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PROCTOLIN AND RELATED *N*-METHYLATED PENTAPEPTIDES SELECTIVELY CONTRACT LOCUST FOREGUT BUT NOT RAT ILEUM

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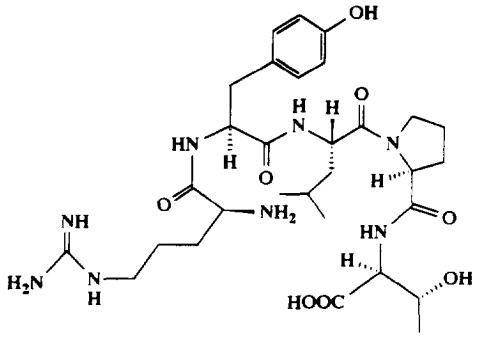
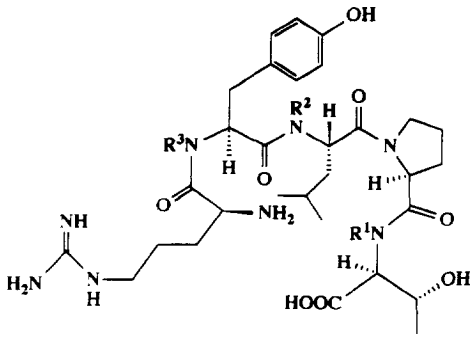
Abstract: Proctolin contracts locust, but contrary to a previous report, not rat gut tissue, in a dose-dependent manner. [*N*-Me-Thr]- and [*N*-Me-Leu]-, but not [*N*-Me-Tyr]-proctolin were also agonists on locust foregut. We propose that proctolin contains a *trans*-Pro and exists in a cyclic conformation based upon solution NMR studies.

The modified peptides of Konopinska *et al.*[1,2] and Gray *et al.*[3] demonstrate that proctolin (**1**) is a lead compound for the design of highly modified peptides and peptoids which may have metabolic stability. In this *Letter*, we report the results of our experiments to characterise pharmacologically and spectroscopically proctolin (**1**) using bioassay techniques with tissues isolated from locust (*Schistocerca gregaria*) foregut and rat ileum. We also report the effects of three *N*-methylated proctolin analogues on both of these tissues. We propose that proctolin (**1**) exists in a cyclic conformation, an inverse γ-turn as evidenced by ^{1}H NMR spectroscopy.

The interactions of peptides and other small molecules with invertebrate neurotransmitter receptors is being studied, in detail, as a possible mode for the selective design of pest control agents[4-6]. We are investigating the agonist-receptor interactions of the pentapeptide proctolin (H.Arg.Tyr.Leu.Pro.Thr.OH, RYLPT) (**1**) which is potentially selective for invertebrates. Our current research into the structure-activity relationships (SAR) of the proctolin receptor is focused upon the affinity and specificity of the receptor probes as a function of modifications to the peptide backbone. In particular, the possibility of ensuring specificity of interaction by varying the structure of the (peptoid) ligand is both theoretically feasible and practically viable. Thus, modified peptides have been designed recently and synthesized as selective endothelin antagonists[7] and a combined inhibitor of angiotensin converting enzyme and neutral endopeptidase-24.11[8]. Isosteric amide bond replacements have been evaluated in potent HIV protease inhibitors which are peptide mimetics[9]. Linear and cyclic pentapeptide enkephalin analogues, which display exceptional potency and specific pharmacological activities, have been reported recently[10-13]. In order to establish that the pentapeptide proctolin (**1**) is selective for invertebrate receptors, we examined its activity on locust and rat gut tissues. The importance of these experiments is highlighted by a report from Penzlin *et al.*[14] which claimed vertebrate potency for (**1**) at low concentrations.



