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NOVEL INHIBITORS OF ENKEPHALIN-DEGRADING ENZYMES II: N^5-SUBSTITUTED-4-THIOXOHYDANTOIC ACIDS AS AMINOPEPTIDASE INHIBITORS

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Some 2-substituted-(2′-aminophenyl)-4-thioxohydantoic acids (*o*-amino PTC-amino acids) have antinociceptive activity when administered (icv) alone (IC_{50} = 0.04–0.87 μM/animal) and show a striking prolongation of the antinociceptive action of (D-Ala-^{2} D-Leu^5)-enkephalin (DADL) in combination. The effects are thought to be mediated via opioid receptors since they are naloxone-reversible. Although inhibitors of the enkephalin degrading puromycin-insensitive, bestatin-sensitive aminopeptidase (possibly aminopeptidase M) their action is weak (IC_{50} = 32μM leucine, 536μM, glycine) and they might be considered to have a direct antinociceptive effect on opioid receptors. The titled compounds constitute novel 'lead' compounds for the development of potent aminopeptidase M inhibitors.

KEY WORDS: *o*-Amino PTC-amino acids, inhibition, amino peptidase M, Enkephalin-degrading enzymes.

INTRODUCTION

Leu^5- and Met^5-enkephalin are putative neurotransmitters in the central nervous system and are thought to be involved in the control of pain perception[1]. Thus, exogenously administered synthetic enkephalin analogues produce analgesia in rodents[2] and in humans[3]. The enkephalins are degraded mainly by aminopeptidases which cleave the tyrosylglycinyl bond and enkephalinase (neutral endopeptidase EC 3.4.24.11) which cleaves the glycinylphenylalanyl bond[4–6]. Cleavage of the Tyr^1-Gly^2 bond of enkephalins by aminopeptidases was the first identified mode of deactivation of the enkephalins by tissue homogenates[7–10]. Evidence that aminopeptidases may have an *in vivo* role in the degradation of enkephalins came with the synthesis of aminopeptidase-resistant analogues of enkephalin ie. D-Ala^2-Met^5-enkephalin (DAME) which possessed considerably greater potency than Met^5-enkephalin due to the resistance of D-Ala^2-enkephalin analogues to degradation by aminopeptidases[2,11]. Discovery of inhibitors of enkephalin-degrading aminopeptidases, bacitracin,[11] puromycin[12] and bestatin[13] provided further evidence of aminopeptidase involvement in the endogenous metabolism of enkephalins. These inhibitors increased both the potency and duration of action of exogenously administered enkephalins. The observation that puromycin could not significantly protect the integrity of endogenously

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released enkephalin from degradation by brain slices[14,15] was in direct contrast to the ability of bestatin to protect such endogenously released enkephalin[15] and provided evidence that bestatin produces a more complete spectrum of inhibition of enkephalin degradation by aminopeptidases than puromycin. Complementary to this evidence was the observation that bestatin had a significantly greater ability than puromycin to potentiate the activity of enkephalin exogenously administered in mice[15–17].

The first aminopeptidase to be isolated from mammalian brain tissues[18–21] was a soluble arylamidase capable of hydrolysing a variety of substrates, including Met^5- and Leu^5-enkephalin, and which was sensitive to inhibition by both bestatin and puromycin. In a search for an aminopeptidase involved in the synaptic deactivation of endogenously released enkephalins Hersh[22] isolated two membrane bound aminopeptidases from rat brain; a puromycin-sensitive enzyme (aminopeptidase MII) with similar characteristics to the previously identified arylamidase and a puromycin-insensitive enzyme (aminopeptidase MI) with a relatively low affinity for the enkephalins. More recently, studies on the location of enkephalin-degrading aminopeptidase enzymes in specific astroblast, astrocyte and neuronal cell populations[23,24] have shown that an arylamidase resistant to bacitracin but sensitive to both puromycin and bestatin is located in the mitochondria so that it is unlikely that this enzyme plays a role in the deactivation of synaptically released enkephalins. The location of puromycin-sensitive and puromycin-insensitive aminopeptidases on the neuronal and glial cell membrane however is consistent with such a role.

Gros *et al.*,[25] and Matsas *et al.*,[26] subsequently succeeded in purifying enzymes from rat and porcine brains respectively which corresponded to aminopeptidase M (EC 3.4.11.2) (originally[25] considered to be possibly equivalent to MI but later withdrawn[67]).

Aminopeptidase M was found to represent about 10% of the total aminopeptidase activity of rat cerebral membranes. The affinity of this purified puromycin-insensitive but bestatin-sensitive enzyme for the enkephalins was found to be much greater than that originally reported by Hersh[22] for his unpurified enzyme. Hui *et al.*[27,28] have purified an opioid receptor-associated aminopeptidase with very similar properties to aminopeptidase M indicating that this enzyme may have a synaptic role in the deactivation of enkephalins. This is consistent with an earlier report[29] that enkephalin binding to opioid receptors was coupled to subsequent aminopeptidase degradation. The opioid pharmacology of aminopeptidase inhibitors has only been partially investigated, even though 80% of the total enkephalin-hydrolysing activity of rat striatal slices is due to tyrosine release[16]. The hydrolysis of enkephalin released from slice preparations by K^+ depolarisation can be inhibited by bestatin but not puromycin[15]. Bestatin but not puromycin potentiates the antinociceptive effect of Met^5-enkephalin in nociceptive tests *in vivo*[17] whilst both agents have mild antinociceptive activity alone in the mouse abdominal constriction test. However, the antinociceptive activity of bestatin is much greater in combination with the enkephalinase inhibitor thiorphan[17]. Recent studies have demonstrated that bestatin displays similar selectivity to thiorphan in a variety of tests[30], ie. bestatin displays antinociceptive activity in the writhing, hot plate jump and vocalisation tests, but not the tail withdrawal, tail flick or hot plate licking tests. Both thiorphan and bestatin do however display antinociceptive activity in the tail withdrawal, tail flick and hot plate licking tests when they are administered in conjunction with subthreshold doses of morphine or Met^5-enkephalin[30].

