producing 50% inhibition (IC_{50}) were calculated by curve fitting of the % inhibition versus inhibitor concentration (M) using the Hill equation.
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