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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

The novel amidocarbamate derivatives of ketoprofen: synthesis and biological activity

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Abstract A series of novel ketoprofen derivatives **4a–j** bearing both amide and carbamate functionalities were prepared using the benzotriazole method of carboxylic and hydroxy group activation. Selective reduction of ketoprofen produced hydroxy derivative **2**, which in the reaction with one or two moles of 1-benzotriazole carboxylic acid chloride (**1**) gave benzotriazole derivatives **3a** and **3b**, respectively. Compounds **3a** and **3b** with various amines afforded amidocarbamates **4a–j**. Antioxidative screenings revealed that the prepared compounds **3b** and **4a–j** possess excellent lipid peroxidation inhibition at 0.1 mM concentration, higher than 95% for the derivatives bearing aromatic, cycloalkyl or heterocyclic substituents. Two of the compounds, **3b** and **4g**, also show high soybean lipoxygenase inhibition activity (95 and 83.5%, respectively). On the other hand, the amidocarbamate derivatives of ketoprofen show only weak reducing activity against 1,1-diphenyl-2-picrylhydrazyl radicals. No selective antiviral effects were noted for the tested compounds against a broad variety of DNA and RNA viruses. Most compounds were endowed with a moderate (IC_{50}: 10–25 μM) cytostatic activity.

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Keywords Ketoprofen amide · Carbamate · Antioxidant · Peroxidation · Lipoxygenase · Antiviral activity · Cytostatic activity

Introduction

Ketoprofen (Ket) is a non-steroidal anti-inflammatory drug (NSAID) with pronounced analgesic and antipyretic properties. Numerous ketoprofen derivatives have been synthesized in order to minimize side-effects, prolong plasma half-life and increase solubility (Bonina *et al.,* 2002a, b, 2003). Some amides have proved to be useful prodrugs, while the others possess anti-inflammatory activity independent of the parent compound. It has been demonstrated that amidation of NSAIDs improves selectivity towards COX-2 (Kalgutkar *et al.,* 2000), while modification of the carboxylic group to hydroxamic acid leads to inhibition of both cyclooxygenase and 5-lipoxygenase, two enzymes crucial in inflammatory processes (Flynn *et al.,* 1990; Muri *et al.,* 2002). Glycine amides of ketoprofen and several other well-known NSAIDs are significantly less irritating to gastric mucosa, while their anti-inflammatory activities are comparable to their parent drugs (Shanbhag *et al.,* 1992; Singh *et al.,* 1990). Ketoprofen glycinate methyl ester has higher anti-inflammatory and analgesic activity than the parent drug (Dhaneshwar and Chaturvedi, 1994). Ketoprofenamides with heterocyclic residues (2-thiazolinyl, 4-methylpyridyl, 3-hydroxypyridyl, pyridyl, 1,5-dimethyl-2-phenylpyrazolonyl or thiazolyl) also possess significant analgesic and anti-inflammatory activities (Spickett *et al.,* 1976), while ketoprofen 2-hydroxyethylamide and ketoprofen esters with bis-(hydroxyalkylthio)-alkanes are useful in the treatment and prevention of atherosclerosis (Lafon, 1977).

Numerous studies suggest that NSAIDs are promising anticancer drugs as well and may be associated with reduced risk of colon, lung, liver and other types of cancers (Thun *et al.,* 2002; Sivak-Sears *et al.,* 2004).

In our previous research potency of amides and hydroxamic acid derivatives of ketoprofen and related NSAIDs as cytostatic and antioxidant agents was screened (Zovko *et al.,* 2003; Marjanović *et al.,* 2007; Wittine *et al.,* 2009). Our articles and the extensive literature data describe the effect of carboxylic group derivatization. To our knowledge, a modification of both carboxylic and carbonyl functionalities in ketoprofen molecule has not been studied. In this article, a series of novel derivatives of ketoprofen bearing amide and carbamate moieties were prepared, characterized and screened for their antioxidative, cytostatic and antiviral activities.

Results and discussion

Chemistry

Benzotriazolides **3a**,**b** were prepared from the reduced ketoprofen derivative **2** and 1-benzotriazole carboxylic acid chloride (**1**), following our previously developed procedure (Scheme 1) (Butula and Jadrijević Mladar Takač, 2000; Zorc *et al.,* 1993). If the reaction was performed with one equivalent of chloride **1**, product **3a** with free hydroxy group was obtained in 75% yield. When the reaction was carried out with two equivalents of chloride **1**, the main product was benzotriazolide **3b**, in which both carboxylic and hydroxy groups were acylated. Minor amount of product **3a** was detected as well, even if the reaction was performed with the excess of chloride **1**. The reaction was run at room temperature in order to avoid benzotriazolide **3a** polycondensation.

Compounds **4a–j** were prepared by the reaction of benzotriazolide **3b** with two equivalents of an appropriate amine, in the presence of five equivalents of triethylamine (Scheme 1). All reactions were performed in toluene, at room temperature, for 0.5–48 h. Triethylamine formed a water soluble salt with benzotriazole, a by-product of the reaction, which was readily extracted with water.

Structures of compounds **3a**,**b** and **4a–j** were deduced from the analysis of their IR, ^{1}H- and ^{13}C-NMR spectra and confirmed by the elemental analysis. The chemical shifts were consistent with the proposed structures of the novel compounds (Fig. 1, Table 1).