The biological activity of aminopeptidase inhibitors though consistent with in-

creased endogenous levels of enkephalin is generally very weak. In contrast, co-administration of bestatin and either captopril or thiorphan produces considerably greater biological activity[31]. An investigation[32] of the ability of bestatin and thiorphan together with Leu^5-enkephalin to produce analgesia in rodents showed significant analgesia in the tail flick test when administered by icv injection but not when administered via cannulae where only thiorphan produced significant analgesia. It was suggested that under normal circumstances enkephalinase, but not aminopeptidase, plays a physiological role in the degradation of enkephalins.

Besides puromycin, bacitracin and bestatin, a number of other natural products have been found to inhibit one or more of the amino-peptidases implicated in endogenous enkephalin degradation eg. the bacterial peptide amastatin[13], the insect peptide proctolin[28], somatostatin and substance P[13], angiotensin I, neurotensin and bradykinin[33]. Semi synthetic derivatives of bestatin are potent aminopeptidase inhibitors[34] and synthetic inhibitors of enkephalin-degrading aminopeptidases based on modified peptide structures incorporating chelating groups, in particular hydroxamic acid moieties,[33] have also been described. Multiple enzyme inhibitors[36–38] have proved to be particularly effective pharmacological agents. Thus, kelatorphan, the most potent multiple inhibitor described[38], inhibits enkephalinase, enkephalinase B and amino-peptidases. It is noteworthy that it has significantly greater antinociceptive activity than a combination of thiorphan and bestatin[39].

In this paper we describe the discovery of 2-substituted-N^5-(2′-aminophenyl)-4-thioxohydantoic acids (*o*-amino PTC-amino acids) as novel inhibitors of a puromycin-insensitive aminopeptidase (possibly aminopeptidase M) with antinociceptive activity when given in conjunction with exogenous DADL administered icv.

MATERIALS

The following agents were employed in the *in vitro* and *in vivo* studies respectively: [tyrosyl-3,5-^{3}H] enkephalin (5-L-leucine), specific activity 50 Ci/mmol (Amersham International), D-Ala^2-D-leu^5-enkephalin acetate (DADL) (Sigma, UK).

METHODS AND RESULTS

Synthesis

^{1}H nmr spectra were determined on solutions in Me_2SO_{d6} (internal Me_4Si) unless otherwise stated with a Perkin-Elmer R32 instrument (90 MHz). Melting points were determined with an Electrothermal capillary instrument and are corrected. Infra-red spectra were determined in KBr discs using a Perkin-Elmer 681 spectrophotometer. Elemental analyses were determined at the School of Pharmacy, University of London.

Difficulties were encountered in the preparation of pure *o*-nitro-and *o*-amino PTC-amino acids due to contamination with the corresponding 5-substituted-3-(2′-nitrophenyl)-2-thioxo-4-imidazolidinone (*o*-nitro PTH-amino acid) or *o*-amino PTH-amino acid respectively, which are readily formed by ring closure at low pH. The *o*-amino PTC-amino acids were isolated as the sodium salts to decrease this source of contamination.

2-Substituted-N^5-(2′-nitrophenyl)-4-thioxohydantoc acids (o-nitro-PTC-amino acids) The DL-amino acid (5 g) was suspended or dissovled in a mixture of pyridine-water (50 ml:100 ml) and the mixture was adjusted to pH 8.6 with sodium hydroxide solution (2N) and maintained at about 40°. A solution of *o*-nitropheny-lisothiocyanate (10 g) in pyridine (50 ml) was added dropwise with vigorous agitation. The mixture was kept within the range pH 8.6–9.0 by the addition of sodium hydroxide solution (2N). The reaction was judged to be complete when the pH of the mixture was unchanged for 3 min. The solution was extracted with benzene (2 × 100 ml) and diethyl ether (2 × 100 ml). The solution was cooled and adjusted to pH 1–2 with hydrochloric acid (2N). The precipitate was collected, dried and then twice recrystallized from ethanol (95%).

Impure N^5-(2′-Nitrophenyl)-4-thioxohydantoic acid (*o*-nitro PTC-glycine) was obtained as yellowish crystals (78.2%) and had mp 144°–148°; [Found: C, 40.19; H, 4.05; N, 15.71; $C_9H_9N_3O_4S$ requires C, 42.36; H, 3.56; N, 16.47%]; ν_{max} (KBr): 3505 (–OH), 3360, 3300 (–NH), 1700 (–COOH), 1600 (aromatic), 1490 (aromatic or $-NO_2$), 1400 (alkyl-CH), 1350 ($-NO_2$), 1290 (–C=S), 740 cm^{-1} (ortho-disubstituted aromatic); ^1H nmr showed δ ($DMSO_{d6}$): 4.24 (d, 2H, $-CH_2$), 7.40–8.20 (m, 5H, aromatic H and –NH), 8.57 (s, 1H, –NH), 11.00 (s, 1H, –COOH); λ_{max} [acetonitrile (12%)-phosphate buffer (0.02M), pH 7.4, 25°]: 243.0 nm. ($\varepsilon = 1.09 \times 10^4$).

