J. Enzyme Inhibition, 1991, Vol. 5, pp. 133–149
Reprints available directly from the publisher
Photocopying permitted by license only

NOVEL INHIBITORS OF ENKEPHALIN-DEGRADING ENZYMES III: 4-CARBOXYMETHYLAMINO-4-OXO-3 (PHENYLAMINO) BUTANOIC ACIDS AS ENKEPHALINASE INHIBITORS

A. PATEL, H.J. SMITH and R.D.E. SEWELL*

Welsh School of Pharmacy, U.W.C.C., PO Box 13, Cardiff U.K.

(Received 10 December 1990)

4-Carboxymethylamino-4-oxo-3-(4′-aminophenylamino) butanoic acid (**25**), its ethyl ester (**26**) and the corresponding unsubstituted-aryl analogues (**17**) and (**16**) are fairly potent inhibitors of enkephalinase (neutral endopeptidase; EC 3.4.24.11), K_i = 0.14–0.39 μM, with weak inhibitory potency, K_i = 15–75 μM, towards aminopeptidase MII.

In the mouse abdominal constriction test, the esters (**26**) and (**16**) showed systemic inhibitory (anti-nociceptive) activity with ED_{50} values 62 ± 3.05 and 81 ± 1.74 mg/kg respectively. In the mouse tail immersion test, both (**26**) and (**16**) exhibited antinociceptive activity when administered intracerebroventricularly and (**26**) also exhibited a systemic effect which was only partially reversed by naltrexone. The antinociceptive effect seen with (**26**) reflects its ranking *in vitro* as an inhibitor of enkephalinase (K_i = 0.14 μM) but it is possible that this effect is not totally opioid-mediated. Compounds (**26**) and (**16**) represent the first combined inhibitors of enkephalinase and aminopeptidase MII.

KEY WORDS: Enkephalinase, aminopeptidase MII, enkephalin-degrading enzymes, 4-(carboxymethyl-amino)-4-oxo-3(phenylamino) butanoic acids.

INTRODUCTION

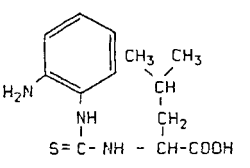
Enkephalins are endogenous pentapeptides thought to function as neurotransmitters or modulators in the central nervous system by stimulating opioid receptors. Their effects are weak and transient due to termination of the enkephalinergic signal by enzymic degradation.[1,2]

Although several peptidases occurring in the CNS are capable of catalysing the hydrolysis of the enkephalins, eg. carboxypeptidase,[3] peptidyl peptidase A[4] (ACE), dipeptidyl aminopeptidase[5] (enkephalinase B), enkephalinase[6] (neutral endopeptidase-24.11) and a variety of fully or partially characterised aminopeptidases of either a soluble[7-10] or membrane bound form,[11-13] aminopeptidase and enkephalinase are considered to account for about 90% of the degradation at the synapse. Enkephalinase is considered to be the more important degradation enzyme due to its location at cerebral synaptic membranes[14] and its coincident distribution and general association with enkephalins in the brain.[15-18] In accord with this view, inhibitors of enkephalinase increase levels of enkephalins in the brain[19] and produce naloxone-reversible analgesia.[20-23] Although several potent inhibitors of enkephalinase are known, e.g., thiorphan,[20]

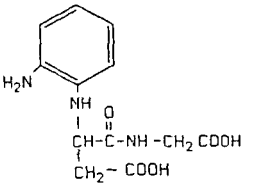
*Correspondence

acetorphan[23] and kelatorphan,[24] they are not orally active as antinociceptive agents. Bioavailability seems to be the overriding factor in production of an antinociceptive effect *in vivo* and SCH 32615 (as the ester SCH 34826)[25] and RU 44004[26] are the only enkephalinase inhibitors to produce an effect on oral absorption. Acetorphan (a prodrug of thiorphan)[27] is active systemically but not orally. It is not possible on the data available to predict the minimum level of *in vitro* enkephalinase inhibitory potency required in an agent to produce a satisfactory *oral* antinociceptic effect (assuming satisfactory absorption on oral administration, stability to first pass liver metabolism and good penetration of the blood-brain barrier). A value of K_i in the low nM region might not be essential for effective oral antinociceptive activity provided the bioavailability of the compound is good and a K_i value in the high nM region may be adequate.

We have shown[28] that some novel 2-substituted-(2′-aminophenyl)-4-thioxohydantoic acids. (**I**; *o*-amino PTC-amino acids) have antinociceptive activity when administered (icv) alone in mice (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) when given in combination. The effects are thought to be mediated via opioid receptors since they are naloxone-reversible. The compounds are inhibitors of the enkephalin-degrading (puromycin-insensitive, bestatin-sensitive) *aminopeptidase* (possibly amino-peptidase M) but their action is weak (IC_{50} = 32 μM, leucine; 536 μM, glycine) so that they might be considered to have a direct antinociceptive effect on opioid receptors.



(**1**)



(**2**)

Using the *o*-amino PTC-amino acid structure as a lead structure we have chemically manipulated the molecule in an attempt to introduce enkephalinase inhibitory activity, i.e., a dual inhibitor. Using molecular graphics (Chem X software/VAX) and modelling on thiorphan, a potent enkephalinase inhibitor (**2**) was suggested as a potential dual inhibitor. Synthesis of (**2**) was unsuccessful but other aryl substituted compounds as well as the unsubstituted-aryl compound were prepared and examined *in vitro* as enkephalinase and aminopeptidase inhibitors.

MATERIALS

[Leu]-enkephalin and D-Ala-D-Leu-enkephalin (DADL) were obtained from Sigma, (^{3}H-tryosyl)-[Leu]-enkephalin (48.5Ci, mol^{-1}) from Amersham International, U.K. Cocktail T from B.D.H., naltrexone from May and Baker, carboxymethylcellulose from ICI and Tween 80 from B.D.H. TLC plates were of the plastic, silica gel type 60 (Merck 5748).

METHODS

Biochemistry

Preparation of a "particulate fraction". A procedure based on the technique used by Hudgin *et al.*[12] as adapted from the original method of Malfroy *et al.*[15] was employed as previously described.[30]

Enkephalinase (EC 3.4.24.11) assay. The particulate fraction (50 μl) was preincubated for 15 min at 25°C with puromycin (10 μl, 1 mM), captopril (10 μl, 10 μM) and putative enkephalinase inhibitors (10 μl), all in Tris-HCl buffer (pH 7.4), in a shaking water bath. Incubations were started by adding ^{3}H[Leu]-enkephalin (10 μl, 0.4 μM in buffer) and 10 μl of buffer for IC_{50} determinations on 10 μl of unlabelled Leu-enkephalin for K_m determinations. Incubations were performed for 15 min at 25°C and terminated by placing the incubation tubes in a boiling water bath for 10 min. After cooling to room temperature 10 μl of a mixture of unlabelled [Leu]-enkephalin (1 mg/ml), Tyr (1 mg/ml), Tyr-Gly (2 mg/ml) and Tyr-Gly-Gly (3 mg/ml) was added to the mixture as a marker and shaken vigorously. The resulting mixture (25 μl) was applied in aliquots (5 μl) onto TLC plates which were then dried. The plates were then developed for 2 h in ethyl acetate : propan-2-ol : water : acetic acid (40 : 40 : 19 : 1), dried, sprayed with ninhydrin reagent (0.5% in ethanol) and heated at 55°C for 15 min. The spots corresponding to [Leu]-enkephalin (R_f = 0.9), Tyr (R_f = 0.7) and Tyr-Gly (R_f = 0.6) were cut out and placed into the same scintillation vial. The spot corresponding to Tyr-Gly-Gly (R_f = 0.5) was cut out and placed in a separate vial. Water (1 ml) and Cocktail T (15 ml) were added to the vials which were then shaken and left to stand for 2 h. The amount of tritiated material in each vial was determined using a LKB1217 Rackbeta liquid scintillation counter.

Aminopeptidase MII assays. The particulate fraction (50 μl) was preincubated for 15 min at 25°C with thiorphan (10 μl, 1 μM) captopril (10 μl, 10 μM) and putative aminopeptidase MII inhibitors (10 μl) all in Tris-HCl buffer (pH 7.4). Incubations were initiated by addition of ^{3}H[Leu]-enkephalin (10 μl, 0.4 μM in buffer) and carried out for 10 min at 25°C. They were heat terminated and after cooling to room temperature 10 μl of a mixture of [Leu]-enkephalin, Tyr, Tyr-Gly and Tyr-Gly-Gly (as described for enkephalinase assay) was added and the incubation mixture was shaken vigorously. The mixture (25 μl) was applied to TLC plates in aliquots (5 μl) and the method of separation and detection of the incubation products followed the method described for the enkephalinase assay. The spots corresponding to [Leu]-enkephalin, Tyr-Gly and Ty-Gly-Gly were cut out and placed in one vial and the spot corresponding to Tyr was placed in a separate vial. Water (1 ml) and Cocktail T (15 ml) were added, the vials were shaken and left to stand for 2 h after which the tritiated content of each vial was determined.

