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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Compounds against inflammation and oxidative insult as potential agents for neurodegenerative disorders

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Abstract Amides of proline, a feature encountered in nootropics, via the carboxylic group of ibuprofen, indomethacin, ketoprofen and naproxen were prepared. Proline carboxylic group was amidated or esterified with potential antioxidant or neuroprotective compounds. Proline was replaced by 4-hydroxyproline, 2-pipecolic acid or omitted, for investigating the contribution of structure to activity. Anti-inflammatory activity was determined, and selected compounds were examined for anti-dyslipidemic action, protection against brain ischaemia/reperfusion and brain penetration.

Keywords Non steroidal anti-inflammatory drugs · Proline conjugates · Inflammation · Brain penetration

Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative condition that affects an increasing number of individuals as life expectancy is rising. Neurofibrillary tangles and senile plaques (Crentsil, 2004), as well as a serious cholinergic deficit in the brain are main pathological features of Alzheimer-type dementia (Palmer, 2002). Inflammatory reactions, surrounding the cerebral microvasculature, are often observed during AD, and inflammation products are found in the central nervous system of AD patients (Eikelenboom and Veerhuis, 1996; Streit *et al*., 2004). Brain capillaries themselves are also affected in the course of AD (De Vries *et al*., 1997). In addition, cerebral ischaemia seems to play an important role in the pathogenesis of AD (Qi *et al*., 2007). Finally, there is a significant role of oxidative stress in the development of AD (Moreira *et al*., 2008). Reactive oxygen species released in the central nervous system after an ischaemic event may influence blood brain barrier permeability. It is already known that the function of blood brain barrier is impaired in AD patients (De Vries *et al*., 1997).

Thus, despite the fact that there is still enough uncertainty about the molecular mechanism in AD pathogenesis, and although a really effective intervention has not evolved yet, the above pathobiochemical changes in the demented brain can be used as a starting point for rational design of plurifunctional molecules for the medicinal treatment of cognition disorders.

In previous investigations, we have reported the design and study of a number of properly modified non steroidal anti-inflammatory drugs (NSAIDs) designed to prevent or restore some pathological changes appearing in AD, via a combination of biological properties that the new molecules would acquire (Siskou *et al*., 2007; Doulgkeris *et al*., 2006). In this study, an effort is made to complete and extend those studies with the synthesis and biological evaluation of some more compounds, to identify favourable structural characteristics. Thus, amides of proline, a feature often encountered in nootropic agents (Ostrovskaya *et al*., 2007), with the carboxylic group of known NSAIDs, i.e., ibuprofen, indomethacin, ketoprofen and naproxen were prepared. Proline carboxylic group was esterified with 2-methoxy-4-methyl phenol and 3-(3-hydroxypropyl)pyridine, expected to offer neuroprotective or neurotrophic activity, or with

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2-nitroxyethanol, that can liberate nitric monoxide. Amidation of the proline carboxylic group with the thiol-containing cysteamine and cysteine ethyl ester, that would present antioxidant properties, was also performed. For further investigation about the contribution of structural features to activity, esters of phenylpropanol and ethylenediol were prepared. Finally, the proline moiety was replaced by 4-hydroxy-proline, 2-pipecolic acid, or it was excluded and the NSAIDs were directly converted to esters or amides using some of the above mentioned alcohols or amines.

The synthesized compounds were tested for anti-inflammatory and antioxidant activity. An effective inhibitor of the in vitro lipid peroxidation was also tested in vivo, by evaluation of lipid peroxidation after rat brain ischaemia and reperfusion. Finally, since the designed site of action of these compounds is the central nervous system, a HPLC method was developed using brain homogenates of rats treated with a number of the most promising compounds, for the determination of blood–brain barrier penetration.

Results and discussion

Chemistry

The synthesized compounds are given in Tables 1, 2 and 3. Their synthesis has been performed by methods we have applied previously (Siskou *et al*., 2007; Doulgkeris *et al*., 2006; Galanakis *et al*., 2004). During the purification of the nitric acid derivative **7** by flash chromatography, a second compound was isolated and identified as the hydrolysis product **8**.

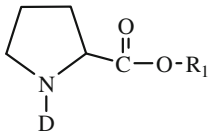
In an effort to react the pipecolic acid amide of indomethacin with cysteine ethyl ester, compound **14** was obtained as the main reaction product. Amide bond hydrolysis of indomethacin has been known to occur in both acid (Garcia *et al*., 2006) and alkaline environment (Suleiman and Najib, 1990) even at 25°C.

Acute inflammation

All tested compounds present anti-inflammatory activity, yet most of them are weaker than or equal to the parent NSAIDs (Tables 4, 5). However, this study reveals some important structural characteristics, which possibly are implicated with this activity. Thus, the nitric ester **7** induces 62% inhibition, better than ibuprofen, but the hydrolysis product **8** only 24%. Given the fact that ibuprofen amide with proline, when amidated with cysteamine, produces 61% inhibition of oedema at the same experiment (Siskou *et al*., 2007), the role of the action of these compounds against reactive oxygen species formed during inflammation can be indicated. This antioxidant action can be mediated via different mechanisms, via NO liberation in the former case, the reductive effect of the –SH group in the latter. We have shown the anti-inflammatory activity of nitric oxide donors before (Ziakas *et al*., 2006) examining the anti-inflammatory potential of various tolfenamic acid nitric ester derivatives. The positive effect of the thiol group is further verified by the ketoprofen derivative **10** (52% inhibition), compared to ketoprofen (42%).

Another point that deserves further investigation is the presence of the 3-pyridyl-propyl group, which seems to contribute to the anti-inflammatory action in several cases. Thus, a simple esterification with 3-pyridyl-propanol

Table 1 Structures of the synthesized compounds **1–8**



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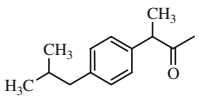
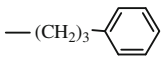
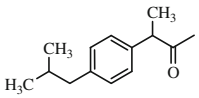
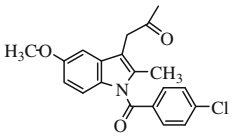
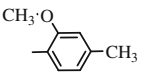
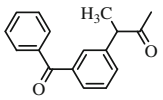
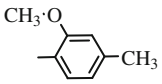
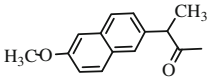
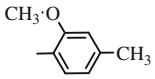
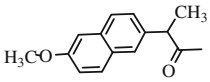
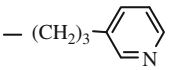
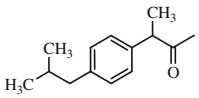
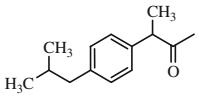
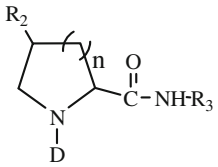
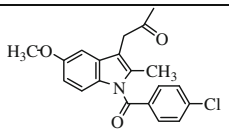
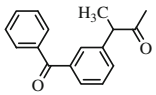
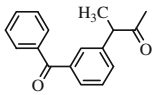
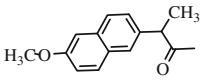
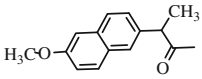
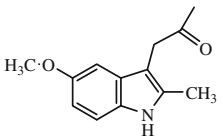
1		
2		$-CH_2CH_2Br$
3		
4		
5		
6		
7		$-CH_2CH_2ONO_2$
8		$-CH_2CH_2OH$

Table 2 Structures of the synthesized compounds **9–14**



No.	D	n	R_2	R_3
9		1	H	–CH(COOC$_2$H$_5$)CH$_2$SH
10		1	H	–CH(COOC$_2$H$_5$)CH$_2$SH
11		1	H	–CH$_2$CH$_2$SH
12		1	H	–CH$_2$CH$_2$SH
13		1	OH	–CH$_2$CH$_2$SH
14		2	H	–CH(COOC$_2$H$_5$)CH$_2$SH

increases the anti-inflammatory activity of ketoprofen (**17**) and ibuprofen (**16**). We have found (Siskou *et al*., 2007) that the activity of the ibuprofen derivative is greatly increased (81%) when a proline moiety is incorporated between ibuprofen and 3-pyridyl-propanol. On the contrary, when the pyridyl group is replaced by phenyl, a great drop in activity is observed (**1**).