The elemental analysis data indicated that *o*-nitro-PTH-glycine existed as an impurity.

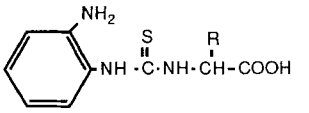
N-[N′-(2′-nitrophenyl)-thiocarbamyl]-pyrrolidine-2-carboxylic acid (*o*-nitro-PTC-proline) was obtained as yellow crystals (69.3%) and had mp 166°–169°; [Found: C, 48.96; H, 4.48; N, 14.38; $C_{12}H_{13}N_3O_4S$ requires C, 48.81; H, 4.44; N, 14.32%]; ν_{max} (KBr): 3290 (–NH), 3000 (broad –OH), 2940 (alkyl–CH) 1700 (–COOH), 1585 (aromatic), 1490 (alkyl-CH), 1380 ($-NO_2$), 1270 (–C=S), 775 cm^{-1} (ortho-disubstituted aromatic); ^{1}Hnmr showed δ ($DMSO_{d6}$): 2.08 (m, 4H, $-CH_2-CH_2$) 3.74 (m, 2H, $-CH_2$), 4.80 (m, 1H, –CH), 7.60 (m, 4H, aromatic H), 9.44 (s, 1H, NH); λ_{max}: [acetonitrile (12%)-phosphate buffer (0.02M), pH 7.4, 25°]: 245.0 nm $\varepsilon = 1.73 \times 10^4$.

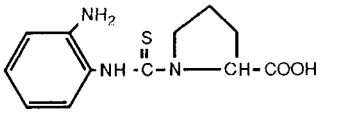
2-Substituted-N^5-[2′-aminophenyl] 4-thioxohydantoic acids (o-amino PTC-amino acids) The 2-substituted-N^5-(2′-nitrophenyl)-4-thioxohydantoic acid (1 g) was suspended in water (20 ml) and finely ground sodium sulphide ($Na_2S\ XH_2O$, 30% Na_2S, 6 g)[40,41] was added with agitation followed by ammonium chloride (0.5 g). The mixture was then heated to 70° and kept at this temperature for 30 min. The mixture was then cooled and the precipitate formed was filtered off. The filtrate was extracted with benzene (2 × 20 ml) and the aqueous layer was then evaporated to dryness under vacuum. The residue was extracted with ethanol (3 × 20 ml), and petroleum ether (80°–100°) was added to the combined extracts until the solution became cloudy. The cloudy solution was kept in the cold overnight, and the precipitate formed was collected and recrystallized from a mixture of ethanol and petroleum ether.

N^5-(2′-Aminophenyl)-4-thioxohydantoic acid sodium salt (*o*-amino-PTC-glycine sodium salt) was obtained as a dark red powder (21.3%) and had mp > 300°; [Found: C, 44.70; H, 4.99; N, 17.30; $C_9H_{10}N_3O_2SNa$ requires C, 43.73; H, 4.09; N, 17.00%]; ν_{max} (KBr): 3560, 3200 (–OH, –NH, $-NH_2$), 3030 (aromatic), 2920 (alkyl –CH), 1650 ($-NH_2$), 1600 ($-COO^-$), 1550 (–NH), 1500 (aromatic), 1470 (–NH or alkyl –CH), 1420 (alkyl –CH), 1300 (–C=S), 740 (cm^{-1}) (ortho-disubstituted aro-

matic); ^{1}H nmr showed δ ($DMSO_{d6}$): 3.71 (d, 2H, $-CH_2$), 4.78 (s, 2H, $-NH_2$), 6.72 (m, 4H, aromatic H), 7.42 (s, 1H, –NH), 9.27 (s, 1H, –NH); λ_{max} [acetonitrile (12%)-phosphate buffer (0.02M), pH 7.4, 25°]: 281.5 nm ($\varepsilon = 3.9 \times 10^3$); TLC (toluene : acetic acid = 2:1) showed one spot with Rf value of 0.61; The Lassaigne's sodium fusion test showed the presence of sulphur.

N-[N′-(2′-aminophenyl)-thiocarbamyl]-pyrrolidine-2-carboxylic acid sodium salt (*o*-Amino-PTC-proline sodium salt) was obtained as a brown powder (19.7%) and had mp > 300° [Found: C, 50.4; H, 4.7; N, 14.6; $C_{12}H_{14}N_3O_2SNa$ requires C, 50.16; H, 4.92; N, 14.62%]; ν_{max} (KBr): 3380 ($-NH_2$), 3240 (–NH), 3030 (aromatic), 2975 (alkyl –CH), 1640 ($-NH_2$), 1590 ($-COO^-$), 1520 (–NH), 1450 (–NH or alkyl –CH), 1400, 1350 (alkyl –CH), 1300 (–C=S), 750 cm^{-1} (ortho-disubstituted aromatic); ^{1}H nmr showed δ ($DMSO_{d6}$): 1.90 (m, 4H, $-CH_2-CH_2-$), 3.52 (m, 2H,$-CH_2$), 4.22 (m, 1H –CH), 4.75 (s, 2H, $-NH_2$), 6.30–7.60 (m, 5H, aromatic H and –NH) (peak at 7.25 disappeared in $DMSO_{d6}$ + D_2O indicating the presence of –NH); λ_{max} [acetonitrile (12%)-phosphate buffer (0.02M), pH 7.4, 25°]: 285.0 nm ($\varepsilon = 3.6 \times 10^3$); TLC (toluene : acetic acid = 2 : 1) showed one spot with Rf value of 0.33. The product was hygroscopic and an aqueous solution gave a precipitate after storage for several days.