Aminopeptidase M (EC 3.4.11.2) assay. A solution of aminopeptidase M (70 μl, 0.06 units/ml) in Tris-HCl buffer (pH 7.4) was preincubated with the putative aminopeptidase inhibitors (10 μl) for 15 min at 25°C in a shaking water bath. Incubations were initiated by adding ^{3}H[Leu]-enkephalin (10 μl, 0.4 μM) and the mixture incubated for 10 min at 25°C. Incubations were terminated by heating for 10 min. After cooling to room temperature, a mixture of unlabelled [Leu]-enkephalin (1 mg/ml) and

Tyr (2 mg/ml) was added and the incubation mixture was shaken. The incubation mixture (25 μl) was applied to TLC plates and the incubation products separated and detected by the method previously described for the enkephalinase assay. The spots corresponding to [Leu]-enkephalin and Tyr were cut out and placed in separate vials. Water (1 ml) and Cocktail T (15 ml) were added, the vials were shaken, left to stand for 2 h and the tritiated content of each vial determined.

Calculation of percentage inhibition. Non-enzymatic degradation of ^{3}H[Leu] enkephalin during the assay procedure was determined by running a sample containing Tris-HCl buffer (pH 7.4) in place of the particulate fraction or enzyme. This background value for the individual products obtained by non-enzymic hydrolysis was deducted from each value obtained for the individual products in the normal assay procedure to give corrected values. The value for the total conversion of the substrate by the enzyme was obtained from the value of the control sample containing ^{3}H[Leu]-enkephalin and the particulate fraction or enzyme in the absence of the putative enzyme inhibitor. Percentage inhibition of the enzyme by the putative inhibitors was calculated from the corrected disintegration per minute (DPM) values using the equation:

$$\% \text{ inhibition} = 1 - \frac{(\text{DPM Fragment/DPM Total (Test)})}{(\text{DPM Fragment/DPM Total (control)})} \times 100 \quad (1)$$

Determination of the Michaelis–Menten constant (K_m). The Michaelis–Menten constant (K_m) was determined graphically using the Lineweaver–Burke plot.[31] The velocity of the reaction is given by:

$$V = (\text{DPM (Product)})/(\text{DPM (Total)}) \times (S)/(t \times P) \quad (2)$$

where V = velocity (μM/min/mg), DPM = disintegrations per min; S = substrate concentration (μM), t = incubation time (min) and P = protein concentration[32] (mg/ml).

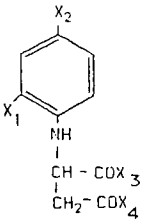
The K_m value for hydrolysis of ^{3}H[Leu]-enkephalin by enkephalinase and aminopeptidase MII was determined graphically by the Lineweaver-Burke method with a substrate concentration range of 5–80 μM for enkephalinase and 5–40 μM for aminopeptidase MII. Each substrate concentration contained 40 nM labelled [Leu]-enkephalin and the remainder consisted of unlabelled [Leu]-enkephalin. Each value of the Lineweaver-Burke plots (not shown) represented the mean ($\pm$ S.E.M.) for four determinations. The K_m values for ^{3}H[Leu]-enkephalin determined by this method were 55 μM for enkephalinase (c.f. 26.1 μM,[30] 22 μM[33] and 9.44 μM[12]) and 16.6 μM (c.f. 33.4 μM,[30] 18.0 μM[34] and 14.86 μM[12]) for aminopeptidase MII.

Preliminary screening for enkephalinase, aminopeptidase MII and aminopeptidase M inhibitory activity. The putative inhibitors were screened for enkephalinase and aminopeptidase MII inhibitory activity at a final concentration of 100 μM. The results for the initial screening are given in Tables I and II. In the screen for aminopeptidase M activity, using a final assay concentration of 1 mM only compound (**18**) showed inhibitory activity at a very low level (16% inhibition).

The IC_{50} values for enkephalinase (EC 3.4.24.11) and aminopeptidase MII inhibition for the different inhibitors studied were determined from plots of % inhibition vs. log concentration (not shown) using a single ^{3}H[Leu]-enkephalin concentration of 40 nM

TABLE I

In vitro Enkephalinase (EC 3.4.24.11, Enk) and Aminopeptidase MII (APMII) inhibitory activity.



Compound	X_1	X_2	X_3	X_4	% Inhibition* Enk	% Inhibition* APMII
3	H	H	NHC_6H_5	NHC_6H_5	10	N/D
4	H	H	OH	OH	64	80
5	H	H	NH_2	OH	63	N/D
6	NO_2	H	OH	OH	83	25
7	NO_2	H	OH	NH_2	62	N/D
8	NO_2	H	NH_2	OH	46	N/D
9	H	NO_2	OH	OH	53	53
10	H	NO_2	OH	NH_2	34	N/D
11	H	NO_2	NH_2	OH	46	N/D
12	H	NH_2	OH	OH	82	80
13	H	NH_2	OH	NH_2	0	N/D
14	H	NH_2	NH_2	OH	68	N/D

*100 μM final concentration. N/D = not determined.

(final concentration) and an inhibitor concentration range of 10 nM to 100 μM. Each point on the % inhibition vs. log concentration plots represented the mean ($\pm$ S.E.M.) of three determinations. Since the substrate concentration used in the assay is in the nanomolar range and the K_m values for both enkephalinase (EC 3.4.24.11) and aminopeptidase MII fall in the micromolar range then K_i and IC_{50} are practically equivalent since $IC_{50} = K_i(1 + S/K_m)$.[35]

Pharmacology

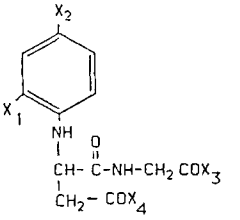
Animal and laboratory conditions: Male albino mice (GB1 variants of an ICI-WSP strain) were maintained at a temperature of 22 $\pm$ 1°C in groups of 12 under normal phase lighting conditions (light 08.00–20.00 h: dark 20.00–08.00 h). All experiments were carried out between 10.00 and 15.00 h at an ambient temperature of 20–24°C. Where possible all drugs were dissolved in sterile sodium chloride (0.9%) or suspended in a mixture of carboxymethylcellulose (0.5%) and Tween 80 (0.2%) in water and all doses are expressed as free acid or base weight.

Nociceptive tests

Mouse abdominal constriction test.[36] In this test, groups of 8 male albino mice (20–25 g) were dosed with either the test compound or vehicle (5.0 ml/kg sc) and left with food and water *ad lib* for 15, 45 or 60 min. Acetic acid (1% v/v 10.0 ml/kg ip) was administered and the number of abdominal constriction responses occurring during 25 min post acetic acid administration was recorded. The mean % protection

TABLE II

In vitro Enkephalinase (EC 3.4.24.11, Enk) and Aminopeptidase MII (APMII) inhibitory activity.



Compound	X_1	X_2	X_3	X_4	% Inhibition† Enk	% Inhibition† APMII	$IC_{50}(\mu M)$‡ Enk*	$IC_{50}(\mu M)$‡ APMII
15	H	H	$OCH_2C_6H_5$	OH	98	N/D	N/D	N/D
16	H	H	OC_2H_5	OH	99	30	0.39 ± 0.015	65 ± 0.13
17	H	H	OH	OH	98	51	0.195 ± 0.002	75 ± 20
18	NO_2	H	OH	OH	97	63	3.5 ± 1.2	80 ± 9.2
19	NO_2	H	OC_2H_5	OH	94	26	8.8 ± 2.29	130 ± 8.4
20	NO_2	H	$OCH_2C_6H_5$	OH	92	N/D	N/D	N/D
21	NO_2	H	OC_2H_5	NH_2	50	N/D	N/D	N/D
22	NO_2	H	OC_2H_5	$OC(CH_3)_3$	73	N/D	N/D	N/D
23	H	NO_2	OH	OH	93	30	22.5 ± 3.5	N/D
24	H	NO_2	OC_2H_5	OH	88	58	N/D	N/D
25	H	NH_2	OH	OH	96	58	0.243 ± 0.03	32 ± 13.1
26	H	NH_2	OC_2H_5	OH	98	65	0.140 ± 0.014	15 ± 1.61

* Equivalent to K_i under the assay conditions.