Proctolin (RYLPT) (**1**)
H.Arg.Tyr.Leu.Pro.Thr.OH



N-Me-Thr-proctolin (**2**) R1=Me; R2=R3=H
N-Me-Leu-proctolin (**3**) R2=Me; R1=R3=H
N-Me-Tyr-proctolin (**4**) R3=Me; R1=R2=H

Foreguts (oesophagus to proventriculus), isolated from *S. gregaria*, were incubated in Clarke Insect Ringer[3] at 18±2°C for 20 min prior to constructing dose response curves for proctolin (**1**), [*N*-Me-Thr]- (**2**), [*N*-Me-Leu]- (**3**) and [*N*-Me-Tyr]-proctolin (**4**) at concentrations ranging from 10 nM to 100 μM, using a 6 min cycle with 2 washes. Proctolin (**1**) caused dose-dependent tissue contraction at concentrations ranging from 10 nM to 5 μM with a maximum response at 1 μM (Fig. 1a). [*N*-Me-Tyr]-Proctolin (**4**, Fig. 1b) was without agonist activity on this tissue. [*N*-Me-Leu]-Proctolin (**3**, Fig. 1c) and [*N*-Me-Thr]-proctolin (**2**, Fig. 1d) caused maximal tissue contractions equivalent to 52% and 78% of the proctolin maximum at 80 μM and 2 μM respectively.

Fig. 1

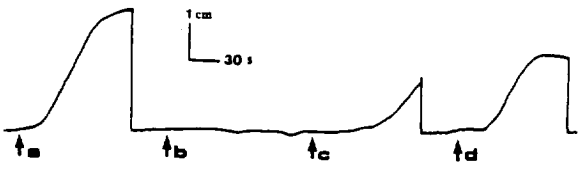
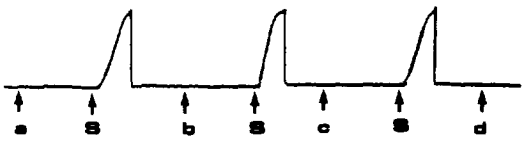


Fig. 2



Rat ileum tissues (3 cm in length taken 20 cm from the pyloroduodenal junction) were incubated in Ringer Tyrodes' solution at 37°C for 20 min prior to testing the agonist effects of proctolin (**1**), three *N*-methylated analogues (**2**), (**3**) and (**4**) as well as serotonin (S) using a 3 min cycle with 1 wash. Proctolin (**1**, Fig. 2a), [*N*-Me-Thr]- (**2**, Fig. 2b), [*N*-Me-Leu]- (**3**, Fig. 2c) and [*N*-Me-Tyr]-proctolin (**4**, Fig. 2d) caused no significant contractile or relaxant effects on this tissue when tested at doses ranging from 10 nM to 1 mM (Fig. 2). However, the tissue showed consistent contractile responses to serotonin (S, 1 μM) before (not shown) and after addition of doses of proctolin (Fig. 2). The failure of proctolin to contract rat ileum preparations, at doses as high as 1 mM, was surprising in view of an earlier report[14] that this peptide caused contraction even at 10 nM. Our demonstration of the selectivity of proctolin for insect rather than mammalian tissue supports the view that proctolin has potential as a lead compound for insecticide design.

Furthermore, neither proctolin nor its analogues had any antagonistic effects on the rat ileum thereby adding to the case that proctolin receptors should be considered as targets for insecticide design. *N*-Methylation of [Leu^3], proctolin analogue (**3**), resulted in significantly reduced activity, an observation which is supported by earlier work[15] on proctolin receptors in *Periplaneta americana*. [*N*-Me-Thr]-Proctolin (**2**) was a more potent agonist than [*N*-Me-Leu]-proctolin (**3**). We conclude from this result that the [Thr^5] amide (NH) plays a relatively insignificant part in the interaction between the parent compound and its receptor, dependent upon our assumption that both ligands bind in a similar conformation and at a shared binding-site. Although [Thr^5] amide can be *N*-methylated with 78% retention of proctolin-like activity, and [Leu^3] amide is sensitive to *N*-methylation (proctolin-like activity reduced to 52%), *N*-methylation of [Tyr^2] (**4**) caused abolition of the myogenic activity displayed by the parent pentapeptide (**1**). The presence of a hydrogen atom on this amide bond may therefore be significant in eliciting the affinity of the ligand at this receptor. Consequently, we have studied the solution conformations of these pentapeptides by high-field NMR.