R = H ; o - amino PTC - glycine

o - amino PTC - proline

R = $CH_2CH(CH_3)_2$; o - amino PTC - leucine

2-(2′-methylpropyl)-N^5-(2′-aminophenyl)-4-thiohydantoic acid sodium salt (o-amino PTC-Leucine sodium salt). The pure form of *o*-nitro PTC-leucine could not be prepared and the corresponding PTH derivative was used in the reduction reaction.

The (±)5-(2′-methylpropyl)-3-(2′-nitrophenyl)-2-thioxo-4-imidazolidinone[42] (2 g) was suspended in water (20 ml) and sodium suphide (Na_2SXH_2O, 30% Na_2S, 6 g) which had been finely ground was added with agitation and the mixture was then maintained at 25° for 3 h. The insoluble residue was filtered off and ammonium chloride (0.5 g) was added to the filtrate. The mixture was then treated according to the procedure described previously. The product of the reduction was obtained as a brown powder (26.2%) and had mp > 300°; λ_{max} (KBr): 3435 ($-NH_2$), 3340 (–NH), 2960 ($-CH_2$), 1570 ($-COO^-$), 1420 (–NH), 1300 (C=S), 740 cm^{-1} (ortho-disubstituted aromatic); ^{1}H nmr showed δ ($DMSO_{d6}$): 0.93 [d, 6H, $-(CH_3)_2$], 1.65 (m, 3H, $-CH_2$–CH), 4.33 (t, 1H, –CH), 5.11 (s, 2H, $-NH_2$), 6.72 (m, 4H, aromatic H), 10.45 (s, 1H, –NH); TLC (toluene : acetic acid = 2:1) showed three spots and a tailing region with R_f values of 0.00, 0.82, 0.91 and 0.49–0.36 respectively; λ_{max} [acetonitrile (12%)–phosphate buffer (0.02M), pH 7.4, 25°]: 280.8 nm. ($\varepsilon = 2.1 \times 10^3$).

Nociceptive Tests

The mouse tail immersion test[43,44] used in this work followed the method described by Sewell and Spencer[45,46]. This procedure employs a stimulus temperature of 48° and is

sensitive to a wide range of agonist and partial agonist opioid analgesics. "% Antinociceptive effect" was derived from the relationship; area under time-latency response curve for drug treated group/area under time-latency response curve for control.

The abdominal constriction test[47] possesses a high sensitivity towards opioid analgesics though it is largely non specific towards accepted analgesics. The term "% protection" is used to describe the ability of a drug to reduce writhing in the test. The hot plate test[48] was used in certain experiments to expand and confirm the data obtained in the tail immersion test. Antinociceptive activity was determined by calculating the percentage increase in nociceptive reaction latency produced by a drug compared to vehicle treatment.

Mean and standard error (mean $\pm$ SE) were calculated and the level of significance was judged by the "two tailed students t-test". The following symbols were used to denote significance levels in all studies: *($p < 0.05$), **($p < 0.01$) ***($p < 0.001$). The "drug alone" group was usually compared statistically with the "vehicle control" group and the "combination" compared with the DADL alone treated animals.

In the evaluation of IC_{50} values and slopes of dose-response relationships, linear regression analysis was used[49].

Male albino mice of GB_1 variants of an ICI strain weighing 18–22 g (triangle Lab. Newton Abbot, Devon) were fed on a "rat and mouse" breeding diet (Grain Harvesters Wigham, Kent) and allowed tap water ad libitum. Food and water supply were withdrawn two hours prior to experimentation.

Animal house and laboratory conditions were maintained on a 12 h–12 h light-dark cycle, the temperature was maintained at 20 $\pm$ 1° and all experiments were carried out between 11.00 and 17.00 h. The test compounds were dissolved in normal saline. Intracerebraventricular injections (icv) in conscious mice followed the method of Haley and McCormick[50] as modified by Brittain and Handley[51].

Effects of o-amino PTC-amino acids on the antinociceptive activity of (D-Ala²-D-Leu⁵)-enkephalin (DADL) Combinations of *o*-amino PTC-amino acids, (sodium salts), and DADL were administered intracerebroventricularly (icv) to mice in the tail immersion test. The results are shown in Table 1.

TABLE I

% Antinociceptive effects of *o*-amino PTC-amino acids, DADL and their combination[a]

Treatment Compounds	% Antinociceptive Effects			
	Compound Alone (100 μg)	DADL (1.0 μg)	Combination of Compound and DADL	Test Period (min.)
o-Amino-PTC-Gly	27.6 ± 15.0	139.3 ± 12.6	225.9 ± 34.4*[c]	135
o-Amino-PTC-Pro	19.0 ± 14.5	137.8 ± 20.5	278.0 ± 41.3**[c]	135
o-Amino-PTC-Leu	56.5 ± 15.3*[b]	137.8 ± 20.5	274.0 ± 14.9***[c]	135

[a] The antinociceptive activities were determined by the mouse tail immersion test. Drugs were administered by the i.c.v. route. Compound pre-treatment 5 min. before enkephalin injection in the combination group.

[b] Significantly different from vehicle control group.

[c] Significantly different from [D-Ala²-D-Leu⁵] Enk treated group.