† 100 μM final concentration.

‡ Mean ± (S.E.M.) for three determinations carried out at 40 nM ^{3}H(Leu] enkephalin concentration.

(±S.E.M.) was given by: % Protection = [1-(Total constrictions (test))/Total constrictions (control))] × 100.

The mouse tail immersion test. This test[37,38] used male albino mice (18–25 g) in groups of 10 and followed the method described by Sewell and Spencer.[39,40] The baseline reaction time for each animal was determined by the immersion of the tail in a water bath maintained at a constant temperature of 48°C by a Grant Instruments circulator. The rection time (latency) was measured to the nearest tenth of a second and up to a 15 s 'cut off' point. The animals were then dosed with vehicle or test compound and the reaction time was determined initially at 10 and 20 min post dosing and then at 20 min intervals up to 180 min post dosing. The "% antinociceptive effect" was calculated from the area under the curve (AUC) of time (min) vs. latency (s) plots i.e., % Antinociceptive effect = [(AUC (test) − AUC (control))/AUC (control)] × 100.

Statistical analysis of comparative antinociceptive effects of different treatment groups were performed using the "two tailed Student's *t*-test" on the data from the mouse abdominal constriction test and with the Mann–Whitney U-test on the data from mouse tail immersion test. The following symbols have been used in Figures and Tables to denote significance levels: *($P < 0.05$), **($P < 0.01$), ***($P < 0.005$) and ****($P < 0.001$).

Test compounds. *In vivo* screening for antinociceptive activity was undertaken on compounds chosen on the basis of their *in vitro* enzyme inhibitory activity (see

Tables I and II) *viz.* the esters (**26**), (**16**) and (**19**), using freshly prepared suspensions for each test as well as the dicarboxylic acid derivatives (**17**), (**25**) and (**18**). During preliminary studies, a fast degradation of compound (**25**) was observed when it was suspended in CMC (0.5%)/Tween 80 (0.2%). Degradation was indicated by a discolouration of the suspension and a lack of antinociceptive activity for doses up to 250 μg/animal/icv.

Synthesis

The compounds tested were prepared in our laboratories and are either described here or elsewhere[29]-4-(Carboxymethylamino)-3-(2′-nitrophenylamino)-4-oxo-butanoic acid (**18**) was prepared by reaction between 2-nitrofluorobenzne and L-aspartic acid to give 2-(2′-nitrophenylamino)butane-1,4-dioic acid which was converted to the anhydride using carbodiimide and then reacted with glycine. The single product obtained was allotted the structure (**18**) rather than the isomeric 4-(carboxymethylamino)-2-(2′-nitrophenylamino)-4-oxo-butanoic acid on the following evidence. L-Asparagine and 2-nitrofluorobenzene gave 3-carbamoyl-2-(2′-nitrophenylamino) propanoic acid which was converted to 4-(ethoxycarbonylmethylamino)-4-oxo-3-(2′-nitrophenylamino) butane carboxamide using ethyl glycinate and DCC. An identical carboxamide was obtained by reacting (**19**), the monethyl ester of (**18**), prepared from the anhydride with ethyl glycinate, with ammonia.

The preferred direction of ring opening of the anhydride is in accord with the previously reported reaction of substituted tetrahydro-2,5-furandiones with nucleophiles.[46] Reaction mainly occurs on the more electrophilic carbonyl-carbon atom adjacent to an electron-withdrawing group although substituent size and solvent are influencing factors.[46]

4-(Carboxymethylamino)-3-(4′-nitrophenylamino)-4-oxo-butanoic acid (**23**) was prepared from 2-(4′-nitrophenylamino)-1,4-dioic acid, formed by reaction between 4-nitrofluorobenzene and aspartic acid, followed by reaction with glycine using DCC. The single product obtained (**23**) was allotted this structure on the basis of the previous observations with the anhydride, 3-(2′-nitrophenylamino) tetrahydro-2,5-furandione, regarding the preferred position for nucleophilic attack since the intermediary tetrahydro-2,5-furandione is expected to be formed in this reaction.[47] This view was confirmed by reaction of 2-(4′-nitrophenylamino)butane-1,4-dioic acid, after treatment with DCC, with ammonia. A mixture of products was obtained from which 3-carbamoyl-3-(4′-nitrophenylamino) propanoic acid was isolated. This acid had different physical and spectral properties from authentic 3-carbamoyl-3-(4′-nitrophenylamino)propanoic acid prepared by reaction of 4-nitrofluorobenzene with asparagine.

Reaction between the dioic acid and ethyl glycinate gave (**24**).

Reduction of the nitro compounds (**23**) and (**24**) with hydrogen and palladium-charcoal as catalyst gave the required amino compounds (**25**) and (**26**) respectively.

4-(Carboxylmethylamino)-4-oxo-3-(phenylamino)butanoic acid (**17**) was prepared from 2-(phenylamino)butane-1,4-dioc acid (obtained by reaction of phenylamine with maleic acid in refluxing acetone) by coupling with glycine after treatment with carbodiimide in acetone. The single product (**17**) obtained was assigned this structure on the basis of previous results regarding the direction of ring opening of the related anhydrides reported here. Furthermore in a similar reaction with ammonia, the dioic acid gave 3-carbamoyl-3-phenylaminopropanoic acid as confirmed by ^{1}H N.m.r. NOE where irradiation of $CONH_2$ or CH separately produced increased absorption

of the CH and NH_2 (as well as NH and Ph) bands respectively. Reaction between the dioic acid and ethyl glycinate gave (**16**).

Experimental

M.p.s. were determined on an Electrothermal instrument and are uncorrected. I.r. spectra on K Br discs were recorded on a Perkin-Elmer 681 spectrophotometer. ^1H n.m.r. spectra in ($^2H_6$)DMSO unless otherwise stated were recorded on a Perkin-Elmer R32 (90 Hmz) spectrometer and are quoted in p.p.m. relative to tetramethylsilane as internal reference. ^{13}C n.m.r. spectra in ($^2H_6$)DMSO were recorded on a Jeol-FX90Q (90 MHz) spectrometer using either tetramethylsilane or ($^2H_6$) dimethylsulphoxide as internal standard. Mass spectra were determined by the SERC Mass Spectrometry Centre at Swansea. Elemental analyses were determined at the School of Pharmacy, London. NOE experiments were conducted by Dr. W.A. Thomas, Roche Products Ltd., Welwyn Garden City, Herts.

Representative spectral data is given although all compounds had satisfactory data. *2-(2'-Nitrophenylamino) butane-1,4-dioic acid.* L-Aspartic acid (10 g, 70 mmol) and sodium hydrogen carbonate (16 g) in water was mixed with 2-nitrofluorobenzene (16 g, 120 mmol) in ethanol (100 ml) and refluxed for 7 h. Saturated sodium chloride solution (100 ml) was then added and the mixture washed with ether (100 ml). The aqueous layer was poured into hydrochloric acid (1 M, 100 ml), the mixture extracted with ethyl acelate (2 × 100 ml) and the extracts washed with sodium chloride solution (10%, 2 × 100 ml), dried ($MgSO_4$) and evaporated to leave *2-(2'-nitrophenylamino)butane-1,4-dioic acid* (12.4 g, 69%), m.p. 174°-176°C. (Found: C, 47.26; H, 3.99; N, 10.99. $C_{10}H_{10}N_2O_6$ requires C, 47.25; H, 3.97; N, 11.02%). ν_{max} 3320, 1720, 1700, 1620, 1520 1350 cm^{-1}. δ_H 13.5–11.5 (2H, bs, COOH), 8.6 (1H, d, J = 9 Hz, NH), 8.08 (1H, d, J = 8 Hz, aromatic), 7.50 (1H, t, J = 8 Hz, aromatic), 7.08 (1H, d, J = 8 Hz, aromatic), 6.70 (1H, t, J = 8 Hz, aromatic), 4.95–4.60 (1H, m, J = 9 Hz, CH–CH_2, 2.98 (2H, d, J = 5 Hz, CH–CH_2. δ_c 172.504 (C = O), 172.254 (C = O), 144.224, 136.854, 132.104, 126.614, 116.304, 114.914 (aromatic), 51.974 (CH), 36.554 (CH_2) ([$^2H_6$] DMSO 39.1).