Lipoxygenase and cycloxygenase activities

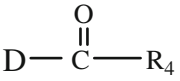
The effect of a number of the synthesized compounds on the in vitro lipoxygenase activity is shown in Table 6. A similar to the in vivo anti-inflammatory effect of the 3-pyridyl-propanol derivatives is also observed with lipoxygenase inhibition. Thus, the three most active lipoxygenase inhibitors are compounds **6**, **16** and **19** (IC_{50} 46, 45 and 28 μM, respectively). Compound **17**, although moderately inhibiting lipoxygenase, strongly inhibits COX-1 and COX-2 (87 and 69%, respectively). Furthermore, the naproxen ester with 3-pyridyl-propanol (**19**) significantly inhibits both COX isoforms (COX-1: 71%; COX-2: 50%), whilst this activity almost disappears with the naproxen derivative **5** (16% inhibition for COX-1, no effect on COX-2), as well as with the indomethacin analogue **3** (16 and 18% for COX isoforms). Naproxen and ketoprofen were included as reference compounds at the same experimental conditions, and found to inhibit COX-1 by 52 and 86% and COX-2 by 88 and 78%, respectively.

It is known that small molecules incorporating a proline moiety esterified with 2-pyridyl-propanol cross the blood–brain barrier and possess neurotrophic action (Steiner *et al*., 1997). By this study, some additional properties of such derivatives are demonstrated, with possible further applications.

In vitro lipid peroxidation

Most of the compounds tested had a minor effect on the peroxidation of rat microsomal membrane lipids, e.g., the nitric ester **7** inhibited the peroxidation reaction by 14% at 1 mM. As expected, the highest activity was observed with the thiol-containing compounds **10** and **20**, with IC_{50} value (after 45 min of incubation) of 440 and 650 μM, respectively, being very much in accordance with the reported (Doulgkeris *et al*., 2006) value of 390 μM for compound **9**. However, compound **14**, derived after amide bond hydrolysis of the indomethacin moiety, was found to be a very potent antioxidant, with IC_{50} value of 8 μM, under the same experimental conditions. It is known that melatonin, being structurally related to **14**, as well as other indole derivatives show some antioxidant activity in vitro. This effect can be attributed to the donation of hydrogen by the indole NH group (Marshall *et al*., 1996).

Table 3 Structures of the synthesized compounds **15–20**



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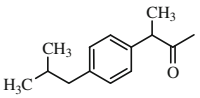
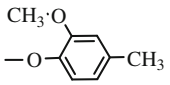
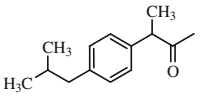
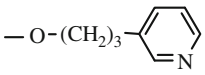
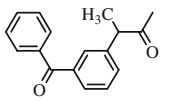
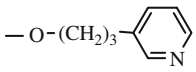
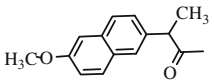
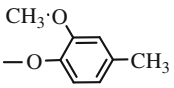
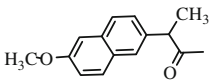
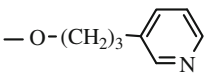
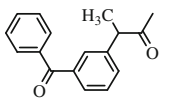
15		
16		
17		
18		
19		
20		–$NHCH_2CH_2SH$

Table 4 Effect of the tested compounds on FCA-induced mice paw oedema

Compound	Dose (mmol/kg)	Percent oedema increase (mean ± SEM)	Percent oedema inhibition
1	0	103.5 ± 25.5	0
	0.30	73.0 ± 7.0**	31
3	0	80.2 ± 7.7	0
	0.15	40.6 ± 3.5**	49
	0.30	36.6 ± 4.5 **	54
7	0	74.1 ± 8.7	0
	0.30	24.8 ± 3.5**	62
8	0	103.5 ± 25.5	0
	0.30	74.7 ± 9.7*	24
9	0	96.8 ± 5.6	0
	0.15	72.7 ± 3.0**	25
	0.30	54.6 ± 1.7**	44
15	0	83.5 ± 6.1	0
	0.30	45.4 ± 7.6**	43
16	0	74.1 ± 10.7	0
	0.30	29.5 ± 6.3**	59
S-Ibuprofen	0	103.5 ± 25.5	0
	0.30	49.6 ± 18.6**	53
Indomethacin	0	81.7 ± 4.9	0
	0.15	26.3 ± 2.8**	68

* $P < 0.01$, ** $P < 0.001$ compared to controls (Student's *t* test)

Brain oxidative stress caused by ischaemia–reperfusion

The effect of the indomethacin derivative **9** on oxidative stress induced in vivo by reperfusion of ischaemic rat brain is determined. We selected this compound for several reasons: it has been found (Doulgkeris *et al.*, 2006) to inhibit considerably in vitro lipid peroxidation, to reduce chronic inflammation induced by Freund's complete adjuvant (FCA) by 77%, whilst it did not produce any gastrointestinal irritation, compared to equimolar dose of indomethacin, which demonstrated serious gastrointestinal toxicity. Furthermore, a similar compound, integrating ibuprofen in the place of indomethacin, has been found (Siskou *et al.*, 2007) to protect the brain from oxidative insult induced by ischaemia–reperfusion. Finally, both ibuprofen and indomethacin have been found able to cross the blood–brain barrier (Parepally *et al.*, 2006).