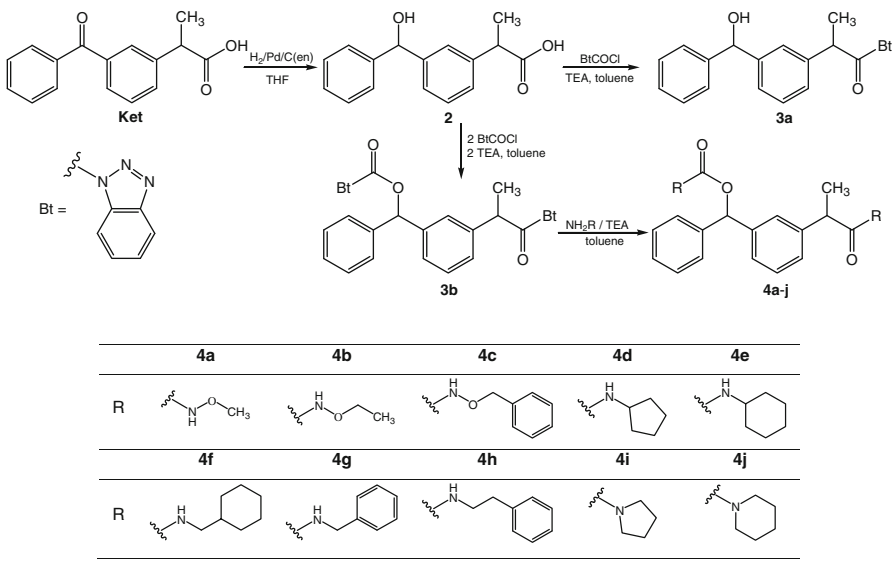
Biological studies

Antioxidant activity

The interaction of the examined compounds with the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was studied. Interaction with DPPH indicates radical scavenging ability in an iron-free system. Interactions were monitored after 20 and 60 min at two concentrations of DPPH (0.05 and 0.1 mM). Ketoprofen, the prototype compound, benzotriazolide **3b** as well as all the tested compounds presented very low interaction values. The results are shown in Table 2.

Soybean lipoxygenase inhibition

Compounds were further evaluated for the inhibition of soybean lipoxygenase (LOX) by the UV absorbance



Scheme 1 Synthesis of compounds **4a–j**

Fig. 1 Atom enumeration of compounds **3a** and **3b**

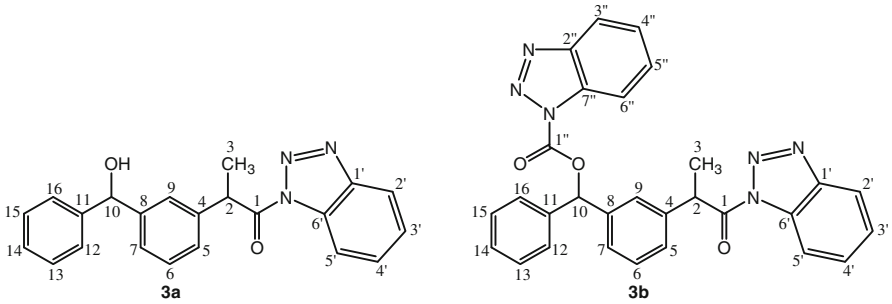
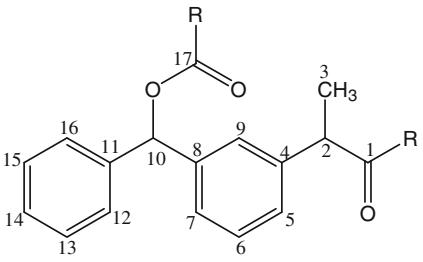


Table 1 ^{1}H- and ^{13}C-NMR data for compounds **4a–j**



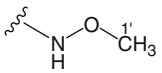
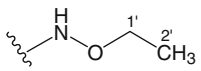
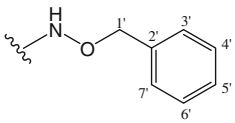
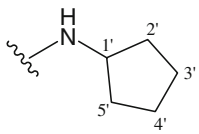
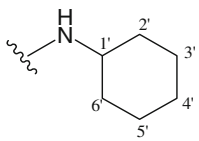
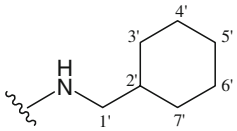
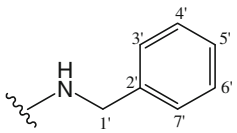
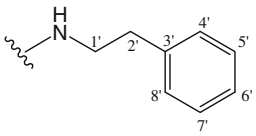
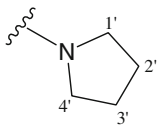
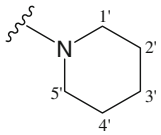
Compd.	R	^{1}H- and ^{13}C-NMR (DMSO-d_6, δ/ppm, J/Hz)
4a		11.22 (s, 1H, NH carbamate), 10.65 (s, 1H, NH amide), 7.39–7.21 (m, 9H, arom.), 6.71 (s, 1H, 10), 3.58[a] and 3.51[b] (2s, 3H, 1′), 3.38 (q, 1H, 2, J = 6.79), 1.31 (d, 3H, 3, J = 7.02) 170.31 (17), 156.20 (1), 142.14, 141.10, 140.98 (4, 8, 11), 128.97, 128.27, 127.21, 126.96, 125.68, 125.42 (5–7, 9, 12–16), 77.15 (10), 64.03[a] and 63.49[b] (1′), 42.49 (2), 18.63 (3)
4b		11.09 (s, 1H, NH carbamate), 10.54 (s, 1H, NH amide), 7.36–7.21 (m, 9H, arom.), 6.69 (s, 1H, 10), 3.81–3.66[a] (m, 3H, 2, 1′) and 3.42–3.33[b] (m, 2H, 1′), 1.30 (d, 3H, 3, J = 6.99), 1.15–1.04 (m, 6H, 2′) 170.37 (17), 156.30 (1), 142.25, 141.13, 141.07 (4, 8, 11), 128.95, 128.23, 127.18, 126.94, 125.62, 125.37 (6–8, 10, 13–17), 77.12 (10), 71.50[a] and 70.87[b] (1′), 42.48 (2), 18.61 (3), 13.87[a], 13.83[b] (2′)
4c		11.21 (s, 1H, NH carbamate), 10.66 (s, 1H, NH amide), 7.38–7.21 (m, 19H, arom.), 6.72 (s, 1H, 10), 4.77[a] and 4.70[b] (2s, 4H, 1′), 3.41 (q, 1H, 3, J = 6.96), 1.31 (d, 3H, 3, J = 6.86) 170.54 (17), 156.43 (1), 142.16, 141.08, 141.06 (4, 8, 11), 136.35, 136.27 (2′), 129.37, 129.26, 128.74, 128.71 (3′–7′), 128.96, 128.25, 127.32, 126.96, 125.74, 125.42 (5–7, 9, 12–16), 77.92[a] and 77.09[b] (1′), 77.33 (10), 42.44 (2), 18.68 (3)
4d		7.87 (d, 1H, NH carbamate, J = 6.90), 7.44 (d, 1H, NH amide, J = 6.90), 7.34–7.17 (m, 9H, arom.), 6.63 (s, 1H, 10), 3.98–3.87[a] and 3.81–3.73[b] (2m, 1H, 1′), 3.55 (q, 1H, 2, J = 6.96), 1.83–1.16 (m, 16H, 2′–5′), 1.28 (d, 3H, 3, J = 7.00) 172.86 (17), 155.26 (1), 143.12, 141.80, 141.65 (4, 8, 11), 128.81, 128.66, 127.94, 127.01, 126.91, 125.87, 125.09 (5–7, 9, 12–16), 76.39 (10), 52.61[a] and 50.65[b] (1′), 45.18 (2), 32.78[a] and 32.61[b] (2′) 32.69 (5′), 23.95[a] and 23.91[b] (3′), 23.73 (4′), 18.99 (3)
4e		7.75 (d, 1H, NH carbamate, J = 7.77), 7.36–7.17 (m, 10H, arom., NH amide), 6.62 (s, 1H, 10), 3.55 (q, 1H, 2, J = 6.94), 3.50–3.40[a] and 3.29–3.17[b] (2m, 1H, 1′), 1.74–1.52, 1.28–0.96 (2m, 20H, 2′–6′), 1.27 (d, 3H, 3, J = 7.01) 172.42 (17), 154.96 (1), 143.16, 141.80, 141.66 (4, 8, 11), 128.78, 128.63, 127.91, 127.01, 126.92, 125.81, 125.10 (5–7, 9, 12–16), 76.37 (10), 50.01[a] and 47.85[b] (1′), 45.23 (2), 33.11 (2′), 32.78 (6′), 25.68[a] and 25.61[b] (3′), 25.06 (4′) 24.97 (5′), 18.95 (3)