Observed nOes for proctolin (**1**) and its *N*-methylated derivatives (**2**), (**3**) and (**4**) were defined as strong with the lower-bound distance set at 0.185-0.250 nm, based upon the area of the cross-peaks, in d^6-DMSO at 20°C. There are distance constraints for the sequence Tyr-Leu-Pro-Thr. The presence of short-range nOes, and the absence of long-range inter-residue nOes are further evidence for a γ-turn across 3 residues, i.e. a small turn rather than larger turns across 4 (β) or 5 (α) residues. Similar strong nOes were detected in CD_3OH at -30°C. Thus, in proctolin (**1**), Tyr CHα gave strong nOes with Tyr 2, Tyr 6 and Leu NH, Leu CHα with Leu Me and Pro CH_2, and Pro CHα with Thr NH. Proctolin (**1**) is conformationally constrained with an inverse γ-turn at Thr NH...Leu CO (intramolecular hydrogen bond), from NH temperature dependence studies, whereas (**3**) displays only a weak γ-turn at Thr NH...Leu CO. As a result of these data, together with NH temperature dependence studies and *J* data, we propose that proctolin (**1**) exists in a similar conformation in d^6-DMSO and in CD_3OH; there was one predominant (90%) isomer. The proline amide was shown to have *trans*-geometry following from a strong nOe between CHα (Leu) and CH_2δ (Pro), but not CHα (Pro), and from Cβ (Pro) 27.8 ppm and Cγ (Pro) 24.2 ppm in ^{13}C NMR. This evidence for a *trans*-proline conformation is significant for the solution structure because of the strong conformation-directing properties of Pro. There was little secondary structure detectable for proctolin (**1**), although there is a possible salt bridge between the terminii Arg NH_2 and Thr COO. This proposal is based upon the results of NMR spectra gathered in the presence of increasing concentrations of $LiClO_4$ (0 to 9 mg/0.5 ml d^6-DMSO) where the Arg NH_2 signal moved 0.3 ppm upfield, the largest observed change in chemical shift. *N*-Me-Leu-Proctolin (**3**) displayed comparable ^{1}H NMR data to proctolin (**1**). *N*-Me-Tyr-Proctolin (**4**) displayed similar strong nOes to (**1**), but, in addition, there were strong nOes between Leu CHα...Tyr CHα, Leu NH...Pro CH_2δ, and ThrNH...Pro CH_2β. Temperature coefficient studies of Arg NH_3 were consistent with a cyclic conformation for analogue (**4**), a salt bridge between Arg NH_3 and Thr COO. The amide NHs (Leu and Thr) are orientated outwards towards solvent and exhibit high temperature coefficients.

In this *Letter*, we have shown that proctolin (**1**) is a potent inducer of myogenic activity in gut tissue isolated from an invertebrate species, but has no such activity on an equivalent mammalian tissue. Significantly, [*N*-Me-Tyr]-proctolin (**4**) was devoid of agonist activity, an observation which supports the concept that the [Tyr^2] amide of the pentapeptide plays a significant role in receptor molecular recognition[16,17]. From these studies, we propose that proctolin receptors are targets for insecticide design as proctolin (**1**) is a powerful stimulant of insect muscle as well as occurring in the insect CNS. Furthermore, proctolin shows no significant effects in vertebrates and, consequently, proctolin analogues may disrupt insect physiology and biochemistry without causing toxic effects in mammals; such studies are in progress in our laboratories.

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