Ischaemia is found to increase significantly ($P < 0.05$ compared to controls) oxidative stress in the brain, expressed as malondialdehyde formation (3.7 ± 0.2, against control 2.0 ± 0.7 nmol/mg brain protein) and treatment with **9** caused a statistically significant ($P < 0.01$ compared to ischaemia; not significant compared to controls) reduction of malondialdehyde formation (3.0 ± 0.2 nmol/mg brain protein). These results can be considered interesting, since, besides providing evidence that this compound can enter the

Table 5 Effect of the tested compounds on carrageenan-induced rat paw oedema

Compound	Dose (mmol/kg)	Percent oedema increase (means ± SEM)	Percent oedema inhibition
4	0	38.4 ± 2.6	0
	0.30	32.7 ± 3.0^{NS}	13
5	0	59.8 ± 12.0	0
	0.30	40.0 ± 3.9*	20
6	0	37.7 ± 9.7	0
	0.30	28.6 ± 7.1*	24
10	0	45.1 ± 5.3	0
	0.30	21.7 ± 3.4**	52
17	0	37.7 ± 9.7	0
	0.30	14.8 ± 7.6*	61
18	0	37.7 ± 9.7	0
	0.30	22.7 ± 3.7*	40
19	0	37.7 ± 9.7	0
	0.30	27.4 ± 4.4*	27
20	0	45.2 ± 5.6	0
	0.30	27.3 ± 3.7*	39
S-Ketoprofen	0	45.2 ± 5.6	0
	0.30	26.2 ± 1.2*	42
S-Naproxen	0	58.5 ± 4.3	0
	0.30	28.7 ± 4.2*	51

NS not significant (Student's *t* test)

* $P < 0.01$, ** $P < 0.001$ compared to controls

Table 6 In vitro evaluation of lipoxygenase (LOX) inhibition

Compound	LOX inhibition IC_{50} (μM)
1	60
4	60
6	46
7	240
8	320
10	170
15	65
16	45
17	145
18	90
19	28
Ibuprofen	200
Ketoprofen	220
Naproxen	220

brain, it is known that ischaemia–reperfusion injury is mainly due to radical attack (Rodrigo *et al*., 2005), and further that ischaemic neuronal changes are implicated in dementia (Pluta and Amek, 2008).

Gastrointestinal toxicity

Nonsteroidal anti-inflammatory drugs induce gastric mucosal lesions in part by the activation of inflammatory cells and the production of proinflammatory cytokines. In particular, indomethacin causes serious gastric ulcers through various processes, including generation of reactive oxygen species, initiation of lipid peroxidation, infiltration of leucocytes, induction of apoptosis, in addition to inhibition of prostaglandin synthesis (Bech *et al*., 2000). These effects are dose dependent and in many cases severe enough to limit their use. In this study, we examined the appearance of gastrointestinal toxicity after the administration of the indomethacin derivative **3**, one of the most potent synthesized anti-inflammatory agents, and compound **17**, a ketoprofen derivative with high anti-inflammatory action and a potent inhibitor of both cyclooxygenase isoforms. At doses equimolar to those that the parent drugs induced 50% mortality, both compounds had mild effects on the gastrointestinal tract (Table 7), compound **3** being less toxic. Since these compounds have no antioxidant activity and do not seem to possess any tissue protective properties, we could only attribute their low gastrointestinal toxicity to the masking of the carboxylic group. The parent non steroidal anti-inflammatory drug may gradually be liberated after hydrolysis. The higher gastrointestinal toxicity of the ketoprofen derivative **17**, compared to the indomethacin analogue, could be explained by the easier hydrolysis of an ester than an amide bond. Additional gastric irritation by **17** may be due to the fact that it has been found to be a quite strong COX-1 inhibitor, whilst **3** has only weak cyclooxygenase inhibitory action.

Measurement of compounds in brain and blood

Although, we have computational and indirect experimental evidence that the compounds of the above general structure can reach the brain, we decided to get a direct proof, by experimentally determining them in rat brain by HPLC. We start this examination by a preliminary assessment of four compounds (Table 8) previously reported to possess remarkable antioxidant, anti-inflammatory activities and low gastrointestinal toxicity (Siskou *et al*., 2007; Doulgkeris *et al*., 2006).

Furthermore, the ibuprofen derivative ethyl 2-(1-(2-(4-isobutylphenyl)propanoyl)pyrrolidine-2-carboxamido)-3-mercaptopropanoate (compound **ii**, Table 8) has been found to protect rat brain from oxidative stress and glutathione depletion after ischaemia–reperfusion injury (Siskou *et al*., 2007).

All compounds are found able to enter the brain relatively soon, and to remain there at measurable concentrations for more than 2 h. The ibuprofen derivatives **i** and **ii** seem to follow a different pattern of the presence in the brain,

Table 7 Gastrointestinal toxicity of two new compounds, indomethacin and ketoprofen

Compound	Dose (μmol/kg)	Mortality (%)[a]	Perforating ulcers (%)[b]	Body weight change[c]	Incidence of melena (%)[d]
3	84	0	0	+11	0
Indomethacin	84	50	80	−15	100
17	200	33	50	−13	60
Ketoprofen	200	50	85	−24	100

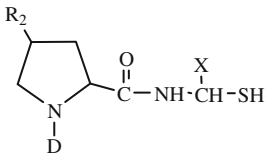
[a] Dead per total × 100

[b] Percent of animals developing perforating intestinal ulcers

[c] In g/100 g body weight. Standard deviation of the weight change is always within 10% of the average value

[d] Percent of animals developing melena defecation

Table 8 Determination of compounds in rat brain



Compound			30 min		60 min		120 min	
D	R_2	X	μg/g brain (± SEM)	%[a]	μg/g brain (± SEM)	%[a]	μg/g brain (± SEM)	%[a]
i. Ibuprofen	H	H	9.78 ± 1.28	4.9	10.22 ± 2.56	5.2	35.17 ± 4.95	17.8
ii. Ibuprofen	H	$COOCH_2CH_3$	1.59 ± 0.61	0.6	40.77 ± 4.55	15.6	27.84 ± 2.77	10.7
iii. Naproxen	H	H	7.66 ± 1.93	3.3	1.94 ± 0.26	0.9	1.53 ± 0.05	0.7
iv. Naproxen	OH	H	26.55 ± 3.62	11.0	5.70 ± 0.86	2.4	0.71 ± 0.12	0.3

[a] Percentage brain penetration

compared with the naproxen derivatives **iii** and **iv**: the concentrations of the former are gradually increased with time, whereas the latter reaches the brain within the first 30-min post injection and a decrease is observed in the next 90 min. Although it has been observed, in retrospective clinical studies, that chronic treatment with non steroidal anti-inflammatory drugs may protect against Alzheimer's disease, there are not enough studies concerning their concentration in the brain. Both ibuprofen and naproxen have been reported to have access to the brain, however, it has been claimed (Mannila *et al*., 2005) that ibuprofen brain penetration is insufficient for its use in Alzheimer's disease. Better results were obtained with ibuprofen derivatives having higher lipophilicity, such as ibuprofen glycerides (Deguchi *et al*., 2000). Regarding the above, we consider the performed molecular modifications a significant improvement for better brain penetration.

We also determined the blood concentrations of 1(2-(6-methoxynaphthalen-2-yl)propanoyl)-4-hydroxypyrrolidine-2-carboxylic acid 2-mercaptoethylamide (compound **iv**, Table 8), which has the lowest lipophilicity of the four, by the same method and at the same time intervals. It was found that the ratio of brain to blood concentration is 0.04 2-h post injection, which drops to 0.004 1 h later, possibly due to redistribution.

Conclusion

Disorders, such as Alzheimer's disease, could be treated effectively with compounds designed to act at different causes and stages of their pathogenesis. The described molecules could become useful for developing agents that may slow the progression or delay the onset of neurodegenerative diseases.

Experimental

Materials and methods

All commercially available reagents (from Aldrich-Chemie, Steinheim, Germany; Merck, Darmstadt, Germany) were of the appropriate purity. 2-Thiobarbituric acid, soybean

lipoxidase and diagnostic kits for cycloxygenase 1 and 2 determinations were purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA).