Table 1 continued

Compd.	R	^{1}H- and ^{13}C-NMR (DMSO-d_6, δ/ppm, J/Hz)
4f		7.86 (t, 1H, NH carbamate, $J = 5.48$), 7.44-7.16 (m, 10H, arom., NH amide), 6.61 (s, 1H, 10), 3.59 (q, 1H, 2, $J = 6.88$), 2.87-2.80 (m, 4H, 1′), 1.72-1.53 (m, 10H, 2′, 3′, 7′), 1.41-1.28, 1.20-1.04, 0.88-0.72 (3m, 12H, 4′–6′), 1.29 (d, 3H, 3, $J = 7.00$) 173.42 (17), 155.93 (1), 143.10, 141.78, 141.59 (4, 8, 11), 128.80, 128.62, 127.96, 127.02, 125.85, 125.10 (5–7, 9, 12–16), 76.48 (10), 47.07[a] and 45.25[b] (1′), 45.45 (2), 38.13[a] and 37.86[b] (2′), 30.80[a] and 30.77[b] (3′), 30.71 (7′), 26.49, 25.83 (4′–6′), 19.02 (3)
4g		8.49–8.44 (dd, 1H, NH carbamate, $J = 3.71$, $J = 5.25$), 8.04-8.00 (m, 1H, NH amide), 7.38–7.13 (m, 19H, arom.), 6.67 (s, 1H, 10), 4.24–4.19 (m, 4H, 1′), 3.66 (q, 1H, 2, $J = 6.83$), 1.34 (d, 3H, 3, $J = 6.90$) 173.54 (17), 156.10 (1), 142.92, 141.68, 141.53 (4, 8, 11), 140.07, 139.92 (2′), 128.88, 128.77, 128.05, 127.25, 126.99, 126.09, 125.26 (5–7, 9, 12–16), 128.73, 128.69, 127.51, 127.40, 127.15 (3′–7′), 76.82 (10), 45.51 (2), 44.27[a] and 42.51[b] (1′), 19.07 (3)
4h		8.01 (t, 1H, NH carbamate, $J = 5.05$), 7.54 (t, 1H, NH amide, $J = 5.48$), 7.34–7.08 (m, 19H, arom.), 6.64 (s, 1H, 10), 3.55 (q, 1H, 2, $J = 6.88$), 3.28–3.20 (m, 4H, 1′), 2.66–2.50 (m, 4H, 2′), 1.28 (d, 3H, 3, $J = 7.16$) 173.40 (17), 155.73 (1), 142.94, 141.71, 141.54 (4, 8, 11), 139.87, 139.70 (3′), 128.83, 127.98, 127.12, 127.00, 125.97, 125.19 (5–7, 9, 12–16), 129.10, 128.75, 128.68, 126.53, 126.44 (4′–8′), 76.54 (10), 45.47 (2), 42.39[a] and 40.73[b] (1′), 35.79[a] and 35.49[b] (2′), 19.10 (3)
4i		7.39–7.17 (m, 9H, arom.) 6.67 (s, 1H, 10), 3.87 (q, 1H, 2, $J = 6.62$), 3.57–3.46, 3.30–3.14[a] and 3.03–2.95[b] (3m, 1′, 4′), 1.89–1.62 (m, 8H, 2′, 3′), 1.27 (d, 3H, 3, $J = 6.77$) 171.51 (17), 153,46 (1), 142.55, 142.04, 141.81 (4, 8, 11), 129.15, 128.91, 127.99, 127.04, 126.79, 125.94, 125.10, (5–7, 9, 12–16), 76.85 (10), 46.45[a] and 46.18[b] (1′), 46.07[a] and 46.00[b] (4′), 43.71 (2), 25.98[a] and 25.72[b] (2′), 24.90[a] and 24.16[b] (3′), 20.31 (3)
4j		7.34–7.17 (m, 9H, arom.), 6.66 (d, 1H, 10, $J = 2.07$), 4.05 (q, 1H, 2, $J = 6,65$), 3.74–3.70, 3.59–3.43[a] and 3.21–3.01[b] (3m, 8H, 1′, 5′), 1.59–1.31, 1.18–1.07, 0.62–0.48 (3m, 12H, 2′–4′), 1.24 (dd, 3H, 3, $J = 3.15$, $J = 3.59$) 171.04 (17), 153,87 (1), 143.37, 142.06, 141.64 (4, 8, 11), 129.25, 128.90, 127.99, 127.08, 126.87, 126.66, 125.36, 125.16, 124.64 (5–7, 9, 12–16), 77.28 (10), 46.27[a] and 44.89[b] (1′), 42.81[a] and 42.79[b] (5′), 41.85 (2), 25.76[a] and 25.73[b] (2′), 25.51[a] and 24.40[b] (3′), 24.24 (4′), 21.10 (3)