3-(2'-Nitrophenylamino)tetrahydro-2,5-furandione. 2-(2'-Nitrophenylamino)butane-1,4-dioic acid (2 g, 8 mmol) and 1-(3-dimethylaminopropyl) 3-ethyl carbodiimide hydrochloride (1.7 g, 9 mmol) in dichloromethane (50 ml) were stirred for 1 h. The dichloromethane solution was then washed succesively with water (2 × 25 ml), hydrochloric acid (1.0 M, 25 ml) and water (2 × 25 ml) and then evaporated. The residue was washed with dry diethyl ether (2 × 10 ml) and dried in a vacuum oven to leave *3-(2'-Nitrophenylamino) tetrahydro-2,5-furandione* (0.9 g, 48.4%), m.p. 175°–178°C. ν_{max} 3390, 1870, 1785, 1620, 1580, 1510, 1360 cm^{-1}. δ_H 8.4 (1H, d, J = 9 Hz, NH), 7.55 (1H, t, J = 8 Hz, aromatic) 7.10 (1H, d, J = 8 Hz, aromatic) 6.8 (1H, dd, J = 8 Hz, aromatic) 5.45 (1H, q, J = 9 Hz, CH–CH_2), 3.5 (1H, dd, J_{AX} = 9 Hz, J_{BX} = 9 Hz, CH–CH_2), 3.05 (1H, dd, J_{AX} = 9 Hz, J_{BX} = 9 Hz, J_{AB} = 18 Hz, CH–CH_2), 8.05 (1H, d, J = 8 Hz, aromatic).

4-oxo-4-(carboxymethylamino)-3-(2'-nitrophenylamino)butanoic acid (**18**). The anhydride (5 g, 21 mmol) in acetone (20 ml) was mixed with glycine (1.6 g, 21 mmol) and potassium hydroxide (2.4 g, 42.8 mmol) in water (20 ml) and the mixture stirred overnight. The acetone was then removed, the residue acidified to pH2 with hydrochloric acid (5M) and the mixture extracted with ethyl acetate (2 × 50 ml). The combined extracts were washed with sodium chloride solution (10% w/v, 50 ml), dried ($MgSO_4$) and the solvent removed to give *4-oxo-4(carboxymethylamino)-3-(2'-nitrophenylamino)butanoic acid* as a bright yellow oil (5.0 g, 75.9%). (Found: C, 46.46; H, 4.36; N, 13.36. $C_{12}H_{13}N_3O_7$ requires C 46.30; H, 4.21, N, 13.50%). ν_{max} (neat) 3350, 1720, 1670, 1620, 1510, 1350 cm^{-1}. δ_H 8.7–6.63 (6H, m, aromatic, NH amine, NH amide), 7.65 (1H, m, CH), 3.78 (2H, d, J = 6.0 Hz, NH–CH_2), 2.85 (2H, d, J = 6.0 Hz, CH–CH_2). δ_c ([$^2H_6$] DMSO/$CDCl_3$). 171.649 (C = O), 170.735 (C = O), 169.577 (C = O), 143.863, 136.429, 132.225, 126.192, 116.199, 114.676 (aromatic), 53.072 (CH), 40.947 (CH_2), 37.169 (CH_2) (TMS, 0.0).

4-(Ethoxycarbonylmethylamino)-4-oxo-3-(2'-nitrophenylamino)butanoic acid (***19***). The anhydride (10.5 g, 40 mmol) in tetrahydrofuran (25 ml), glycine ethyl ester hydrochloride (6.2 g, 44 mmol) in the minimum amount of water and triethylamine (9.0 g, 89 mmol) in tetrahydrofuran (5 ml) were mixed and stirred overnight at room temperature. The solvent was then removed and hydrochloric acid (1.0 M, 200 ml) added to the residue. The bright yellow sticky solid which separated out was extracted with ethyl acetate (2 × 100 ml) and the combined extracts washed with sodium chloride (10% w/v, 100 ml) dried ($MgSO_4$) and the solvent removed. The residue was triturated with diethyl ether to give a bright yellow solid which on drying gave 4-(ethoxycarbonylmethylamino)-4-oxo-3-(2'-nitrophenylamino)butanoic acid (11.0 g, 69.6%), m.p. 134–136°C (Found: C, 49. 67; H, 5.08; N, 12.33. $C_{14}H_{17}O_7N_3$ requires C, 49.55; H, 5.05; N, 12.39%) ν_{max} (KBr), 3360, 3300, 1735, 1700, 1660, 1625, 1510, 1350 cm^{-1}. δ_H 12.9–12.1 (1H, broad singlet, COOH), 8.6 (1H, t, J = 5 Hz, NH–CH_2), 8.4 (1H, d, J = 8 Hz, aromatic), 8.1 (1H, d, J = 8 Hz, NH), 7.55 (1H, t, J = 8 Hz, NH), 7.55 (1H, t, J = 8 Hz, aromatic), 7.0 (1H, d, J = 8 Hz, aromatic), 6.75 (1H, t,

J = 8 Hz, aromatic), 4.6 (1H, q, J = 6 Hz, CH-CH_2), 4.05 (2H, q, J = 6 Hz, CH_2CH_3), 3.83 (2H, d, J = 5 Hz, NH-CH_2), 2.75 (2H, d, J = 5 Hz, CH-CH_2), 1.28 (3H, t, J = 7 Hz, CH_2CH_3).

2-(4'-Nitrophenylamino)butane-1,4-dioic acid. 4-Fluorobenzene (16 g, 120 mmol), L-aspartic acid (10 g, 70 mmol) and sodium hydrogen carbonate (20 g) in ethanol — water (1 : 2, 300 ml) were refluxed for 30 h. Saturated sodium chloride solution (100 ml) was then added and the mixture washed with diethyl ether (100 ml). The aqueous layer was poured into hydrochloric acid (1 M, 200 ml) and after adjustment to pH 2.5 was extracted with ethyl acetate (3 × 100 ml). The extract was washed with sodium chloride solution (10%, 2 × 100 ml), dried ($MgSO_4$) and evaporated to give *2-(4'-nitrophenylamino)butane-1,4-dioic acid* (9 g, 58%), m.p. 119–120°C (Found C, 47.45; H, 4.05; N, 11.23. $C_{10}H_{10}N_2O_6$ requires C, 47.25; H, 3.97; N, 11.02%).

4-(Carboxymethylamino)-4-oxo-3-(4'-nitrophenylamino)butanoic acid. (**23**). 2-(4'-Nitrophenylamino) butane-1,4-dioic acid (4.6 g, 18 mmol) and DCC (4.1 g, 20 mmol) in tetrahydrofuran (50 ml) were stirred for 4 h. Glycine (1.36 g, 18.1 mmole) and sodium hydroxide (1.45 g, 36.3 mmole) in water (20 ml) were then added and the mixture stirred overnight, filtered and evaporated. Water (100 ml) was added to the residue and the mixture filtered and the filtrate adjusted to pH2 with hydrochloric acid and extracted with ethyl acetate (2 × 50 ml). The extract was washed with sodium chloride solution (10%, 50 ml), dried ($MgSO_4$) and evaporated. The oily residue was extracted with diethyl ether (3 × 50 ml) and the extract left to stand overnight when yellow needles were deposited *4-(carboxymethylamino)-4-oxo-3-(4'-nitrophenylamino)butanoic acid*, m.p. 153–155°C. (Found: C, 46.03; H, 4.43; N, 13.37. $C_{12}H_{13}N_3O_7$ requires C, 46.30; H, 4.21; N, 13.50%).

4-Carboxymethylamino-4-oxo-3-(1',4'-phenyldiamino)butanoic acid (*25*). The nitrobutanoic acid (**23**) (3.2 g, 10.3 mmol) was dissolved in aqueous ethanol (70%, 25 ml) and shaken with palladium on activated charcoal (10%, 0.3 g) and hydrogen at atmospheric pressure. On completion the mixture was warmed and filtered through celite. The filtrate on standing deposited a solid which was collected, washed with ethanol and dried to give *4-carboxymethylamino-4-oxo-3-(1,4-phenyldiamino)butanoic acid* (0.9 g, 31%), m.p. 186–189°C. (Found: C, 51.05: H, 5.55; N, 14.82: (M^+–H_2O), 263.0895. $C_{12}H_{15}N_3O_5$ requires C, 51.24; H, 5.38; N, 14.94%: (M^+–H_2O). 263.0896.