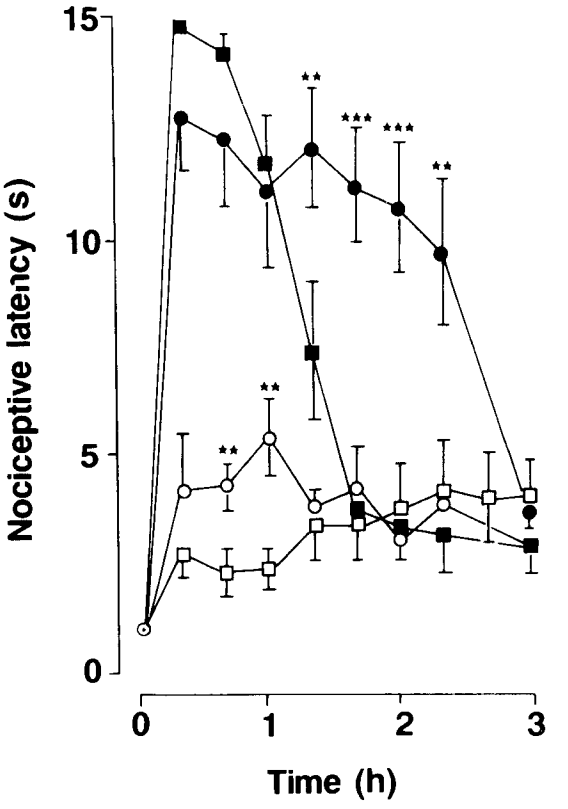


FIGURE 1 Effects of o-amino-PTC-glycine on the analgesic activity of DADL in the mouse tail immersion test. ○ - o-amino-PTC-glycine (100 μg, i.c.v.); ■ - DADL (1 μg, i.c.v.); ● - combination of o-amino-PTC-glycine (100 μg, i.c.v.) and DADL (1 μg, i.c.v.); □ - saline vehicle (10 μl i.c.v.).

The o-amino PTC-amino acids displayed little nocicetive activity alone but enhanced the antinociceptive activity of DADL. o-Amino PTC-glycine did produce significant antinociception 40 to 60 min after administration (Figure 1) but also extended the duration of DADL antinociception up to 3 h. This prolongation of antinociception in combination with DADL was also noted up to 3 h with o-amino PTC-leucine (Figure 2) and more markedly beyond 3 h with o-amino PTC-proline (Figure 3). The antinociceptive potencies of thiorphan and o-amino PTC-proline, alone and in combination with DADL were compared in the mouse tail immersion test (Table II). Thiorphan when administered alone did not show any activity in this test (c.f. Roques *et al.*[52]). The o-amino PTC-proline appears to be more active in producing analgesia alone or potentiating the effects of DADL than thiorphan in this test.

The mouse abdominal constriction test was employed to determine the IC_{50} values of centrally administered thiorphan and the o-amino PTC-amino acids. The results are shown in Figure 4.

It is seen that o-amino PTC-proline (IC_{50} = 11.4 μg/animal). o-amino PTC-glycine (IC_{50} = 120 μg) and o-amino PTC-leucine (IC_{50} = 252 μg) are approximately 25,2

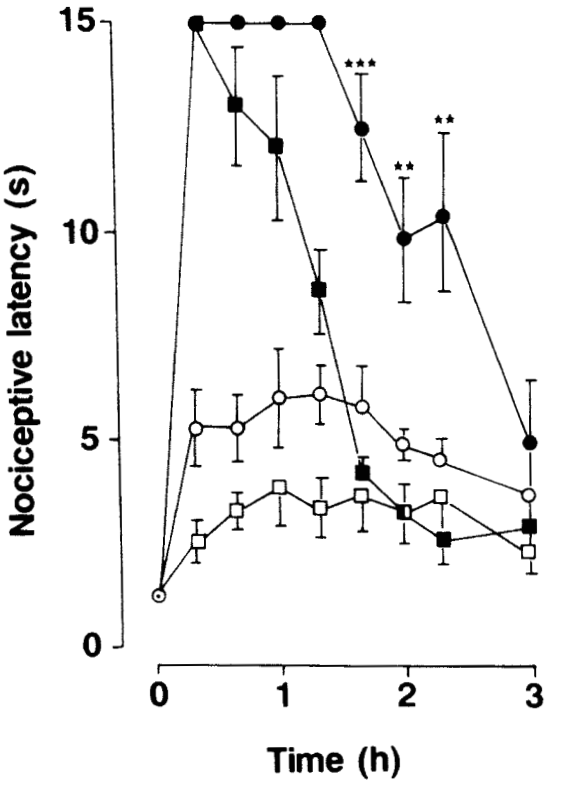


FIGURE 2 Effects of *o*-amino-PTC-leucine on the analgesic activity of DADL in the mouse tail immersion test; ○ - *o*-amino-PTC-leucine (100 μg, i.c.v.); ■ - DADL (1 μg, i.c.v.); ● - combination of o-amino-PTC-leucine (100 μg, i.c.v.) and DADL (1 μg, i.c.v.); □ - saline vehicle (10 μl i.c.v.)

and 1.05 fold more active than than thiorphan (IC_{50} = 265 μg) in protecting against the abdominal constriction response.

Peripheral injection (sc) of the PTC compounds in the mouse abdominal constriction test did not produce any observable protection (results not shown).

The ability of naloxone to antagonise *o*-amino PTC-proline and DADL analgesia was demonstrated in the mouse tail immersion test and is shown in Table III. The antagonistic effect of naloxone on the protective activity of *o*-amino PTC-proline was also shown using the mouse abdominal constriction test. Thus, the IC_{50} value of *o*-amino PTC-proline administered with naloxone was increased from 11.9 μM/animal (0.04 μM) to 58.5 μg/animal (0.2 μM) and to 125.3 μg/animal (0.42 μM) by 1 and 5 mg/kg doses of the antagonist respectively (Figure 5).