[a] Signals of the carbamate atoms

[b] Signals of the amide atoms

based enzyme assay (Pontiki and Hadjipavlou-Litina, 2007). Lipoxygenases oxidize certain fatty acids at specific positions to hydroperoxides, precursors of leukotrienes, which contain a conjugated triene structure, i.e. soybean lipoxygenase converts linoleic to 13-hydroperoxylinoleic acid. Leukotrienes play an important role as mediators of a variety of inflammatory and allergic processes (Kühn *et al.,* 1990). Inhibitors of LOX have attracted attention initially as potential agents for the treatment of inflammatory and allergic diseases but their therapeutic potential has now been expanded to certain types of cancer and cardiovascular diseases (Pontiki and Hadjipavlou-Litina, 2005). Most of the LOX inhibitors are antioxidants or free radical scavengers, since lipoxygenation occurs via a carbon-centred radical (Muller, 1994). Perusal of IC_{50} values shows that compound **3b** is the most active by far, followed by compounds **4g**, **4d** and **4f** (IC_{50} = 21–95 μM). From Table 2 it is obvious that aromatic and cycloalkyl derivatives **4g**, **4d** and **4f** are more potent lipoxygenase inhibitors than the other amidocarbamates.

Inhibition of linoleic acid lipid peroxidation

Azo compounds generating free radicals through spontaneous thermal decomposition are useful for free radical production studies in vitro. The water soluble azo compound 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) has been extensively used as a clean and controllable source of thermally produced alkylperoxyl free radicals. In our studies, AAPH was used as a free radical initiator to follow oxidative changes of linoleic acid to conjugated diene hydroperoxide. The results indicated that all the compounds are excellent inhibitors of lipid peroxidation (LP) (54.5–99.5%), significantly higher than

Table 2 Interaction with DPPH, in vitro inhibition of soybean lipoxygenase (LOX) and lipid peroxidation (LP)

Compd.	DPPH 20 min[a] (%)	DPPH 60 min[a] (%)	DPPH 20 min[b] (%)	DPPH 60 min[b] (%)	LOX inhibition[b] (%)	LP inhibition[c] (%)	LP inhibition[b] (%)	c log P[d]	CMR[d]
3b	2.9	3.7	2.5	5.4	95.0[f]	15.0	98.0	6.05	14.16
4a	n.a.[e]	4.1	1.8	3.2	26.9	2.5	61.0	2.17	9.71
4b	2.5	3.7	2.3	1.1	22.3	n.a.	54.5	3.23	10.64
4c	3.4	5.6	n.a.	n.a.	40.8	49.2	99.3	5.71	14.74
4d	3.2	2.8	n.a.	2.3	69.6[f]	n.a.	95.2	4.76	12.76
4e	2.0	3.1	n.a.	n.a.	22.7	5.4	96.1	5.88	13.69
4f	1.7	3.1	1.6	5.2	56.6[f]	1.4	99.5	7.12	14.62
4g	4.2	5.3	n.a.	2.4	83.8[f]	n.a.	98.4	4.33	14.43
4h	3.6	8.5	n.a.	2.2	18.9	22.5	99.0	6.01	15.36
4i	5.7	6.5	2.6	2.7	12.7	16.4	77.2	4.70	11.84
4j	8.6	10	1.7	4.3	n.a.	25.5	97.4	5.81	12.76
Ketoprofen	6.4	3.1	8.1	7.2	n.d.[g]	n.d.	69.3	n.d.	n.d.
Caffeic acid	n.d.	n.d.	n.d.	n.d.	600	n.d.	n.d.	n.d.	n.d.
NDGA	81	83	93	97	n.d.	n.d.	n.d.	n.d.	n.d.
Trolox	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	63	n.d.	n.d.

Concentrations of the tested compounds: [a] 5×10^{-5} M; [b] 1×10^{-4} M; [c] 1×10^{-5} M

[d] Theoretically calculated values

[e] No activity under the reported experimental conditions

[f] IC_{50} value was also determined: 21 (**3b**), 86 (**4d**), 95 (**4f**), 32 (**4g**), 130 (Ket) μM

[g] Not determined

ketoprofen (69.3%) at 0.1 mM concentration (Table 2). This inhibition was found to be concentration dependent.