Synthesis

Melting points were obtained on a MEL-TEMP II (Laboratory Devices) apparatus and are uncorrected. Infrared (IR) spectra were taken with a FTIR-810M (Shimadzu) Fourier Transform Spectrophotometer. Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a Brucker 400 MHz spectrometer and chemical shifts are reported in parts per million (δ) relative to tetramethysilane. Signals are designated as follows: s, singlet; d, doublet; t, triplet; q, quadruplet and m, multiplet. Elemental analyses were performed with a Perkin-Elmer 2400 CHN analyzer and are within 0.4% of the calculated values.

General method for the preparation of proline/pipecolic acid amides with the NSAIDs

The corresponding NSAID, i.e., (*S*)-ibuprofen ((*S*)-2-(4-isobutylphenyl)propanoic acid), indomethacin (1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indole-3-acetic acid), (*S*)-ketoprofen ((*S*)-2-(3-benzoylphenyl)propanoic acid) or (*S*)-naproxen ((*S*)-(6-methoxy-2-naphthyl)propanoic acid) (48.5 mmol) was dissolved in dichloromethane (50 ml) and maintained at 0°C. Oxalyl chloride (145.5 mmol) was added dropwise, the mixture was kept at 0°C for 30 min and then at room temperature for 3–5 h. The volatile components were distilled off under reduced pressure to give the chlorides as yellow oils or solids (indomethacin acid chloride, Mp 124–126°C, naproxen acid chloride, Mp 92–94°C). The chloride (46.7 mmol) and a 4 N aqueous solution of sodium hydroxide (12 ml) were then added alternatively, at 0°C, to an aqueous solution of L-proline ((*S*)-pyrrolidine-2-carboxylic acid) or *trans*-4-hydroxy-L-proline ((*2S*, *4R*)-4-hydroxypyrrolidine-2-carboxylic acid) or L-pipecolic acid ((*S*)-2-piperidine carboxylic acid), prepared by dissolving the amino acids (46.2 mmol) in 2 N aqueous solution of sodium hydroxide (26 ml). The mixture was stirred at 0°C for 20 min, then at room temperature for 1–6 h, washed with ethyl acetate (3 × 40 ml), acidified with hydrochloric acid 1 N to pH 2, extracted with chloroform (4 × 60 ml) and dried (Na_2SO_4). The solvent was evaporated under reduced pressure and the residue was purified and identified.

1-(2-(4-Isobutylphenyl)propanoyl)pyrrolidine-2-carboxylic acid, ***A*** *(Siskou et al., 2007)*

Mp 112–114°C (from ether and petroleum ether), yield 90%.

1-(2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetyl)pyrrolidine-2-carboxylic acid, ***B*** *(Doulgkeris et al., 2006)*

Mp 120–124°C (from ether and petroleum ether), yield 87%.

1-(2-(3-Benzoylphenyl)propanoyl)pyrrolidine-2-carboxylic acid, ***C***

Oil, yield 85%. IR (neat) 1606, 1670, 1756 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.50 (d, 3H, C**H$_3$**CH; J = 9.0 Hz), δ 1.90–2.30 (m, 4H, C-3 pyrr., C-4 pyrr.), δ 3.30–3.40 (m, 2H, C-5 pyrr.), δ 3.90 (q, 1H CH_3C**H**CO), δ 4.40 (t, 1H, C-2 pyrr.), δ 7.40–7.70 (m, 9H arom.).

1-(2-(6-Methoxynaphthalen-2-yl)propanoyl)pyrrolidine-2-carboxylic acid, ***D***

Mp 158–160°C (from ether and petroleum ether), yield 90%. IR (KBr) 1632, 1735 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.50 (d, 3H, C**H$_3$**CH; J = 8.9 Hz), δ 1.80–2.30 (m, 4H, C-3 pyrr., C-4 pyrr.), δ 3.20–3.60 (m, 2H, C-5 pyrr.), δ 3.70 (m, 1H, C**H**CH_3), δ 3.90 (s, 3H, CH_3O), δ 4.65 (t, 1H, C-2 pyrr.), δ 7.10 (s, 2H arom., C-1 naphth., C-5 naphth.), δ 7.40 (d, 1H arom., C-7 naphth.; J = 11.4 Hz), δ 7.65–7.80 (m, 3H arom.).

1(2-(6-Methoxynaphthalen-2-yl)propanoyl)-4-hydroxypyrrolidine-2-carboxylic acid, ***E***

Mp 152–156°C (from ethyl alcohol and water), yield 73%. IR (KBr) 1632, 1726, 3368 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.60 (d, 3H, C**H$_3$**CH; J = 9.0 Hz), δ 2.10–2.45 (m 2H, C-3 pyrr.), δ 3.15–3.65 (m, 2H, C-5 pyrr.), δ 3.80 (m, 1H, C-4 pyrr.), δ 3.90 (s, 3H, CH_3O), δ 4.35–4.45 (m, 1H C-2 pyrr.), δ 4.75–4.85 (m, 1H, CH_3C**H**), δ 7.10 (s, 1H arom., C-5 naphth.), δ 7.15 (s, 1H arom., C-1 naphth.), δ 7.4 (d, 1Harom., C-7 naphth.; J = 11.4 Hz), δ 7.60–7.70 (m, 3H arom.).

1-(2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetyl)-piperidine-2-carboxylic acid, ***F***

Mp 161–163°C (from cold chloroform), yield 73%. IR (KBr) 1625, 1680, 1718 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.2–1.4 (m. 2H, C-3 piper.), δ 1.50–1.70 (m, 4H, C-4, C-5 piper.), δ 2.00–2.30 (m, 2H, C-6 piper.), δ 2.35–2.40 (s, 3H, indolyl-CH_3), δ 3.60 (s, 2H, CH_2CO), δ 3.70 (s, 3H, OCH_3), δ 5.20 (s, 1H, C-2 piper.), δ 6.70–7.40 (m, 4H arom., C-6 indolyl, C-7 indolyl, C-3 4-chlorobenzoyl and C-5 4-chlorobenzoyl), δ 7.60–8.00 (m, 3H arom., C-4 indolyl, C-2 4-chlorobenzoyl, C-6 4-chlorobenzoyl).

General method for the preparation of esters of the proline derivatives

A solution of the proline derivatives (**A**–**D**) (10 mmol), the corresponding alcohol or phenol (11 mmol), *N*,*N*′-dicyclohexylcarbodiimide (DCC, 11 mmol) and *N*,*N*-dimethylaminopyridine (DMAP, 1 mmol) in dichloromethane (15–20 ml) was maintained at room temperature for 5–10 h. After filtration, the filtrate was washed with water, dried ($MgSO_4$), dichloromethane was evaporated under reduced pressure, the residue was dissolved in dry ethyl acetate, kept at 4°C for 24 h, filtered and concentrated under reduced pressure. The final esters were purified with flash chromatography and identified.

Compound **2** (see below) was further converted to the corresponding nitric ester, compound **7**: compound **2** (10 mmol) was dissolved in acetonitrile (20 ml) and brought to 80°C. A solution of silver nitrate (15 mmol) in acetonitrile was added and the mixture was refluxed for 8 h. The produced silver bromide was filtered off, the filtrate was concentrated under reduced pressure, dichloromethane was added (30 ml), the mixture was washed with water (2×20 ml), dried ($CaCl_2$), the solvent was evaporated and the residue was purified with flash chromatography.