Biochemistry

Aminopeptidase 50 μl "particulate fraction" (see preceding paper) was pre-incubated in a shaking water bath for 15 min at 25°C with final concentrations of thiorphan (100 mM added in 10 μl), captopril (1 μM: added in 10 μl) and putative

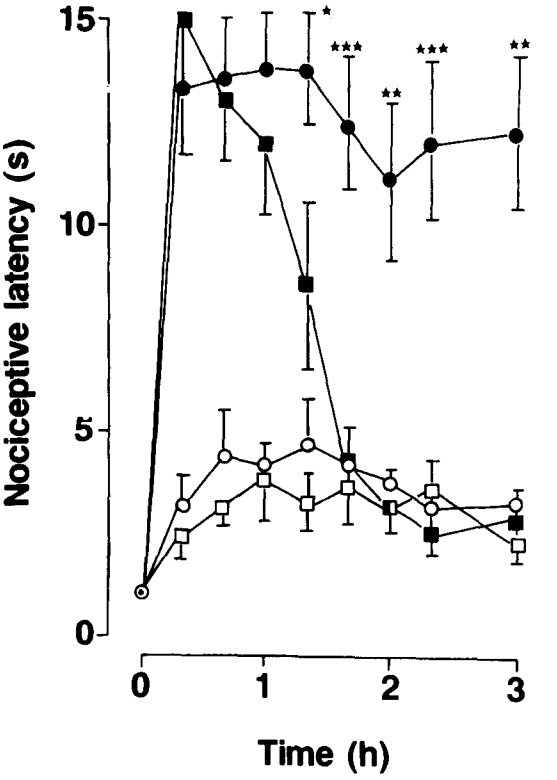


FIGURE 3 Effects of o-amino-PTC-proline on the analgesic activity of DADL in the mouse tail immersion test; ○ - o-amino-PTC proline (100 μg, i.c.v.); ■ - DADL (1 μg, i.c.v.); ● - combination of o-amino-PTC-proline (100 μg, i.c.v.) and DADL (1 μg, i.c.v.); □ - saline vehicle (10 μl i.c.v.)

TABLE II
Effects of thiorphan and o-amino-PTC-proline on DADL-enkephalin antinociception

Compounds Treatment	% Antinociceptive Effects	
	Thiorphan	o-Amino-PTC-proline
Compound Alone (100 μg)	31.9 ± 26.2	132.2 ± 15.0***[a]
DADL alone (1 μg)	114.8 ± 21.3	114.8 ± 21.3
Combination of the compound (100 μg) and DADL (1 μg)	199.5 ± 38.7*[b]	290.0 ± 13.1***[b]
Experiment period (min)	180	180

The antinociceptive activities were determined by the mouse tail immersion test. Drugs were administered by the i.c.v. route. Compounds administered 5 min before DADL.

[a] Significantly different from control group (vehicle treated).

[b] Significantly different from DADL administered alone group.

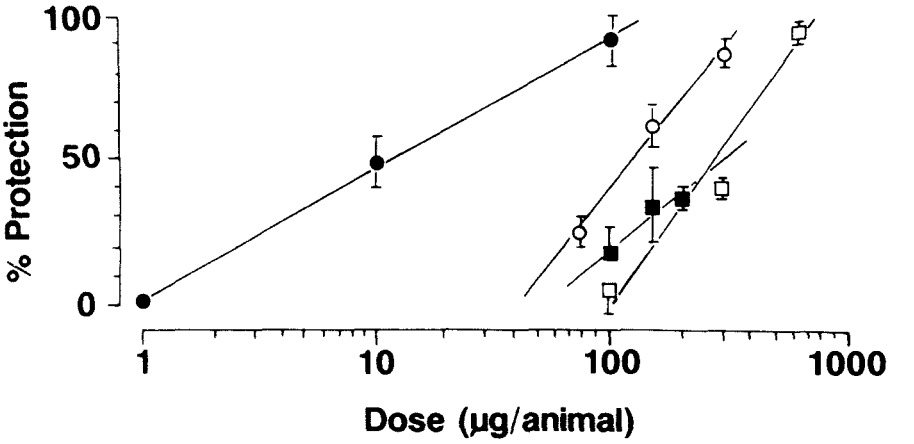


FIGURE 4 Determination of the IC_{50} values for *o*-amino-PTC-proline, *o*-amino-PTC-glycine, *o*-amino-PTC-leucine and thiorphan using mouse abdominal constriction test; ● - *o*-amino-PTC-proline (i.c.v.); ○ - *o*-amino-PTC-glycine (i.c.v.); □ - *o*-amino-PTC-leucine (i.c.v.); ■ - thiorphan (i.c.v.) The tested compound was administered 5 min before the injection of the irritant solution (3% acetic acid).