Regression analysis of the LP inhibition at 100 μM revealed that the overall molar refractivity (CMR) is the main physicochemical parameter influencing the inhibition. The linear CMR model suggests that the compounds with high CMR value will be more active. No correlation for lipophilicity was found.

$$\log\ \mathrm{LP}\% = 0.047(0.018)\ \mathrm{CMR} - 1.317(0.234)$$

$$n = 11,\ r = 0.894,\ r^2 = 0.800,\ q^2 = 0.680,$$
$$s = 0.045,\ F_{1,8} = 31.555,\ \alpha = 0.01$$

Antiviral and cytostatic evaluation

Antiviral evaluation was performed on a broad series of DNA and RNA viruses (as listed under the "Experimental" section). No antiviral effects were noted for any of the tested compounds against any of the viruses evaluated at subtoxic compound concentrations (data not shown). Only **4g** showed minor antiviral activity against vesicular stomatitis virus in HeLa cell cultures (EC_{50}: 3 μM), Sindbis virus (EC_{50}: 11 μM) and Punta Toro virus in Vero cell cultures (EC_{50}: 11 μM). However, the activity was found at compound concentrations close to their cytostatic activities (CC_{50}: 2.7–14 μM) (Table 3), pointing to a toxic rather than a specific antiviral effect. The compounds have also been evaluated for their cytostatic activity against murine leukaemia L1210, murine mammary carcinoma FM3A and human T-lymphoblast CEM cell cultures. The 50% inhibitory concentrations of the test compounds ranked between 2.7 and 422 μM depending on the nature of the compound and the tumour cell line evaluated (Table 3). The majority of the compounds show IC_{50} values around 10–25 μM (i.e. **4c**, **4d**, **4e**, **4f**, **4g**, **4h** and **4j**), pointing to a relatively minor role of the R-substituents on the core structure for cytostatic

Table 3 Cytostatic activity of the test compounds in cell cultures

Compd.	IC_{50} (μM)[a]		
	L1210	FM3A	CEM
3b	38 ± 1	194 ± 13	67 ± 31
4a	334 ± 76	10 ± 128	422 ± 110
4b	262 ± 15	275 ± 70	313 ± 25
4c	9.7 ± 0.2	11 ± 1	14 ± 0
4d	9.8 ± 0.2	12 ± 1	12 ± 2
4e	9.6 ± 0.1	15 ± 1	15
4f	11 ± 0	n.a.[b]	n.a.
4g	10 ± 1	14 ± 1	2.7 ± 0.3
4h	8.2 ± 1.0	13 ± 1	14
4i	41 ± 3	35 ± 5	42 ± 0
4j	15 ± 2	8.0 ± 5.1	13 ± 6

[a] Compound concentration required to inhibit tumour cell proliferation by 50%

[b] No activity under the reported experimental conditions

activity, as long as a bulky lipophilic (cyclic) entity has been present. Also, the presence of the amide groups might play an important role to eventually exert cytostatic potential.

Conclusions

A series of novel ketoprofen amidocarbamate derivatives **4a–j** were prepared and screened for antioxidative, antiviral and cytostatic activities. Antioxidative screenings revealed that the prepared compounds possess excellent LP inhibition. Compounds **3b** and **4g** also showed high soybean lipoxygenase inhibition activity. No selective antiviral effects were noted for the tested compounds against a broad variety of DNA and RNA viruses. Most compounds were endowed with a moderate cytostatic activity.

Experimental

Melting points were determined on a Stuart Melting Point Apparatus SMP3 and were uncorrected. IR spectra were recorded on a FTIR Perkin Elmer Paragon 500 spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Gemini 300 spectrometer, operating at 300 and 75.5 MHz for the ^{1}H and ^{13}C nuclei, respectively. Samples were measured in DMSO-d_6 solutions at 20°C in 5-mm NMR tubes. Chemical shifts (δ) were referred to TMS. Coupling constants (J) are given in Hz. Elemental analysis was determined on CHN-LECO-932. For thin-layer chromatography, precoated Merck silica gel 60 F254 and solvent system cyclohexane/ethyl acetate/methanol (3:1:0.5) were used. Spots were visualized by short-wave UV light and iodine vapour. Column chromatography was performed on Merck silica gel 0.063–0.200 mm with cyclohexane/ethyl acetate (1:2, 2:1 → 1:1, 1:1) as eluents. *O*-ethylhydroxylamine hydrochloride was obtained from Fluka and ketoprofen from PLIVA. All chemicals were purchased from Sigma-Aldrich. All solvents were of analytical grade purity and were dried prior to use.

*1-Benzotriazole carboxylic acid chloride (BtCOCl, **1**)*

Solution of benzotriazole (1.191 g, 10 mmol) and triphosgene (2.523 g, 8.5 mmol) in dry toluene was refluxed for 3 h. The reaction mixture was evaporated under reduced pressure. The crude product was used in the following reactions without further purification (Butula and Jadrijević Mladar Takač, 2000).

*2-(3-(Hydroxy(phenyl)methyl)phenyl)propanoic acid (**2**)*

2-(3-(Hydroxy(phenyl)methyl)phenyl)propanoic acid (**2**) was prepared by the catalytic hydrogenation of ketoprofen using H_2/Pd/C(en)/tetrahydrofurane (Hattori *et al.,* 2001), according to the modified published procedure (Allegretti *et al.,* 2003; 2005).

*2-(3-(Hydroxy(phenyl)methyl)phenyl)propanoic acid benzotriazolide (**3a**)*

To a solution of **2** (2.561 g, 10 mmol) and triethylamine (1.4 ml, 10 mmol) in dry toluene (20 ml), a solution of chloride **1** (1.696 g, 10 mmol) in dry toluene (20 ml) was added dropwise (0.25 h). The reaction mixture was stirred at room temperature for 1 h and washed four times with water. The organic layer was dried over anhydrous sodium sulphate, filtrated and evaporated. Thus, obtained crude product was purified by the trituration with ether. Yield: 2.681 g (75%); mp 96–99°C; IR (KBr): ν_{max} 3342, 3072, 3027, 3003, 2942, 2874, 1738, 1060, 1597, 1486, 1452, 1376, 959, 771, 751, 746, 710 cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ 8.26–8.21 (m, 2H, arom.), 7.80–7.75 (m, 1H, arom.), 7.62–7.57 (m, 1H, arom.), 7.52–7.50 (m, 1H, arom.), 7.33–7.14 (m, 8H, arom.), 5.88 (d, 1H, 11, $J = 4.00$ Hz), 5.65 (d, 1H, 10, $J = 3.83$ Hz), 5.32 (q, 1H, 2, $J = 6.89$ Hz), 1.63 (d, 3H, 3, $J = 6.94$ Hz); ^{13}C-NMR (DMSO-d_6) δ 173.50 (1), 146.82, 145.88, 145.83, 140.01, 131.18 (4, 8, 12, 1′, 6′), 131.39, 129.08, 128.45, 127.14, 127.02, 126.66, 126.63, 126.58, 125.84, 120.53, 114.44 (5–7, 9, 13–17, 2′–5′), 74.49 (10), 45.12 (2), 19.07 (3). Atom enumeration is given in Fig. 1. Anal. Calcd. for $C_{22}H_{19}N_3O_2$: C, 73.93; H, 5.36; N, 11.76. Found: C, 73.64; H 5.39; N, 11.39.