During the preparation and purification procedure, a second, less polar compound was isolated and identified, compound **8**.

3-(Phenyl)propyl 1-(2-(4-isobutylphenyl)propanoyl) pyrrolidine-2-carboxylate, ***1***

Yellow oil, purified by flash chromatography (petroleum ether/ethyl acetate 4/1), yield 74%. IR (neat) 1645, 1739 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.83 (d, 6H, CH($C\mathbf{H_3}$)$_2$; $J = 8.0$ Hz), δ 1.37 (d, 3H, $C\mathbf{H_3}$CHCO; $J = 8.0$ Hz), δ 1.75–2.11 (m, 7H, C-3 pyrr., C-4 pyrr., $CH_2C\mathbf{H_2}CH_2$, C**H**(CH_3)$_2$), δ 2.37 (d, 2H, $C\mathbf{H_2}$CH(CH_3)$_2$; $J = 8.0$ Hz), δ 2.58 (t, 2H, phenyl-CH_2), δ 3.17–3.45 (m, 2H, C-5 pyrr.), δ 3.67 (q, 1H, $CH_3C\mathbf{H}CO$), δ 4.07 (t, 2H, $C\mathbf{H_2}O$), δ 4.48 (t, 1H, C-2 pyrr.), δ 7.00–7.22 (m, 9H arom.). Anal. ($C_{27}H_{35}NO_3$), C, H, N.

2-Bromoethyl 1-(2-(4-isobutylphenyl)propanoyl) pyrrolidine-2-carboxylate, ***2***

Colourless oil purified by flash chromatography (petroleum ether/ethyl acetate 4/1), yield 85%. IR (neat) 1645, 1743 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.83 (d, 6H, CH($C\mathbf{H_3}$)$_2$; $J = 8.0$ Hz), δ 1.35 (d, 3H, $C\mathbf{H_3}$CHO; $J = 8.0$ Hz), δ 1.77–2.12 (m, 5H, C-3 pyrr., C-4 pyrr., C**H**(CH_3)$_2$), δ 2.38 (d, 2H, $C\mathbf{H_2}$CH(CH_3)$_2$; $J = 8.0$ Hz), δ 3.19 (m, 2H, C-5 pyrr.), δ 3.29–3.54 (m, 2H, CH_2Br), δ 3.66 (q, 1H, $CH_3C\mathbf{H}CO$), δ 4.08 (t, 1H, C-2 pyrr.), δ 4.30–4.39 (m, 2H, $C\mathbf{H_2}O$), δ 7.03 (d, 2H arom.; $J = 12.0$ Hz), δ 7.13 (d, 2H arom.; $J = 12.0$ Hz). Anal. ($C_{20}H_{28}BrNO_3$), C, H, N.

2-Methoxy-4-methylphenyl 1-(2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl) acetyl)pyrrolidine-2-carboxylate, ***3***

Yellow solid purified by flash chromatography (petroleum ether/ethyl acetate 1/1), mp 76–78°C, yield 41%. IR (KBr) 1630, 1645, 1765 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.90–2.20 (m, 4H, C-3 pyrr. C-4 pyrr.), δ 2.20–2.50 (m, 6H, phenyl-CH_3, indolyl-CH_3), δ 3.50–3.65 (m, 2H, C-5 pyrr.), δ 3.70 (s, 2H, CH_2CO), δ 3.75 (s, 6H, phenyl-OCH_3, indolyl-OCH_3), δ 4.80 (t, 1H, C-2 pyrr.), δ 6.60–7.00 (m, 6H, indolyl-3H arom., 2-methoxy-4-methylphenyl-3H arom.), δ 7.40 (d, 2H arom., C-3 4-chlorobenzoyl, C-5 4-chlorobenzoyl; $J = 6.5$ Hz), δ 7.65 (d, 2H arom., C-2 4-chlorobenzoyl, C-6 4-chlorobenzoyl; $J = 9.0$ Hz). Anal. ($C_{32}H_{31}ClN_2O_6$), C, H, N.

2-Methoxy-4-methylphenyl 1-(2-(3-benzoylphenyl)propanoyl)pyrrolidine-2-carboxylate, ***4***

Colourless oil purified by flash chromatography (petroleum ether/ethyl acetate 1/1), yield 40%. IR (neat) 1606, 1653, 1774 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.45 (d, 3H, $C\mathbf{H_3}$CH; $J = 9.0$ Hz), δ 1.90–2.20 (m, 4H, C-3 pyrr. C-4 pyrr.), δ 2.40 (s, 3H, phenyl-CH_3), δ 3.30 (m, 2H, C-5 pyrr.), δ 3.70 (s, 3H, CH_3O), δ 3.90 (q, 1H, $CH_3C\mathbf{H}CO$), δ 4.50 (t, 1H C-2 pyrr.), δ 6.50–7.00 (m, 3H, 2-methoxy-4-methyl-phenyl-3H arom.), δ 7.40–7.70 (m, 9H arom.). Anal. ($C_{29}H_{29}NO_5$), C, H, N.

2-Methoxy-4-methylphenyl 1-(2-(6-methoxynaphthalen-2-yl)propanoyl)pyrrolidine-2-carboxylate, ***5***

White solid purified by flash chromatography (petroleum ether/ethyl acetate 4/3), mp 148–150°C, yield 51%. IR (KBr) 1645, 1770 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.50–1.58 (m, 3H, $C\mathbf{H_3}$CHCON), δ 1.90–1.95 (m, 2H, C-4 pyrr.), δ 2.00–2.30 (m, 2H, C-3 pyrr.), δ 2.35 (s, 3H, CH_3-phenyl), δ 3.40–3.50 (m, 2H, C-5 pyrr.), δ 3.85 (s, 6H, CH_3O-phenyl, CH_3O-naphth.), δ 4.50 (t, 1H C-2 pyrr.), δ 4.75–4.80 (q, 1H, $CH_3C\mathbf{H}CON$.), δ 6.70–7.70 (m, 9H arom.). Anal. ($C_{27}H_{29}NO_5$), C, H, N.

3-(Pyridin-3-yl)propyl 1-(2-(6-methoxynaphthalen-2-yl)propanoyl)pyrrolidine-2-carboxylate, ***6***

Purified by flash chromatography (ethyl acetate/acetonitrile 10/1) as viscous oil, yield 50%. IR (Nujol) 1611, 1750 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.54 (d, 3H, $C\mathbf{H_3}$CH; $J = 9.0$ Hz), δ 1.86 (m, 2H, $CH_2C\mathbf{H_2}CH_2$), δ 1.90–2.16

(m, 4H, C-3 pyrr. C-4 pyrr.), δ 2.30 (t, 2H, pyridyl-CH_2), δ 3.30–3.55 (m, 2H, C-5 pyrr.), δ 3.75 (s, 3H, CH_3O), δ 3.89 (q, 1H, CH_3**C**HCO), δ 4.11 (t, 2H COO**CH_2**), δ 4.55 (m, 1H C-2 pyrr.), δ 7.05 (d, 1H arom., C-7 naphth.; $J = 11.4$ Hz), δ 7.20–7.70 (m, 6H arom., C-1 naphth., C-3 naphth., C-8 naphth., C-9 naphth., C-2 pyridyl, C-6 pyridyl), δ 8.37 (m, 3H arom., C-4 naphth. C-2 pyridyl, C-6 pyridyl). Anal. ($C_{27}H_{30}N_2O_4 \times 0.5\ CH_3CN$), C, H, N.