4-(Ethoxycarbonylmethylamino)-4-oxo-3-(4'-nitrophenylamino)butanoic acid (**24**). To 2-(4'-nitrophenylamino)butane-1,4-dioic acid (6 g, 23.6 mmol) in tetrahydrofuran (100 ml) was added DCC (5.4 g, 26.2 mmol) and the mixture stirred for 4 h. Glycine ethyl ester hydrochloride (3.3 g, 23.6 mmol) and triethylamine (4.8 g, 47.4 mmol) in tetrahydrofuran-water (1 : 1, 20 ml) were then added and the resultant mixture stirred overnight, filtered, evaporated and the residue mixed with sodium hydrogen carbonate solution (10%, 100 ml). After filtration the solution was acidified (pH 2.9) with hydrochloric acid and extracted with ethyl acetate (3 × 50 ml). The extracts were washed with sodium chloride solution (10%, 2 × 50 ml), dried ($MgSO_4$) and evaporated. Diethylether (20 ml) was added to the residue which, on standing, deposited a yellow solid. Crystallisation (ethanol) gave *4-(ethoxycarbonylmethylamino)-4-oxo-3-(4'-nitrophenylamino)butanoic acid* (2.5 g, 31%). m.p. 120–123°C. (Found: C, 49.71; H, 5.03, N, 12.52. $C_{14}H_{17}N_3O_7$ requires C, 49.55; H, 5.05; N, 12.39%).

4-(Ethoxycarbonylmethylamino)-4-oxo-3-(1',4'-phenyldiamino)butanoic acid (**26**). The nitrobutanoic acid (**24**) (2 g, 5.9 mmol) was reduced in the usual manner. The suspension was filtered and the solid obtained suspended in water (50 ml) and hydrochloric acid (5 M, 0.6 ml) added. The mixture was filtered through celite and sodium hydroxide solution (0.12 g, 5 ml) added. Crystals were obtained of *4-(ethoxycarbonylmethylamino)-4-oxo-3-(1',4'-phenyldiamino)butanoic acid* (0.9 g, 49%). m.p. 210–212°C (dec). (Found: C, 54.45; H, 6.20; N, 13.71: M^+, 309.1326. $C_{14}H_{19}N_3O_5$ requires C, 54.36; H, 6.19; N, 13.59%: M^+, 309.1325).

2-(Phenylamino)butane-1,4-dioic acid. Maleic acid (20 g, 200 mmol) and phenylamine (64 g, 690 mmol) in dry acetone (150 ml) were refluxed for 9 h. The solid formed was collected, washed with diethylether (2 × 50 ml), suspended in ethyl acetate (200 ml) and extracted with sodium hydroxide solution (0.5 M, 2 × 100 ml). The aqueous extract was acidified (pH2), washed with dichloromethane (100 ml) and extracted with ethyl acetate (3 × 100 ml). The extract was washed with sodium chloride solution (10%, 2 × 100 ml), dried (Na_2SO_4) and evaporated to give *2-(phenylamino)butane-1,4-dioic acid*, (15.1 g, 42%), m.p. 148–150°C. (Found: C, 57.31, H, 5.23; N, 6.64: M^+, 209.0689. $C_{10}H_{11}NO_4$ requires C, 57.41; H, 5.30; N, 6.70%: M^+, 209.0688.

4-(Carboxymethylamino)-4-oxo-3-(phenylamino)butanoic acid (**17**). 2-(Phenylamino)butane-1,4-dioic acid. (2 g, 9.6 mmol) and 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (2g, 10.6 mmol) in dry acetone (20 ml) were stirred for 1 h. Glycine (0.8 g, 10.7 mmol) and potassium hydroxide (0.6 g) in water (5 ml) were added and the mixture stirred for 4 h. The mixture was then evaporated, hydrochloric acid added and the mixture extracted with ethyl acetate. The extract was washed with sodium chloride solution (10%), dried ($MgSO_4$) and evaporated to leave *4-(carboxymethylamino)-4-oxo-3-(phenylamino) butanoic acid*, (1.1 g, 43%), m.p. 65–70°C (dec). (Found: C, 54.16; H, 5.26; N, 10.41: M^+, 266.1033. $C_{12}H_{14}N_2O_5$ requires C, 54.13; H, 5.30; N, 10.52%: M^+, 266.1035).

4-(Ethoxycarbonylmethylamino)-4-oxo-3-(phenylamino)-butanoic acid (*16*). 2-(Phenylamino)-butan-1,4-dioic acid (5.0 g, 23.9 mmol), DCC (5.4 g, 26.2 mmol) in tetrahydrofuran (30 ml) were placed in a round

bottomed flask and stirred for 1 h. Glycine ethyl ester hydrochloride (3.34 g, 23.9 mmol) and triethylamine (4.84 g, 47.8 mmol) in water (20 ml) were added and the mixture was stirred at room temperature for 4 h. The suspension was filtered and the solvent removed. Hydrochloric acid (10%, 100 ml) was added to the residue and the mixture was filtered and extracted with ethyl acetate (2 × 50 ml). The ethyl acetate fraction was washed with sodium chloride solution (10% w/v) dried (Na_2SO_4) and the solvent volume reduced. On standing a buff coloured solid separated out of 4-(ethoxycarbonylmethylamino)-4-oxo-3-(phenylamino) butanoic acid (1.7 g, 24.16%) m.p. 122–125°C (Found: C, 57.1; H, 6.19; N, 9.61%. M^+, 294.1226; $C_{14}N_{18}O_5N_2$ requires C, 57.13; H, 6.17; N, 9.52%. M^+, 294.1225).

RESULTS

Biochemistry

N-aryl substituted aspartic acid and its amides exhibited low activity towards enkephalinase and amino peptidase MII (Table 1). Amidification of either of the carboxyic acid groups (**5**, **7**, **8** and **14**) had little effect on the inhibitory activity of the corresponding dicarboxylic acid (**4**, **6** and **12** respectively). Furthermore, substitution of NH_2 or NO_2 in the aryl ring of the dicarboxylic acid had only a small effect on potency.

Potent enkephalinase inhibitors were obtained when the N-arylaspartic acids were amidified on C–l with a glycine residue (Table 1). More detailed investigation of the dicarboxylic acids (**18**, **25** and **17**) showed that esterification of the glycinate residue (**19**, **26** and **16** respectively) had little effect on the enkephalinase inhibitory activity. However, whereas substitution of a *p*-amino group in the ring in either the acid (**25**) or ester (**26**) had little effect on the inhibitory potency on the unsubstituted aryl acid (**17**) or ester (**16**), a nitro-substituent in either the ortho- or para-position reduced activity by about 20–100 fold respectively.

All the compounds tested were weak inhibitors of aminopeptidase MII. The most potent enkephalinase inhibitor, the ester (**26**), was also the most potent aminopeptidase inhibitor: with IC_{50} ($K_i = 15 \pm 1.61\,\mu M$.

The most potent combined enkephalinase-aminopeptidase MII inhibitors, (**26**) and (**17**), did not exhibit inhibitory activity towards aminopeptidase M at a concentration of 100 μM.

Pharmacology

Mouse abdominal constriction test

Effects of pretreatment time. Compounds (**26**) and (**16**) showed antinociceptive activity in initial studies of 100 mg/kg sc when administered 15 min prior to the acetic acid (1%) in the mouse abdominal constriction test. Compound (**19**) however displayed no antinociceptive activity under the same conditions possibly due to absorption effects and this was investigated by a time-response study. Compound (**26**) showed a peak antinociceptive effect (63%) at 15 min indicating rapid absorption and a short duration of action. (**16**) showed an effect (54%) at 45 min indicating rapid absorption (Figure 1) but a more sustained duration of action than (**26**). The peak effect (67%) for (**19**) was obtained after 60 min attributable to a slower absorption.

To investigate the effects of absorption further, the antinociceptive activity of (**18**) was determined at two dose levels (100 and 200 mg/kg/sc) at two pretreatment times of 15 and 60 min. In this experiment, parallel log dose-response plots of (**18**) obtained for 15 and 60 min pretreatment time would indicate the importance of absorption

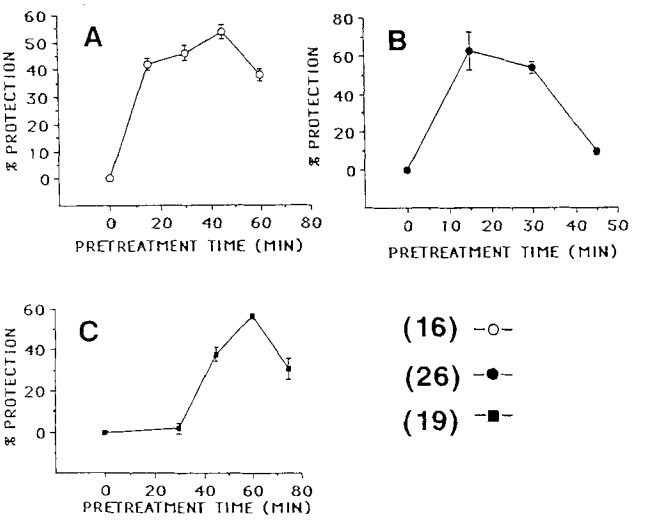


FIGURE 1 Effect of differing pretreatment times on the % protective activity of compounds (**16**) (graph A), (**26**) (graph B) and (**19**) (graph C) administered subcutaneously (100 mg/kg) in the mouse abdominal constriction test.

effects on the antinociceptive activity of (**18**). However this was not found to be the case as the slopes of the two log dose-response plots (15 min = 0.12, 60 min = 0.4) were significantly different (not shown). This result raised the possibility of (**19**) and (**18**) being converted to a more active metabolite *in vivo*. However, the evidence in the above experiments was insufficient to establish (**19**) and (**18**) as prodrugs.