*2-(3-(N-benzotriazolcarbonyloxy)(phenyl)methyl) phenyl)propanoic acid benzotriazolide (**3b**)*

To a solution of **2** (2.561 g, 10 mmol) and triethylamine (3.9 ml, 28 mmol) in dry toluene (50 ml), a solution of chloride **1** (5.080 g, 28 mmol) in dry toluene (50 ml) was added dropwise (0.25 h). The reaction mixture was stirred at room temperature for 1 h and washed four times with water. The organic layer was dried over anhydrous sodium sulphate, filtrated and evaporated. Thus, obtained crude products was purified by the trituration with ether. Yield: 3.52 g (70%); mp 124–127°C; IR (KBr): ν_{max} 3091, 3032, 2980, 2937, 1764, 1732, 597, 1486, 1451, 1398, 1250, 1036, 951, 781, 760, 748, 708, 583 cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ 8.28–7.98 (m, 3H, arom.), 7.78–7.28 (m, 15H, arom.), 7.24 (s, 1H, 10), 5.38 (q, 1H, 2, $J = 6.84$ Hz), 1.66 (d, 3H, 3, $J = 6.94$ Hz); ^{13}C-NMR (DMSO-d_6) δ 173.28 (1), 147.91 (1″), 145.80, 145.77, 140.72, 140.02, 139.17, 131.73 (4, 8, 11, 1′, 6′, 2″, 7″), 131.36, 131.11, 129.83, 129.20, 128.29, 127.36, 127.23, 126.97, 126.79, 126.55, 126.38, 120.70, 120.45, 114.39, 113.67 (5–7, 9, 12–16, 2′–5′, 3″–6″), 81.48 (10), 45.04 (2), 18.98 (3).

Atom enumeration is given in Fig. 1. Anal. Calcd. for $C_{29}H_{22}N_6O_3$: C, 69.31; H, 4.41; N, 16.72. Found: C, 69.39; H 4.63; N, 16.99.

*(3-(1-(Methoxycarbamoyl)ethyl)phenyl)(phenyl)methyl methoxycarbamate (**4a**)*

A solution of benzotriazolide **3b** (0.251 g, 0.5 mmol), *O*-methylhydroxylamine hydrochloride (0.092 g, 1.1 mmol) and triethylamine (0.35 ml, 2.5 mmol) in toluene (5 ml) was stirred at room temperature for 10 h. The reaction mixture was extracted with brine (5 × 10 ml), 1% hydrochloric acid (1 × 5 ml) and washed with water till pH 7. The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure. Thus, obtained the crude product was purified by the column chromatography (eluent cyclohexane/ethyl acetate 1:2). Yield: 0.143 g (80%); oil; IR (film): ν_{max} 3455, 3217, 3065, 2978, 2938, 1725, 1668, 1606, 1489, 1454, 1254, 1115, 1043, 705 cm^{-1}. Anal. Calcd. for $C_{19}H_{22}N_2O_5$: C, 63.67; H, 6.19; N, 7.82. Found: C, 63.75; H 6.03; N, 7.48.

*(3-(1-(Ethoxycarbamoyl)ethyl)phenyl)(phenyl)methyl ethoxycarbamate (**4b**)*

A solution of benzotriazolide **3b** (0.251 g, 0.5 mmol), *O*-ethylhydroxylamine hydrochloride (0.107 g, 1.1 mmol) and triethylamine (0.35 ml, 2.5 mmol) in toluene (5 ml) was stirred at room temperature for 25 h. The reaction mixture was extracted with brine (5 × 10 ml), 1% hydrochloric acid (1 × 5 ml) and washed with water till pH 7. The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure. Thus, obtained the crude product was purified by the column chromatography (eluent cyclohexane/ethyl acetate 2:1 → 1:1). Yield: 0.140 g (73%); oil; IR (film): ν_{max} 3454, 3219, 3064, 2981, 2937, 2891, 1724, 1715, 1668, 1606, 1494, 1454, 1384, 1253, 1113, 1041, 704 cm^{-1}. Anal. Calcd. for $C_{21}H_{26}N_2O_5$: C, 65.27; H, 6.78; N, 7.25. Found: C, 65.34; H 6.43; N, 7.29.

*(3-(1-(Benzyloxycarbamoyl)ethyl)phenyl)(phenyl)methyl benzyloxycarbamate (**4c**)*

A solution of benzotriazolide **3b** (0.251 g, 0.5 mmol), *O*-benzylhydroxylamine hydrochloride (0.175 g, 1.1 mmol) and triethylamine (0.35 ml, 2.5 mmol) in toluene (5 ml) was stirred at room temperature for 48 h. The reaction mixture was extracted with brine (5 × 10 ml), 1% hydrochloric acid (1 × 5 ml) and washed with water till pH 7. The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure. The crude product was purified by the column chromatography (eluent cyclohexane/ethyl acetate 1:1). Yield: 0.115 g (45%); mp 42–45°C; IR (KBr): ν_{max} 3215, 3064, 3032, 2974, 2936, 1722, 1665, 1605, 1495, 1454, 1249, 1107, 1027, 749, 699 cm^{-1}. Anal. Calcd. for $C_{31}H_{30}N_2O_5$: C, 72.92; H, 5.92; N, 5.49. Found: C, 73.00; H 6.04; N, 5.77.