TABLE III

% Antinociceptive effects of *o*-Amino-PTC-Proline, DADL and their combinations with or without naloxone[a]

	% Antinociceptive Effects		
	DADL (1 µg, i.c.v.)	o-Amino-PTC-Pro (100 µg, i.c.v.)	DADL + *o*-Amino-PTC-Pro
plus saline s.c.	169.3 ± 18.6	74.8 ± 23.3	350.0 ± 12.3
plus naloxone (1 mg/kg, s.c.)	86.4 ± 8.6*	37.7 ± 23.3*	192.8 ± 30.0*
plus saline s.c.	211.0 ± 21.9	134.9 ± 33.2	505.5 ± 16.1
plus naloxone (5 mg/kg, s.c.)	66.5 ± 34.4**	88.9 ± 37.7**	161.0 ± 28.6**

[a] Data obtained in mouse tail immersion test. Naloxone was administered 30 min before the injection of the tested compound. *o*-Amino-PTC-Pro was administered 5 min before DADL.
Significance of difference is indicated from group treated with *o*-amino-PTC-Pro or DADL.

aminopeptidase inhibitors (10 µl) at appropriate concentrations in 50 mM Tris HCl buffer (pH 7.4).

Incubations were started by the addition of ^{3}H-Leu^5-enkephalin (10 µl, 40 nM final concentration) in buffer with a suitable concentration of unlabelled Leu^5-enkephalin (10 µl) in buffer (for K_m determinations) or 10 µl of untreated buffer (IC_{50} determinations). The final incubation volume was 100 µl. Incubations lasted 10 min at 25°C and were terminated by the addition of 10 µl of 3N hydrochloric acid. Suitable blanks were obtained by adding hydrochloric acid prior to incubation.

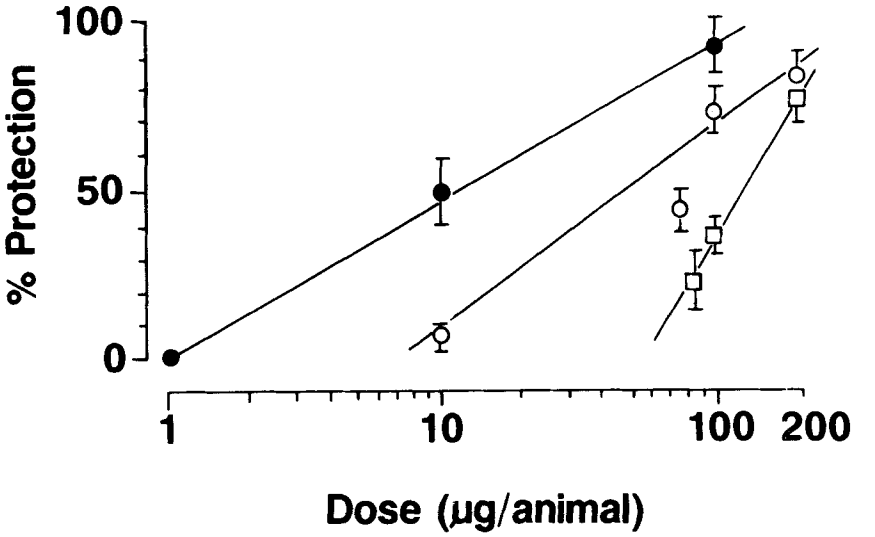


FIGURE 5 Naloxone antagonism of the antinociceptive activity of o-amino-PTC-proline in mouse abdominal constriction test; ● - o-amino-PTC-proline (i.c.v.); ○ - o-amino-PTC-proline (i.c.v.) + - naloxone (1 mg/kg, s.c); □ - o-amino-PTC-proline (i.c.v.) + naloxone (5 mg/kg, s.c). Naloxone was administered 30 min before the injection of o-amino-PTC-proline. o-Amino-PTC-proline was administered 5 min before the irritant solution (3% acetic acid).

After termination of incubations, 10 µl of a mixture of unlabelled Leu^5-enkephalin and its metabolites, Tyr, Tyr-Gly, Tyr-Gly-Gly were added (final concentraiton of each in 120 µl, 0.1–0.5 mg/ml) to the incubation mixture. 50 µl of incubation mixture and suitable reference spots were then applied in 10 µl aliquots to a TLC plate (plastic, silica gel 60 Merck 5748) and thoroughly dried.

Plates were then developed in ethyl acetate:propan-2-ol:water:acetic acid (40:40:19:1), full development taking approximately 2.5 h. After development the plates were removed and allowed to dry, then sprayed with ninhydrin reagent (0.5% in acetone) and heated at 55°C for 15 min. Spots corresponding to Leu^5-enkephalin and its tyrosine containing metabolites were cut out and placed in plastic scintillation vials. To each vial 1 ml of water was added followed by 15 ml of cocktail T (BDH). The vials were sealed, well agitated and then left to stand for 2 h. The vials were then counted in a LKB 1217 Rackbeta liquid scintillation counter and the amount of tritiated compound associated with each vial calculated.

Puromycin-insensitive aminopeptidase 50 µl of the "particulate fraction" was pre-incubated in a shaking water bath for 15 min at 25°C with final concentrations of thiorphan (100 nM; added in 10 µl), captopril (1 µM; added in 10 µl), puromycin (50 µM; added in 10 µl) and putative aminopeptidase inhibitors (10 µl) at appropriate concentrations in 50 mM Tris HCl buffer along with 10 µl ^{3}H-Leu^5-enkephalin, and the previous method for aminopeptidase followed.

Determination of kinetic parameters of enzymes and inhibitors The K_m and V_{max}

values of the neuropeptidases were calcuated from Lineweaver-Burke plots (not shown) for each triplicated experiment and then overall means calculated.

The IC_{50} values for the inhibitors were obtained by plotting log (inhibitory concentration) *vs*% inhibition. The IC_{50} was calculated separately for each individual experiment and then an overall mean calculated for the triplicated experiment.

The K_m and V_{max} values for Leu^5-enkephalin (7–100 μM) hydrolysis by aminopeptidase were 33.4 $\pm$ 4.3 μM and 497 $\pm$ 57 p mol/min/mg protein respectively.