2-Nitroxyethyl 1-(2-(4-isobutylphenyl)propanoyl) pyrrolidine-2-carboxylate, ***7***

Colourless oil, purified by flash chromatography (petroleum ether/ethyl acetate 4/1 and then 3/1), yield 35%. IR (neat) 1641, 1748 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.86 (d, 6H, CH(C**H$_3$**)$_2$; $J = 8.0$ Hz), δ 1.38 (d, 3H, C**H$_3$**CHCO; $J = 8.0$ Hz), δ 1.75–1.92 (m, 4H, C-3 pyrr., C-4 pyrr.), δ 2.15 (m, 1H, C**H**$(CH_3)_2$), δ 2.41 (d, 2H, C**H$_2$**CH$(CH_3)_2$; $J = 8.0$ Hz), δ 3.20–3.48 (m, 2H, C-5 pyrr.), δ 3.69 (q, 1H, CH_3C**H**CO), δ 4.34 (t, 2H, C**H$_2$**O), δ 4.44–4.63 (m, 3H, C-2 pyrr., CH_2ONO_2), δ 7.06–7.16 (m, 4H arom.). Anal. ($C_{20}H_{28}N_2O_6$), C, H, N.

2-Hydroxyethyl 1-(2-(4-isobutylphenyl)propanoyl) pyrrolidine-2-carboxylate, ***8***

Colourless oil, purified by flash chromatography (petroleum ether/ethyl acetate 4/1), yield 25%. IR (neat) 1632, 1743, 3432 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.85 (d, 6H, CH(C**H$_3$**)$_2$; $J = 8.0$ Hz), δ 1.37 (d, 3H, C**H$_3$**CHCO; $J = 8.0$ Hz), δ 1.78–2.17 (m, 6H, C-3 pyrr., C-4 pyrr., C**H**$(CH_3)_2$,OH), δ 2.40 (d, 2H, C**H$_2$**CH$(CH_3)_2$; $J = 8.0$ Hz), δ 3.20–3.26 (m, 2H, C-5 pyrr.), δ 3.43–3.53 (q, 1H, CH_3C**H**CO), δ 3.62 (m, 2H, C**H$_2$**OH), δ 4.16–4.31 (m, 2H, C**H$_2$**O), δ 4.44 (m, 1H, C-2 pyrr.), δ 7.07 (d, 2H arom.; $J = 12.0$ Hz) δ 7.15 (d, 2H arom.; $J = 12.0$ Hz). Anal. ($C_{20}H_{29}NO_4$), C, H, N.

General method for the preparation of amides of the proline/pipecolic acid derivatives

A solution of compounds **B**–**F** (10 mmol) in dry *N*,*N*-dimethylformamide (10 ml) was maintained at 0°C under nitrogen and 1,1′-carbonyldiimidazole (CDI, 10.4 mmol) was added. When the vigorous release of carbon dioxide subsided, the mixture was further stirred at room temperature until no more gas was released. Then, L-cysteine ethyl ester [(*R*)-ethyl 2-amino-3-mercaptopropanoate] hydrochloride (10.1 mmol) was added to compounds **B**, **C** and **F**, or cysteamine (2-aminoethanethiol) hydrochloride (10.1 mmol) was added to compounds **C**, **D** and **E**, and the reaction mixture was stirred at room temperature for 24 h. Then, water (20 ml) was added, the mixture was extracted with chloroform (3 × 30 ml) and dried ($MgSO_4$). The solvent was evaporated under reduced pressure and the residue was purified and identified.

Ethyl 2-(1-(2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl) acetyl)pyrrolidine-2-carboxamido)-3-mercaptopropanoate, ***9*** *(Doulgkeris et al., 2006).*

Purified by flash chromatography (petroleum ether/ethyl acetate 1/2) as yellow solid, mp 109–111°C, yield 30%.

Ethyl 2-(1-(2-(3-benzoylphenyl)propanoyl)pyrrolidine-2-carboxamido)-3-mercaptopropanoate, ***10***

Colourless oil, purified by flash chromatography (petroleum ether/ethyl acetate 3/2 and then 1/1), yield 30%. IR (neat) 1606, 1636, 1662, 1717, 2555 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.25 (t, 3H, C**H$_3$**CH_2O), δ 1.50 (d, 3H, C**H$_3$**CH; $J = 8.5$ Hz), δ 1.55 (t, 1H, SH), δ 1.90–2.30 (m, 4H, C-3 pyrr. C-4 pyrr.), δ 2.90–3.10 (m, 2H, C**H$_2$**SH), δ 3.20–3.40 (m, 2H, C-5 pyrr.), δ 3.70–3.80 (m, 1H, CH_3C**H**), δ 3.90–4.00 (q, 2H, CH_3C**H$_2$**O), δ 4.15–4.30 (m, 1H C-2 pyrr.), δ 4.72 (t, 1H, C**H**CH_2SH), δ 7.40–7.80 (m, 9H arom.). Anal. ($C_{26}H_{30}N_2O_5S$), C, H, N.

1-(2-(3-Benzoylphenyl)propanoyl)-N-(2-mercaptoethyl)pyrrolidine-2-carboxamide, ***11***

Colourless oil, purified by flash chromatography (petroleum ether/ethyl acetate 3/2 and then 1/1), yield 35%. IR (neat) 1606, 1640, 1662, 2555 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.35 (t, 1H, SH), δ 1.50 (d, 3H, C**H$_3$**CH; $J = 9.0$ Hz), δ 1.80–2.20 (m, 4H, C-3 pyrr. C-4 pyrr.), δ 2.30–2.70 (m, 2H, C**H$_2$**SH), δ 3.30–3.80 (m, 4H, C-5 pyrr., C**H$_2$**CH_2SH), δ 3.90–4.00 (q, 1H, C**H**CH_3), δ 4.50 (m, 1H, C-2 pyrr.), δ 7.30–7.90 (m, 9H arom). Anal. ($C_{23}H_{26}N_2O_3S \times 0.3\ CH_3COOCH_2CH_3$), C, H, N.

1-(2-(6-Methoxynaphthalen-2-yl)propanoyl)-N-(2-mercaptoethyl)pyrrolidine-2-carboxamide, ***12*** *(Doulgkeris et al., 2006)*

Purified by flash chromatography (petroleum ether/ethyl acetate 1/3) as white solid, mp 151–152°C, yield 42%.

1-(2-(6-Methoxynaphthalen-2-yl)propanoyl)-N-(2-mercaptoethyl)-4-hydroxypyrrolidine-2-carboxamide, ***13*** *(Doulgkeris et al., 2006)*

Purified by flash chromatography (ethyl acetate/ethanol 95/5) as white solid, mp 140–141°C, yield 29%.