*(3-(1-(Cyclopentylcarbamoyl)ethyl)phenyl)(phenyl)methyl cyclopentylcarbamate (**4d**)*

A solution of benzotriazolide **3b** (0.251 g, 0.5 mmol), cyclopentylamine (0.109 ml, 1.1 mmol) and triethylamine (0.35 ml, 2.5 mmol) in toluene (5 ml) was stirred at room temperature for 1 h. The reaction mixture was extracted with brine (5 × 10 ml), 1% hydrochloric acid (1 × 5 ml) and washed with water till pH 7. The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure. The crude product was triturated with ether several times. Yield: 0.167 g (77%); mp 132–135°C; IR (KBr): ν_{max} 3305, 3269, 3065, 2962, 2870, 1700, 1651, 1606, 1545, 1452, 1249, 1040, 1017, 702 cm^{-1}. Anal. Calcd. for $C_{27}H_{34}N_2O_3$: C, 74.62; H, 7.89; N, 6.45. Found: C, 74.39; H 7.76; N, 6.28.

*(3-(1-(Cyclohexylcarbamoyl)ethyl)phenyl)(phenyl)methyl cyclohexylcarbamate (**4e**)*

A solution of benzotriazolide **3b** (0.251 g, 0.5 mmol), cyclohexylamine (0.126 ml, 1.1 mmol) and triethylamine (0.35 ml, 2.5 mmol) in toluene (5 ml) was stirred at room temperature for 5 h. The reaction mixture was extracted with brine (5 × 10 ml), 1% hydrochloric acid (1 × 5 ml) and washed with water till pH 7. The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure. The crude product was triturated with ether several times. Yield: 0.173 g (75%); mp 139–142°C; IR (KBr): ν_{max} 3303, 3265, 3066, 2933, 2854, 1695, 1650, 1603, 1547, 1450, 1235, 1042, 702 cm^{-1}. Anal. Calcd. for $C_{29}H_{38}N_2O_3$: C, 75.29; H, 8.28; N, 6.06. Found: C, 75.55; H 8.01; N, 6.16.

*(3-(1-(Cyclohexanemethylcarbamoyl)ethyl)phenyl) (phenyl)methyl cyclohexanemethylcarbamate (**4f**)*

A solution of benzotriazolide **3b** (0.251 g, 0.5 mmol), cyclohexanemethylamine (0.143 ml, 1.1 mmol) and triethylamine (0.35 ml, 2.5 mmol) in toluene (5 ml) was stirred at room temperature for 0.5 h. The reaction mixture was extracted with brine (5 × 10 ml), 1% hydrochloric acid (1 × 5 ml) and washed with water till pH 7. The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure. The crude product

was triturated with ether several times. Yield: 0.184 g (75%); mp 128–129°C; IR (KBr): ν_{max} 3340, 3278, 3064, 2922, 2851, 1707, 1654, 1604, 1551, 1449, 1249, 702 cm^{-1}. Anal. Calcd. for $C_{31}H_{42}N_2O_3$: C, 75.88; H, 8.63; N, 5.71. Found: C, 75.58; H 8.22; N, 5.99.

*(3-(1-(Benzylcarbamoyl)ethyl)phenyl)(phenyl)methyl benzylcarbamate (**4g**)*

A solution of benzotriazolide **3b** (0.251 g, 0.5 mmol), benzylamine (0.120 ml, 1.1 mmol) and triethylamine (0.35 ml, 2.5 mmol) in toluene (5 ml) was stirred at room temperature for 0.5 h. The reaction mixture was extracted with brine (5 × 10 ml), 1% hydrochloric acid (1 × 5 ml) and washed with water till pH 7. The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure. The crude product was triturated with ether several times. Yield: 0.127 g (53%); mp 107–109°C; IR (KBr): ν_{max} 3316, 3267, 3087, 3063, 3032, 2932, 1684, 1641, 1606, 1551, 1519, 1454, 1248, 699 cm^{-1}. Anal. Calcd. for $C_{31}H_{30}N_2O_3$: C, 77.80; H, 6.32; N, 5.85. Found: C, 77.66; H 6.48; N, 6.01.

*(3-(1-(Phenylethylcarbamoyl)ethyl)phenyl)(phenyl)methyl phenylethylcarbamate (**4h**)*

A solution of benzotriazolide **3b** (0.251 g, 0.5 mmol), phenylethylamine (0.139 ml, 1.1 mmol) and triethylamine (0.35 ml, 2.5 mmol) in toluene (5 ml) was stirred at room temperature for 0.6 h. The reaction mixture was extracted with brine (5 × 10 ml), 1% hydrochloric acid (1 × 5 ml) and washed with water till pH 7. The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure. The pure product was obtained after trituration with ether. Yield: 0.190 g (75%); oil; IR (KBr): ν_{max} 3417, 3313, 3063, 3028, 2972, 2932, 2872, 1713, 1699, 1660, 1650, 1604, 1538, 1517, 1496, 1454, 1248, 1030, 749, 700 cm^{-1}. Anal. Calcd. for $C_{33}H_{34}N_2O_3$: C, 78.23; H, 6.76; N, 5.53. Found: C, 78.47; H 6.44; N, 5.70.