Comparison of routes of administration. The effects of bioavailability on the activities of (**18**) and (**17**) and their respective ethyl esters (**19**) and (**16**) was investigated by determining their antinociceptive activity in the mouse abdominal constriction test using icv and sc routes of administration. The % protection against abdominal constrictions given by (**18**) (51.9 ± 2.45) and (**19**) (36.63 ± 1.96) reflects the order of *in vitro* enzyme inhibitory potency when administered icv (see Figure 2). However, with the subcutaneous route of administration, the order of antinociceptive potency is reversed with the ethyl ester (**19**) (37.5 ± 2.46) showing greater antinociceptive

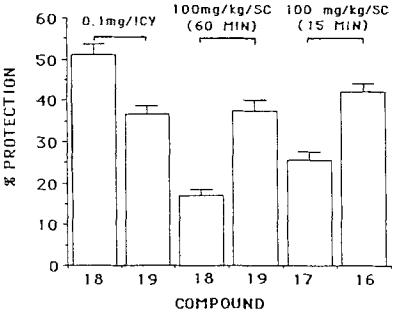


FIGURE 2 Comparison of the intracerebroventricular and subcutaneous routes of administration of compounds (**18**), (**19**), (**17**) and (**16**) in relation to their % protective activity in the mouse abdominal constriction test.

TABLE III
ED_{50} values (sc administration) determined by the mouse abdominal constriction test.

Compound	Peak Pretreatment Time (min)	ED_{50} (mg/kg)	Max % Protection
26	15	62 ± 3.05	66.59 ± 1.45
16	45	81 ± 1.74*	44.0 ± 1.72
17	15	144 ± 4.80	63.91 ± 3.41
19	60	125 ± 5.0	-

*indicates an extrapolated value.

activity than the dicarboxylic acid derivative (**18**) (16.98 ± 1.48). A similar effect was obtained for the ethyl ester (**16**) (42.0 ± 2.26) and dicarboxylic acid (**17**) (25.8 ± 2.02) when administered sc. This may be ascribed to a better bioavailability of the ethyl esters (**19**) and (**16**) possibly due to higher lipophilicity, compared to their dicarboxylic acid derivatives (**18**) and (**17**) when administered subcutaneously.

Determination of ED_{50} values. The ED_{50} values were determined using the mouse abdominal constriction test (Table III). The log dose-response plots for (**26**), (**16**) and (**17**) were parallel at lower doses but all showed maximum antinociceptive ceiling effects at higher doses. The log dose-response plot for (**19**) was not parallel to the plots for (**26**), (**16**) and (**17**) and did not indicate a ceiling effect up to a dose of 200 mg/kg/sc when 75% protection against the writhing response was obtained.

The ED_{50} values obtained for (**19**) (125 ± 5.0 mg/kg), (**26**) (62 ± 3.05 mg/kg) and (**16**) (81 ± 1.74 mg/kg) reflects the rank order of both enkephalinase and aminopeptidase enzyme inhibitory potency obtained from *in vitro* studies (see Table II). The high ED_{50} value for (**17**) (144 ± 4.80) was considered to reflect the poor bioavailability of this compound when administered subcutaneously.

Mouse tail immersion test. Compounds (**18**) (97.38 ± 12.8% antinociceptive activity), (**26**) (180.28 ± 46.31%) and (**16**) ()147.9 ± 16.32%) all showed antinociceptive activity in the mouse tail immersion test (Figure 3) when administered icv and confirmed the order of inhibitory potency obtained in the mouse abdominal constriction test. The duration of action of each compound was indicated by the time (min) vs. latency (s) plots. Compound (**18**) exhibited a slightly delayed onset of antinociceptive activity, showing a peak effect bewtween 80–100 min post dosing (icv) in a manner similar to the effect obtained in the mouse abdominal constriction test (see Figure 3A). The peak antinociceptive effects for (**26**) and (**16**) were at 20 min post dosing (icv) and a sustained antinociceptive activity up to 180 min post dosing was observed for (**26**) (Figures 3B, 3C).

No significant antinociceptive effect (−18.6 ± 11.21%) was observed for (**16**) (100 mg/kg) when administered subcutaneously (Figure 4A). Compound (**16**) (100 mg/kg/sc) in combination with DADL (0.3 ug/animal/icv) showed a significantly greater antinociceptive effect (60.17 ± 9.41) compared to the antinociceptive effect (13.65 ± 9.57) obtained with DADL alone. Thus compound (**16**) potentiated but did not prolong the antinociceptive effect of DADL. The potentiated effect of (**16**) (100 mg/kg/sc) and DADL (0.3 μg/animal/icv) was found to be naltrexone (2 mg/kg.ip) reversible (see Figure 4B).

Compound (**26**) (100 mg/kg/sc) produced a moderate antinociceptive effect (141.24 ± 30.03%) comparable to the antinociceptive effect (151.81 ± 15.75%) of

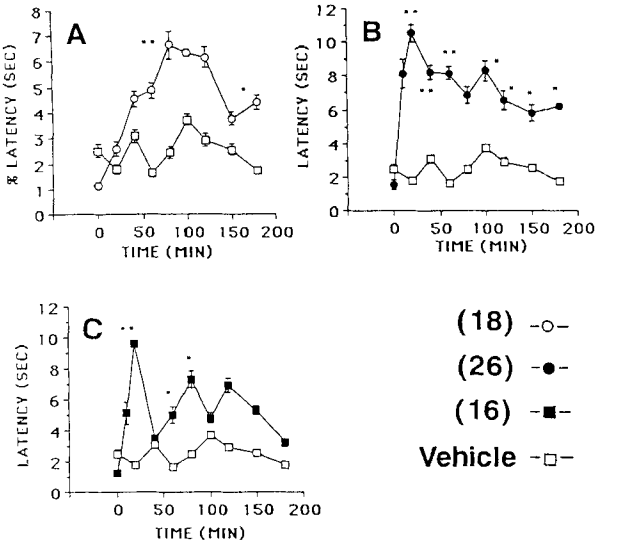


FIGURE 3 Antinociceptive effects of compounds (**18**) (graph A), (**26**) (graph B) and (**16**) (graph C) administered intracerebroventricularly (0.1 mg/animal) in the mouse tail immersion test.

DADL (0.1 μg/animal/icv) (Figure 5A). A significant antinociceptive effect (208.59 ± 21.25%) was obtained with a combination of (**26**) (100 mg/kg/sc) and DADL (0.1 μg/animal/icv), however it was difficult to distinguish the effect as additive or potentiation due to the antinociceptive effect observed for (**26**) when administered alone. The antinociceptive effect (125.77 ± 15.47%) of (**26**) was partially reversed (43.11 ± 8.55%) by naltrexone (2 mg/kg/ip) (see Figure 5B).

DISCUSSION

From a knowledge of the structure of its substrates and inhibitors it has been proposed[20] that the active site of enkephalinase consists of two hydrophobic sites (S_1' and S_2'), a positively charged arginine residue to interact with the C-terminal

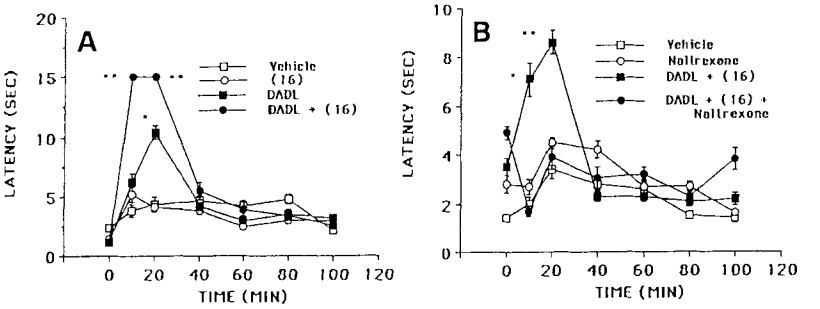


FIGURE 4 *A.* Potentiation of the antinociceptive effects of DADL (0.3 mcg/animal, icv at 0 min) by pretreatment (45 min) with compound (**16**) (100 mg/kg s.c.) in the mouse tail immersion test. *B.* Reversal by naltrexone (2.0 mg/kg i.p.) of the potentiation of DADL antinociception (0.3 mcg/animal i.c.v.-both at time 0 min) by pretreatment with compound (**16**) (100 mg/kg s.c.).