Ethyl 2-(1-(2-(5-methoxy-2-methyl-1H-indol-3-yl)acetyl)piperidine-2-carboxamido)-3-mercaptopropanoate, ***14***

After the reaction of compound **F** with L-cysteine ethyl ester according to the general method, compound **14** was isolated by flash chromatography (petroleum ether/ethyl acetate 1/1) as the main reaction product. Yellow solid, mp 54–57°C, yield 49%. IR (KBr). 1624, 1670, 1739, 2556 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.30 (t, 3H, C**H**$_3$CH$_2$), δ 1.40–1.70 (m, 7H, C-3 piper. C-4 piper., C-5 piper., SH), δ 2.35 (s, 3H, indolyl-CH_3), δ 2.80–3.30 (m, 6H, CH_2CO C-6 piper., C**H**$_2$SH), δ 3.80 (s, 3H, CH_3O), δ 4.10–4.15 (m, 3H, C**H**$_3$CH$_2$O) δ 4.70–4.90 (m, 2H, C-2 piper., CHCO), δ 6.60 (s, 1H arom., C-4 indolyl), δ 6.80–7.10 (m, 2H arom., C-6 indolyl, C-7 indolyl), δ 7.90 (m, 1H, NHCO). Anal. ($C_{23}H_{31}N_3O_5S$), C, H, N.

Preparation of esters and amide of NSAIDs According to the described general methods, the following derivatives of NSAIDs were synthesized. Their IR spectra had a peak at 1740–1760 cm^{-1}.

2-Methoxy-4-methylphenyl 2-(4-isobutylphenyl)propanoate, **15** White solid, purified by flash chromatography (petroleum ether/ethyl acetate 5/1), mp 27–29°C, yield 51%. ^1H NMR ($CDCl_3$) δ 0.92 (d, 6H, CH(C**H**$_3$)$_2$; $J = 8.0$ Hz) δ 1.61 (d, 3H, C**H**$_3$CHCO; $J = 8.0$ Hz), δ 1.88 (m, 1H, C**H**(CH$_3$)$_2$), δ 2.32 (s, 3H, phenyl-CH_3), δ 2.48 (d, 2H, C**H**$_2$CH(CH$_3$)$_2$; $J = 8.0$ Hz), δ 3.69 (s, 3H, CH_3O), δ 3.98 (q, 1H, CH$_3$C**H**CO), δ 6.67–6.83 (m, 3H arom., C-3 phenyl, C-5 phenyl, C-6 phenyl), δ 7.14 (d, 2H arom.; $J = 12.0$ Hz), δ 7.34 (d, 2H arom.; $J = 12.0$ Hz). Anal. ($C_{21}H_{26}O_3$), C, H, N.

3-(Pyridin-3-yl)propyl 2-(4-isobutylphenyl)propanoate, **16** Yellow oil, purified by flash chromatography (petroleum ether/ethyl acetate 3/1), yield 60%. ^1H NMR ($CDCl_3$) δ 0.87 (d, 6H, CH(C**H**$_3$)$_2$), δ 1.50 (d, 3H, C**H**$_3$CHCO; $J = 8.0$ Hz), δ 1.77–1.96 (m, 3H, C**H**(CH$_3$)$_2$, CH$_2$C**H**$_2$CH$_2$), δ 2.43–2.56 (m, 4H, C**H**$_2$CH(CH$_3$)$_2$, pyridyl-CH_2), δ 3.71 (q, 1H, CH$_3$C**H**CO), δ 4.01–4.14 (m, 2H, CH_2O), δ 7.09–7.25 (m, 5H arom., C_6H_4, C-5 pyridyl), δ 7.34 (d, 1H arom., C-4 pyridyl), δ 8.34–8.45 (m, 2H arom. C-2 pyridyl, C-6 pyridyl). Anal. ($C_{21}H_{27}NO_2$), C, H, N.

3-(Pyridin-3-yl)propyl 2-(3-benzoylphenyl)propanoate, **17** Yellow oil, purified by flash chromatography (petroleum ether/ethyl acetate 3/7), yield 60%. ^1H NMR ($CDCl_3$) δ 1.54 (d, 3H, C**H**$_3$CH), δ 1.90 (m, 2H, CH$_2$C**H**$_2$CH$_2$), δ 2.56 (t, 2H, pyridyl-CH_2), δ 3.80(q, 1H, CH$_3$C**H**), δ 4.09 (t, 2H, C**H**$_2$O), δ 7.16–7.78 (m, 11H arom., C_6H_5, C_6H_4, C-4 pyridyl, C-5 pyridyl), δ 8.35 (s, 1H arom., C-2 pyridyl), δ 8.42 (d, 1H arom. C-6 pyridyl; $J = 9.0$ Hz). Anal. ($C_{24}H_{23}NO_3$), C, H, N.

2-Methoxy-4-methylphenyl 2-(6-methoxynaphthalen-2-yl)propanoate, **18** White solid, purified by flash chromatography (petroleum ether/ethyl acetate 4/1), mp 84–85°C, yield 48%. ^1H NMR ($CDCl_3$) δ 1.68 (d, 3H, C**H**$_3$CH; $J = 8.0$ Hz), δ 2.30 (s, 3H, phenyl-CH_3), δ 3.65 (s, 3H, phenyl-OCH_3), δ 3.91 (s, 3H, naphthyl-OCH_3), δ 4.12 (q, 1H, CH$_3$C**H**), δ 6.66–7.75 (m, 9H arom.). Anal. ($C_{22}H_{22}O_4$, $\times 0.8$ H_2O), C, H, N.

3-(Pyridin-3-yl)propyl 2-(6-methoxynaphthalen-2-yl)propanoate, **19** White solid, purified by flash chromatography (petroleum ether/ethyl acetate 1/4), mp 55–56°C, yield 52%. ^1H NMR ($CDCl_3$) δ 1.52 (d, 3H, C**H**$_3$CH; $J = 8.0$ Hz), δ 1.82–1.85 (m, 2H, CH$_2$C**H**$_2$CH$_2$), δ 2.45 (t, 2H, pyridyl-CH_2), δ 3.80 (q, 1H, CH$_3$C**H**), δ 4.01 (s, 3H, CH_3O), δ 4.05 (m, 2H, CH_2O), δ 7.05–7.35 (m, 8H arom., $C_{10}H_6$, C-4 pyridyl, C-5 pyridyl), δ 8.30 (s, 1H arom., C-2 pyridyl), δ 8.40 (d, 1H arom. C-6 pyridyl; $J = 9.0$ Hz). Anal. ($C_{22}H_{25}NO_3$), C, H, N.

N-(2-Mercaptoethyl)-2-(3-benzoylphenyl)-2-propanamide, **20** Colourless oil, prepared, according to the general method, from *S*-ketoprofen and cysteamine, and purified by flash chromatography (petroleum ether/ethyl acetate 2/1), yield 40%. ^1H NMR ($CDCl_3$) δ 1.15 (t, 1H, SH), δ 1.55 (d, 3H, C**H**$_3$CH; $J = 8.0$ Hz), δ 2.65 (m, 2H, C**H**$_2$SH), δ 3.65 (m, 2H, C**H**$_2$CH$_2$SH), δ 3.8 (q, 1H C**H**CH$_3$), δ 7.30–7.70 (m, 9H arom.). Anal. ($C_{18}H_{19}N_2S$), C, H, N.