*(3-(1-Oxo-1-(pyrrolidin-1-yl)propan-2-yl)phenyl)(phenyl)methyl pyrrolidine-1-carboxylate (**4i**)*

A solution of benzotriazolide **3b** (0.251 g, 0.5 mmol), pyrrolidine (0.092 ml, 1.1 mmol) and triethylamine (0.35 ml, 2.5 mmol) in toluene (5 ml) was stirred at room temperature for 0.5 h. The reaction mixture was extracted with brine (5 × 10 ml), 1% hydrochloric acid (1 × 5 ml) and washed with water till pH 7. The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure. The crude product was purified by the column chromatography (eluent cyclohexane/ethyl acetate 1:1). Yield: 0.152 g (75%); oil; IR (film): ν_{max} 3061, 3030, 2973, 2875, 1700, 1643, 1634, 1588, 1454, 1416, 1126, 1096, 764, 709 cm^{-1}. Anal. Calcd. for $C_{25}H_{30}N_2O_3$: C, 73.86; H, 7.44; N, 6.89. Found: C, 73.57; H 7.67; N, 6.80.

*(3-(1-Oxo-1-(piperidin-1-yl)propan-2-yl)phenyl)(phenyl)methyl piperidine-1-carboxylate (**4j**)*

A solution of benzotriazolide **3b** (0.251 g, 0.5 mmol), piperidine (0.109 ml, 1.1 mmol) and triethylamine (0.35 ml, 2.5 mmol) in toluene (5 ml) was stirred at room temperature for 0.75 h. The reaction mixture was extracted with brine (5 × 10 ml), 1% hydrochloric acid (1 × 5 ml) and washed with water till pH 7. The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure. After trituration with ether afforded the pure product. Yield: 0.179 g (80%); mp 106–109°C; IR (KBr): ν_{max} 3050, 3028, 2971, 2940, 2852, 1694, 1628, 1587, 1469, 1426, 1258, 1236, 1148, 1083, 1026, 707, 699 cm^{-1}. Anal. Calcd. for $C_{27}H_{34}N_2O_3$: C, 74.62; H, 7.89; N, 6.45. Found: C, 74.36; H 7.63; N, 6.55.

Interaction with DPPH

To a solution of DPPH (0.05 mM) in absolute ethanol an equal volume of 0.1 or 0.05 mM ethanolic solution of the tested compound was added (Pontiki and Hadjipavlou-Litina, 2007). After 20 and 60 min the absorbance was recorded at 517 nm and compared to the appropriate standard NDGA (Table 2). Ethanol was used as a control. Each in vitro experiment was performed at least in triplicate and the standard deviation of absorbance was <10% of the mean.

Soybean lipoxygenase inhibition

DMSO solution of the tested compound was incubated with sodium linoleate (0.1 mM) and 0.2 ml of soybean lipoxygenase solution ($1/9 \times 10^{-4}$ w/v in saline) at room temperature (Pontiki and Hadjipavlou-Litina, 2007). The conversion of sodium linoleate to 13-hydroperoxylinoleic acid was recorded at 234 nm and compared to the standard inhibitor caffeic acid, according to the procedure previously reported.

Inhibition of linoleic acid lipid peroxidation

Oxidation of linoleic acid to conjugated diene hydroperoxide in an aqueous dispersion is monitored at 234 nm (Re *et al.*, 1999). AAPH was used as a free radical initiator. Ten microliters of the 16 mM linoleic acid dispersion was

added to the UV cuvette containing 0.93 ml of 0.05 M phosphate buffer, pH 7.4 prethermostated at 37°C. The oxidation reaction was initiated at 37°C under air by the addition of 50 μl of 40 mM AAPH solution. Oxidation was carried out in the presence of compounds (10 μl, final concentration 0.1 mM). In the assay with no antioxidant lipid oxidation was measured in the presence of the same level of DMSO. The rate of oxidation was monitored at 37°C by recording the increase of absorption at 234 nm caused by conjugated diene hydroperoxides. The results were compared to the standard inhibitor trolox.

Antiviral and cytostatic activity assays

Murine leukaemia L1210, murine mammary carcinoma FM3A and human T-lymphocyte CEM cells were suspended at 300,000–500,000 cells/ml of culture medium, and 100 μl of a cell suspension was added to 100 μl of an appropriate dilution of the test compounds in wells of 96-well microtiter plates. After incubation at 37°C for 2 (L1210, FM3A) or 3 (CEM) days, the cell number was determined using a Coulter counter. The IC_{50} was defined as the compound concentration required to inhibit cell proliferation by 50%.

The antiviral assays, other than the anti-HIV assays, were based on inhibition of virus-induced cytopathicity in HEL [herpes simplex virus type 1 (HSV-1) (KOS), HSV-2 (G), vaccinia virus and vesicular stomatitis virus], Vero (parainfluenza-3, reovirus-1, Sindbis, Coxsackie B4, and Punta Toro virus), HeLa (vesicular stomatitis virus, Coxsackie virus B4, and respiratory syncytial virus), feline kidney Crandell cells [feline coronavirus (FeCoV)] (FIPV strain) and feline herpes virus (FeHV) or MDCK [influenza A (H1N1, H3N2) and influenza B] cell cultures. Most viruses have been obtained from ATCC (Rockville, MD). HIV-1(III_B) was provided by R. C. Gallo (at that time at NIH, Bethesda, MD) and HIV-2(ROD) was provided by L. Montagnier (at that time at the Pasteur Institute, Paris, France). FCoV (FIPV) and FeHV were kindly provided by H. Egberink, Utrecht, The Netherlands. Confluent cell cultures in microtiter 96-well plates were inoculated with 100 CCID50 of virus (CCID50 being the virus dose to infect 50% of the cell cultures). After a 1 h virus adsorption period, residual virus was removed, and the cell cultures were incubated in the presence of varying concentrations (200, 40, 8, … μM) of the test compounds. Viral cytopathicity was recorded as soon as it reached completion in the control virus infected cell cultures that were not treated with the test compounds. The methodology of the anti-HIV assays was as follows: human CEM ($\sim 3 \times 10^5$ cells/ml) cells were infected with 100 CCID50 of HIV-1(IIIB) or HIV-2(ROD)/ml and seeded in 200 μl wells of a microtiter plate containing appropriate dilutions of the test compounds. After 4 days of incubation at 37°C, HIV-induced CEM giant cell formation was examined microscopically.

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