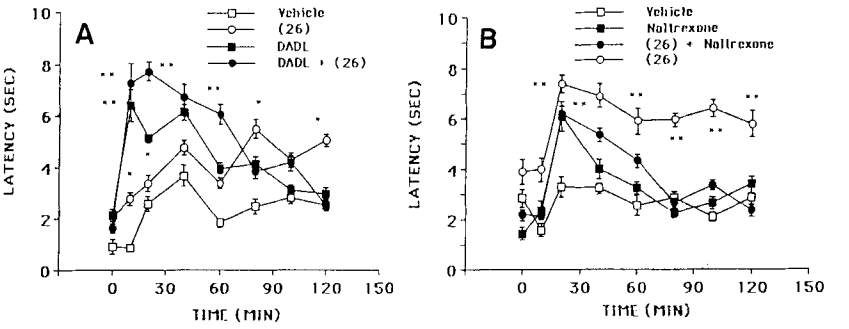


FIGURE 5 *A*. Antinocicpetive effect of compound (**26**) (100 mg/kg/s.c.) alone and in combination with DADL (0.1 mcg/animal i.c.v.) in the mouse tail immersion test. *B*. Reversal by naltrexone (2 mg/kp i.p.) of the antinociceptive effect of compound (**26**) (100 mg/kg s.c.).

carboxyl moiety, a hydrogen donor group interacting with the C-terminal amide linkage and zinc to complex with the scissile amide bond carbonyl oxygen atom. The majority of the inhibitors have the general structure X–P_1'–P_2'–COOH, where X contains the zinc binding ligand and P_1' and P_2' correspond to amino acid residues interacting with the S_1' and S_2' hydrophobic subsites of enkephalinase respectively.[41]

We found that the N-aryl substituted aspartic acids and their monoamide derivatives showed low inhibitory activity towards enkephalinase with little difference in activity between the amide and corresponding carboxylic acid (Table I). Since the spatial difference of the dicarboxylic acid groups in insufficient to span the required distance between the Zn^{++} and guanidinium ion binding sites on the enzyme, as predicted by substrate and inhibitor specificities, it seems likely that either the Zn^{++} binding or guanidinium ion site binds the carboxylate of the inhibitor. This would account for little loss in activity in conversion of the dicarboxylic acid to the monoamide. The guanidinium ion site seems the most likely candidate for binding since substitution of the aryl ring with either NH_2 or NO_2 has little effect on binding which does not indicate a high involvement of the S_1' pocket (see later) but rather less specific binding in the area of the S_2' pocket.

Extension of the N-aryl aspartic acid molecule by amidification in the C–l position with a glycine residue increased the inhibitory potency towards enkephalinase (Table II). This is to be expected since molecular modelling showed a good match of the relevant binding groups of (**2**) and the potent inhibitor thiorphan. Substitution of a *p*-amino group in the aryl ring had little effect on inhibitory potency (c.f. (**25**) and (**17**)) whereas a nitro group in either the ortho (**18**) or para-position (**23**) reduced the activity 18- and 112-fold respectively. These changes would suggest that a specific binding site, S_1', is used by the aryl substituents which imposes steric limitations to the size of the aryl group.

Esterification of the glycinate residue in the dicarboxylic acids (**18**), (**25**) and (**17**) had a marginal effect on activity. This is unexpected since amidification or esterification of the C-terminal carboxylic acid in other groups of inhibitors can lead to a 30 or 6 fold respectively decrease in activity.[42] The pro-drug acetorphan which releases the potent inhibitor thiorphan in the brain has little *in vitro* activity against purified enkephalinase.[43] However, after penetration to the brain it is de-esterified to thiorphan by tissue esterases. It is possible that the esters (**19**), (**26**) and (**16**) are similarly

converted to the more active acids by esterases present in the crude particulate fraction used in this work during the 15 min pre-incubation period. Alternatively, the loss of binding with the guanidinium ion on the enzyme and the carboxylate ion in a salt-linkage could be offset by hydrogen-bonding to the ester carbonyl oxygen atom, the latter strength of binding depending on the relative orientation (i.e., fit) of the ester group which will vary with the nature of the restraints imposed by the nature of the terminal amino acid. The restraints in a glycine residue would be expected to be less than those for other α-substituted amino acid residues.

In the mouse abdominal constriction test, the esters (**26**), (**16**) and (**19**) showed antinociceptive activity when administered subcutaneously, although the effects of (**19**) were delayed due to slower absorption (Figure 1). The ethyl esters (**19**) and (**16**) had better bioavailability profiles than the respective acids (**18**) and (**17**) as determined by comparison of their antinociceptive effects on administration by the icv and sc routes in the abdominal constriction test (Figure 2). The ED_{50} values for (**26**), (**16**) and (**19**) (Table III) were 62 ± 3.05, 81 ± 1.74 and 125 ± 5.0 mg/kg and reflected the rank order of (**26**) > (**16**) > (**19**) as both enkephalinase and amino peptidase MII inhibitors from the *in vitro* studies (Table II).

In the mouse tail immersion test on administration icv the esters (**26**), (**16**) showed a percentage protection effect of 180.28 ± 46.31 and 147.9 ± 16.32 respectively, with a peak effect at 20 min and for (**26**) a sustained antinociceptive effect for 180 min. The acid (**18**) showed reduced activity (97.38 ± 12.8) and a delayed onset of antinociceptive activity with a peak effect after 90 min.

Compound (**16**), when administered sc, potentiated but did not prolong the antinociceptive action of DADL, an effect that was naltrexone reversible and thus opioid-mediated (Figure 4). Compound (**26**) produced a moderate antinociceptive effect alone and significantly increased the antinociceptive effect in the combination with DADL although it was not possible to distinguish this increase as additive or a potentiation due to the extent of antinociceptive activity produced by (**26**) itself (Figure 5A). Moreoever, the antinociceptive effect of (**26**) alone was only partially reversed by naltrexone (Figure 5B). It might be a possibility that the antinociceptive mechanism of (**26**) and (**16**) involves a factor(s) other than enzyme inhibition since the two compounds do not significantly prolong the antinociceptive effects of DADL. This view is supported by the observation that naltrexone only partially reverses the antinociceptive effects of (**26**) (see Figure 5B).

Nociceptive tests with enkephalin-degrading enzyme inhibitors are commonly carried out using the mouse abdominal constriction test and low temperature mouse hot plate test. The mouse tail immersion test is generally not used as the majority of enkephalinase inhibitors including thiorphan[44] and SCH34826[25] have been reported to be inactive in this test. It has been suggested that under the conditions of the tail immersion test, the enkephalin-containing endogenous pain suppression system is inactive hence enkephalinase inhibition is not detected in this test. In contrast, in nociceptive tests where the noxious stimuli is prolonged, as in the mouse abdominal constriction, and low temperature hot plate test, the endogeneous opioid system is activated enabling enkephalinase inhibitors to exhibit antinociceptive effects.[45]

In the data presented, the results for compounds (**19**), (**26**) and (**16**) from the mouse abdominal constriction test, indicate the same rank order of potency as their *in vitro* inhibitory activity for enkephalinase and aminopeptidase MII. Another notable observation is that these compounds also exhibited the same rank order in their antinociceptive effects when administered icv alone in the mouse tail immersion test.

This direct effect has not been reported for other more potent enkephalin-degrading enzyme inhibitors. Furthermore, compound (**26**) uniquely exhibited antinociceptive activity when administered subcutaneously in the mouse tail immersion test, an effect which was only partially reversible by naltrexone. This result is in contrast to that for compound (**16**), which displayed only 3-4 fold less potency for enkephalinase and aminopeptidase MII when compared to (**26**), yet was inactive when administered subcutaneously.