The *o*-amino PTC-amino acids (1 mM) were screened for inhibitory activity against aminopeptidase. The leucine and glycine analogues tested showed a small, but distinct inhibitory effect providing about 10% inhibition of the enzyme. The known inhibitor puromycin had IC_{50} 8.4 $\pm$ 0.9 μM (cf. Schwartz *et al*,[4] 1 μM).

The *o*-amino PTC-amino acids inhibited the puromycin-insensitive aminopeptidase and the IC_{50} values for the leucine and glycine analogues tested were 32.2 $\pm$ 3 μM and 536 $\pm$ 46 μM respectively, Bestatin, a known inhibitor of the enzyme, had IC_{50} = 1.09 $\pm$ 0.17 μM.

DISCUSSION

The *o*-amino PTC-amino acids in the mouse tail immersion test alone produced little significant antinociceptive activity when administered i.c.v. but significantly potentiated the antinociceptive action of DADL (icv) which is resistant to the action of aminopeptidase[54] (Table I, Figures 1–3). The proline analogue was more effective in potentiating the action of DADL (Table II) than thiorphan (icv) a potent inhibitor of enkephalinase[55], in this test. Thiorphan did not produce any significant effect alone, in agreement with Roques *et al.*[52].

In the more sensitive but less specific mouse abdominal constriction test the three *o*-amino PTC-amino acids when administered icv showed significant antinociceptive activity and the glycine, leucine and proline analogues had IC_{50} values of 120 μg/animal, 252 μg/animal and 11.4 μg/animal respectively (Figure 4). They were all more potent than thiorphan (IC_{50} = 265 μg/animal) in this test.

Naloxone, a specific opiate antagonist[56], has been used in conjunction with (D-Ala^2-Met^5)-enkephalin (DAME)[52,57] to assess the involvement of enkephalinergic mechanisms in the observed antinociceptive effects of the combination. Naloxone was shown to antagonise the protection produced by *o*-amino PTC-proline alone and in combination with DADL in the mouse abdominal constriction test (Figure 5) with an increase in the IC_{50} value of 5–10 fold depending on the dose level of naloxone. Naloxone also antagonised the analgesia produced by a similar combination in the mouse tail immersion test (not shown). Taken together these observations suggest that the antinociceptive action of the *o*-amino PTC-amino acids, alone and in combination with DADL, may be mediated via opioid receptors and peptides.

The antinociceptive properties of the enkephalins may be readily demonstrated when they are administered directly into the CNS[52,59]. The analgesia produced is both weak and short lived due to rapid hydrolysis of the enkephalins *in vivo*[7]. A large number of peptidases are found in the CNS many of which are capable of catalysing the hydrolysis of enkephalins, viz. carboxypeptidase A[60], acetylcholinesterase[66], ACE[62], enkephalinase (EC 3.4.24.11)[63], enkephalinase B (DAP III)[64], and a variety of fully or partially characterised aminopeptidases of either a soluble or membrane bound form (see introduction). However, from the distribution of these enzymes in

the brain relative to the synapses where the enkephalins are released only enkephalinase and plasma membrane aminopeptidases are considered responsible for the synaptic deactivation of the enkephalins[24,27,28,29,63,65,67].

On the premise that the *o*-amino PTC-amino acids exerted their antinociceptive activities alone and in combination with DADL by inhibition of enkephalin-degrading enzymes (thus allowing either a build up of enkephalins alone or preservation of DADL responsible for the effect) the compounds were screened at 1 mM concentration as inhibitors of enkephalinase, enkephalinase B (see previous paper for methods) and puromycin-sensitive amino peptidase from the particulate fraction of rat brain homogenates as well as ACE from guinea-pig lung (by Dr. J. Nixon, Roche Products Ltd, Welwyn).

The compounds only exerted a significant inhibitory effect on aminopeptidase and the degree of inhibition was small (10%) at 1 mM concentration. Further investigation revealed that when the particulate fraction was incubated with puromycin an appreciable aminopeptidase activity (about 10%) remained, and this was attributed to a puromycin-insensitive aminopeptidase. This enzyme was inhibited by bestatin, an effective inhibitor of aminopeptidase M[26] (IC_{50} = 1.09 ± 0.17 μM, Leu^5-enkephalin as substrate), confirming the presence of a puromycin-insensitive, bestatin-sensitive aminopeptidase activity in the particulate fraction as the target for the *o*-amino PTC-amino acids. *o*-Amino PTC-leucine and *o*-amino PTC-glycine were weak inhibitors of this enzyme with IC^{50} values of 32.2 ± 2.3 μM, and 536 ± 46 μM respectively.

Although the *o*-amino PTC-amino acids are capable of inhibiting the degradation of enkephalins it is unlikely that their antinociceptive effects originate exclusively from this source since: (1) the inhibitory effect on a small fraction of membrane bound aminopeptidase is small, (2) the antinociceptive activity of DADL is prolonged for an extended period by these compounds yet DADL is relatively stable to animopeptidase action, (3) *o*-amino acids when administered alone show low antinociceptive activity in the tail immersion test yet bestatin (also an inhibitor of aminopeptidase M) does not[65]. It seems likely that the *o*-amino PTC-amino acids constitute a new chemical class of agents with a possibly novel mechanism of action.

The *o*-amino PTC-amino acids described here represent a 'lead' structure for the future development of analgesic drugs or, more importantly, for the development of potent aminopeptidase M inhibitors as tools in the study of enkephalins.

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