Effect on acute inflammation For the in vivo experiments, male Fischer-344 rats (200–280 g) or Balb-C mice (20–30 g) were used. Animals were kept and treated according to the European Communities Council Directive of 24 November 1986 (86/609/EEC). They were housed in controlled rooms, humidity 50–60%, temperature 23°C, with a 12 h light/dark cycle, and free access to standard laboratory chow and tap water.

FCA Acute inflammation was induced by the id injection of FCA (0.05 ml/paw) into the right hind paw of mice, the left paw serving as control. The test compounds, suspended in water with few drops of Tween 80, were given ip (0.15–0.30 mmol/kg body weight) 5 min before the FCA injection. Three hours later, the hind paws were excised and weighted separately. The produced oedema was estimated as paw weight increase compared to the control animals that received only the liquid vehicle (Hadjipetrou-Kourounakis *et al*., 1992).

Carrageenan paw oedema An aqueous solution of carrageenan was prepared (1% w/v) and 0.1 ml of this was injected into the right hind paw of male rats, the left paw serving as control. The tested compounds (dissolved or suspended in water with a few drops of Tween 80) were given ip (0.3 mmol/kg of body weight) 5 min before the

carrageenan injection. After 3 h, the hind paws were excised and weighed separately. The produced oedema was estimated as paw weight increase (Hadjipetrou-Kourounakis *et al*., 1992).

In vitro effect on lipoxygenase activity The reaction mixture (total volume 3 ml) contained 100 µl of the test compounds at various concentrations, dissolved in 60% aqueous ethanol (sample), or 100 µl of the solvent (reference) and 200 µl of soybean lipoxygenase (250 u/ml) in 2.6 ml of Tris buffer (pH 9). The reaction was initiated by the addition of 100 µl sodium linoleate (0.1 mM) in the sample mixture, an equal volume of buffer being added to the reference solution, and monitored for 7 min at 28°C, by recording the absorbance of a conjugated diene structure at 234 nm, due to the formation of 13-hydroperoxylinoleic acid (Siskou *et al*., 2007).

In vitro evaluation of cyclooxygenase activity (isoforms 1 and 2) The effect of compounds **3**, **5**, **17** and **19** on COX-1 and COX-2 activity was measured using ovine COX-1 and human recombinant COX-2 enzymes included in the 'COX Inhibitor Screening Assay' kit provided by Cayman. The assay directly measures PGF_{2a} produced by $SnCl_2$ reduction of COX-derived PGH_2. The prostanoid product is quantified via enzyme immunoassay using a broadly specific antibody that binds to all the major prostaglandin compounds. The compounds were added to the reaction mixture at a final concentration of 200 µM. Arachidonic acid concentration, 0.1 µM; COX, 10^{-9} IU/ml for both isoforms (Ziakas *et al*., 2006).

In vitro lipid peroxidation Hepatic microsomal fraction from untreated rats was prepared. The incubation mixture contained heat inactivated (90°C for 90 s) microsomal fraction, corresponding to 2.5 mg protein/ml (final concentration) or 4 mM fatty acid residues, ascorbic acid (0.2 mM) in Tris–HCl/KCl buffer (50 mM/150 mM) and the test compounds dissolved in dimethyl sulphoxide. The peroxidation reaction was initiated by the addition of a freshly prepared $FeSO_4$ solution (10 µM) and the mixture was incubated at 37°C. Aliquots (0.3 ml) were taken at various time intervals for 45 min. Lipid peroxidation was assessed spectrophotometrically (535 against 600 nm) by the determination of 2-thiobarbituric acid reactive material. All compounds as well as dimethyl sulphoxide were tested and found not to interfere with the assay (Siskou *et al*., 2007).

Effect on brain oxidative stress caused by ischaemia–reperfusion Compound **9** (0.4 mmol/kg body weight) was administered ip once per day for 4 days and once more 2 h before the initiation of ischaemia. The common carotid arteries were exposed and clamped with aneurysm clips to induce incomplete cerebral ischaemia. Animals were subjected to 45 min of carotid occlusion, then the clips were removed and brain was reperfused for 90 min. Animals were subsequently sacrificed, brains were removed quickly, homogenised (1 g in 10 ml cold isotonic KCl solution) for 1 min and stored at −80°C for further evaluation. Lipid peroxidation of rat brain homogenates was determined fluorometrically as 2-thiobarbituric acid reactive substance. The degree of lipid peroxidation was expressed as malondialdehyde formation (nmol/mg brain protein), using malondialdehyde bis(dimethyl acetal) as reference.

Gastrointestinal toxicity Compounds **3**, **17** as well as indomethacin and ketoprofen were administered sc to rats once daily for 4 days. Perforating gastrointestinal ulcers, melena defecation, body weight change and mortality were recorded 24 h after the last injection (Doulgkeris *et al*., 2006).

Measurement of compounds in brain and blood Rats were divided into three groups and the test compounds (0.6 mmol/kg) were administered ip. Brains were quickly removed 30, 60 and 120-min post injection and homogenized in 1.15% potassium chloride solution.

In another set of experiments, compound **iv** (Table 8) was administered as described above and blood was taken from the aorta, under light ether anaesthesia, 30, 60 and 120-min post injection. Blood was centrifuged (3000×*g*, 15 min) and compound **iv** was determined in the plasma.

The compounds were isolated from the brain samples by protein precipitation and solid phase extraction. Brain homogenates (300 µl) were acidified with 2 M hydrochloric acid (300 µl) vortexed for 5 min, acetonitrile (4.0 ml) was added, mixed for 2 min and centrifuged for 10 min (7500×*g*). The supernatants were diluted with ultrapure water (2.0 ml) and applied to the preconditioned and equilibrated C18 solid phase extraction cartridges (Discovery DSC-18; Supelco, Bellefonte, PA, USA) (Mannila *et al*., 2005). The cartridges were first washed with methanol (2.0 ml) and then with water (2.0 ml). The samples were eluted with acetonitrile (3.0 ml), evaporated to dryness under a nitrogen stream at 50°C and reconstituted in acetonitrile (0.5 ml).

The concentrations of the examined compounds in brain samples were measured by a Dionex isocratic RP-HPLC system with UV-detection at 230 nm. The HPLC system consisted of a D-6000A interface, L-6200A intelligent pump, L-4500 diode array detector, L-7350 column oven, D-7000 chromatography data station software (version 3.1) and a Zorbax SB-C18 (4.6 × 150 mm, 5 µm) analytical column (Agilent Technologies Inc., Little Falls, Wilmington, DE, USA). Loop injection volume was 10 and 30 µl for plasma and brain samples, respectively. The mobile

phase was composed of 20 mM phosphate buffer solution (pH 2.5) and acetonitrile in volume ratios of 55:45 and 46:54 for plasma and brain samples, respectively. The calibration curves of the plasma and the brain methods were linear over a range of 0.2–50 and 0.2–6 μg/ml, respectively. The precision of the plasma and the brain sample methods were studied at a concentration level of the lower limit of quantification, and expressed as coefficients of variation (CV%); 4.6% ($n = 5$) and 8.9% ($n = 4$) for spiked samples containing 5 and 0.2 μg/ml, respectively. The recovery was over 84% in all methods studied.

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