This data raises several questions regarding the effect of these compounds on endogenous opoid-mediated analgesia. Firstly, these compounds are less potent enkephalinase inhibitors in comparison to inhibitors such as thiorphan and kelatorphan and yet they exhibit antinociceptive activity when administered alone in the tail immersion test, an effect not seen with thiorphan.[44] Secondly, all three compounds, (**19**), (**26**) and (**16**), display antinociceptive activity in the mouse tail immersion test, in which it has been proposed that the endogenous enkephalinergic system is inactive or devoid of tone.[45] Finally, it is possible that these compounds mediate their antinociceptive effects through direct action on opioid receptors, in a manner analogous to the narcotic analgesics which are highly active in the mouse tail immersion test.[39]

Although the data presented here does not clearly establish the antinociceptive effects of compounds (**26**) and (**16**) as purely being due to their enkephalim-degrading enzyme inhibitory activity, the compounds are worthy of further investigation since it remains to be established whether their antinociceptive effects in the mouse tail immersion test are due to a direct action on opioid receptors, an event which could lead to the development of novel analgesic compounds. In terms of enkephalin-degrading enzyme inhibitors, compounds (**26**) and (**16**) represent the first combined inhibitors of enkephalinase and aminopeptidase MII, although the aminopeptidase MII inhibitory activity is in the micromolar range. These compounds could be utilised as 'lead' compounds for the development of more potent inhibitors which in turn would be useful as biochemical and pharmacological tools for the investigation of the role of aminopeptidase MII in the degradation of endogenously-released enkephalin.

Acknowledgements

We wish to thank The Royal Pharmaceutical Society for the award of a Scholarship to A.P.

References

1. Meek, J.L., Yang, H.-Y.-T. and Costa, E. (1979) *Neuropharmacol.*, **16,** 151–154.
2. Stine, S.M., Yang, H.-Y.-T. and Costa, E. (1980) *Brain Res.*, **188,** 295–299.
3. Pert, C.B., Pert, A., Chang, J.K. and Fong, B.T.W. (1976) *Science*, **194,** 330–332.
4. Erdos, E.G., Johnson, A.R. and Boyden, N.T. (1978) *Biochem. Pharmacol.*, **27,** 843–848.
5. Gorenstein, C. and Snyder, S.H. (1979) *Life Sci.*, **25,** 2065–2070.
6. Malfroy, B., Swerts, J.P., Guyon, A., Roques, B.P. and Schwartz, J.C. (1978) *Nature (Lond.)*, **276,** 523–526.
7. Geary, L.E., Wiley, K.S., Scott, W.L. and Cohen, M.L. (1982) *J. Pharm. Exp. Ther.*, **221,** 104–111.
8. Hayashi, M. (1978) *J. Biochem.*, **84,** 1363–1372.
9. Hersh, L.B. and McKelvy, J.F. (1981) *J. Neurochem.*, **36,** 171–178.
10. Traficante, L.J., Rotrosen, J., Siekierski, J., Tracer, H. and Gershon, S. (1980) *Life Sci.*, **26,** 1697–1706.
11. Hersh, L.B. (1981) *Biochemistry*, **20,** 2345–2350.
12. Hudgin, R.L., Charleson, S.E., Zimmerman, M., Mumford, R. and Wood, P.L. (1981) *Life Sci.*, **29,** 2593–2601.
13. Hue, K.-S., Wang, Y.-T. and Lajtha, A. (1983) *Biochemistry*, **22,** 1062–1067.

14. De La Baume, S., Patey, G. and Schwartz, J.-C. (1981) *Neurosci.*, **6,** 315–321.
15. Malfroy, B., Swerts, J.P., Roques, B.P. and Schwartz, J.-C. (1978) *Nature* (*Lond.*), **276,** 523–526.
16. Schwartz, J.-C., Malfroy, B. and De La Baume, S. (1981) *Life Sci.*, **29,** 1715–1740.
17. Llorens, C., Malfroy, B., Schwartz, J.-C., Gacel, G., Roques, B.P., Roy, J., Morgat, J.L., Javoy-Agid, F. and Agid, Y. (1982) *J. Neurochem.*, **39,** 1081–1089.
18. Waksman, G., Hamel, E., Bouboutou, R., Besseliever, R., Fournie-Zaluski, M.-C. and Roques, B.P. (1984) *C.R. Acad. Sci.* (*Paris*), **299,** 613–615.
19. Zhang, A.-Z., Yang, H.-Y.T. and Costa, E. (1982) *Neuropharmacol.*, **21,** 625–630.
20. Roques, B.P., Fournie-Zaluski, M.C., Soroca, E., Lecomte, J.M., Malfroy, B., Llorens, C. and Schwartz, J.-C. (1980) *Nature* (*Lond.*), **288,** 286–288.
21. Chipkin, R.E., Latranyi, M.B. and Iorio, L.C. (1982) *Life Sci.*, **31,** 1189–1192.
22. Greenberg, R. and O'Keefe, E.H. (1982) *Life Sci.*, **31,** 1185–1188.
23. Lecomte, J.-M., Costentin, J., Valaiculescu, A., Chaillet, P., Marcais-Collado, H., Llorens-Cortes, C., Leboyer, M. and Schwartz, J.-C. (1986) *J. Pharmacol. Exp. Ther.*, **237,** 937–944.
24. Fournie-Zaluski, M.C., Coulaud, A., Bouboutou, R., Chaillet, P., Devin, J., Waksman, G., Costentin, J. and Roques, B. (1985) *J. Med. Chem.*, **28,** 1158–1169.
25. Chipkin, R.E., Berger, J.G., Billard, W., Iorio, L.C., Chapman, R. and Barnett, A. (1988) *J. Pharmacol. Exp. Ther.*, **245,** 829–838.
26. Petit, R., Munt, P., Vevert, J.P. and Delevallée (1989) *International Narcotics Research Conference*, Quebec, July 9–14, 1989, p-77.
27. Lecomte, J.M., Costentin, J., Valaiculesci, A., Chaillet, P., Llorens-Cortes, C., Leboyer, M. and Scwhartz, J.-C. (1986) *J. Pharmacol. Exp. Ther.*, **237,** 937–944.
28. Yu, Z., Williams, P.S., Smith, H.J., Sewell, R.D.E., Patel, A. and Gonzalez, J.P. (1989) *J. Enz. Inhib.*, **3,** 103–117.
29. Patel, A. (1990) Ph.D. Thesis, University of Wales.
30. Williams, P.S., Sewell, R.D.E., Smith, H.J. and Gonzalez, J.P. (1989) *J. Enz. Inhib.*, **3,** 91–101.
31. Lineweaver, H. and Burke, D. (1934) *J. Amer. Chem. Soc.*, **56,** 658–666.
32. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.*, **193,** 265–275.
33. Fournie-Zaluski, M.C., Perdrisot, R., Gacel, G., Swertz, J.P., Roques, B.P. and Schwartz, J.C. (1979) *Biochem. Biophys. Res. Commun.*, **91,** 130–135.
34. Gros, C., Giros, B. and Schwartz, J.-C. (1985) *Biochemistry*, **24,** 2179–2185.
35. Cheng, Y.-C. and Prusoff, W.H. (1973) *Biochem. Pharmacol.*, **22,** 3099–3108.
36. Taber, R.I. (1974) In *Narcotic Antagonists* (Eds. Braude, M., Harris, C., May, L.B., Smith, J.P. and Villaneal, J.E.) vol. 8. Raven Press; New York.
37. Ben-Bassat, J., Peretz, E. and Sulman, F.G. (1959) *Arch. Intern. Pharmacodyn.*, **122,** 434–447.
38. Grotto, M. and Sulman, F.G. (1967) *Arch. Intern. Pharmacodyn.*, **165,** 152.
39. Sewell, R.D.E. and Spencer, P.S.J. (1976) *Neuropharmacology*, **15,** 683.
40. Sewell, R.D.E. and Spencer, P.S.J. (1976) *Br. J. Pharmacol.*, **51,** 140.
41. Schwartz, J.-C. (1989) In *Design of Enzyme Inhibitors as Drugs* (Eds. Sandler, M. and Smith, H.J.) p. 206, Oxford; Oxford University Press.
42. Llorens, C., Gacel, G., Swertz, J.P., Perdrisot, R., Fournie-Zaluski, M.C., Schwartz, J.-C. and Roques, B.P. (1980) *Biochem. Biophys. Res. Commun.*, **96,** 1710–1716.
43. Lecomte, J.M., Costentin, J., Valaiculesci, A., Chaillet, P., Llorens-Cortes, C., Leboyer, M. and Schwartz, J.-C. (1986) *J. Pharmacol. Exp. Ther.*, **237,** 937–944.
44. Chipkin, R.E., Latranyi, M., Iorio, L.C. and Barnett, A. (1982) *Eur. J. Pharmacol.*, **83,** 283–288.
45. Chipkin, R.E. (1986) *Drugs of the Future*, **11,** 593–606.
46. Emery, A.R. and Gold, V.J. (1950) *J. Chem. Soc.*, **2,** 1455–1460.
47. Gish, D.T., Katsoyannis, P.G., Hess, G.P. and Stedman, R.J. (1956) *J. Amer. Chem. Soc.*, **78,** 5954.