Full Paper

Investigations Concerning the Correlation of COX-1 Inhibitory and Hydroxyl Radical Scavenging Activity

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The aim was to study the COX-1 inhibiting efficacy in context with hydroxyl radical scavenging properties of compounds bearing a carboxylic acid and ester function, respectively. In general, the acids are more potent radical scavengers than the corresponding esters but there is no clear correlation with their COX-1 inhibiting potencies. A feasible scavenging mechanism of carboxylic acids is discussed.

Keywords: Carboxylic acids / COX-1 / Hydroxyl radical scavenging / SAR

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Introduction

A wide range of drugs including NSAIDs, glucocorticoids or immunosuppressive drugs are used for therapy of chronic inflammatory diseases like rheumatoid arthritis, Crohn's disease and others. An additional strategy could be the scavenging of reactive oxygen species (ROS).

In Alzheimer's disease, the accumulation of iron in the hippocampus and cerebral cortex leads to an increased formation of $^{\bullet}$OH through the Fenton reaction [1] and for this reason to an increase of oxidative damage in brain tissue.

In rheumatoid synovitis, the cavity pressure is raised and exceeds the capillary perfusion pressure. This causes a collapse of blood vessels leading to a higher generation of ROS. Super oxide anion and hydrogen peroxide do not damage most of macromolecules but can be easily converted into the highly reactive hydroxyl radical which oxidizes IgGs, lipids and lipoproteins [2].

The formation of hydroxyl radicals is also increased in the progression of Parkinson's disease. High levels of hydrogen peroxide are formed through degradation of dopamine by monoamine oxidases in the substantia nigra (SN). High levels of iron can be found in SN, too. This presence of iron leads to an increased formation of hydroxyl radicals through the Fenton reaction leading to an increased nigral cell degeneration [3].

We describe the synthesis of pyrrole and isothiazole derivatives with carboxylic acid and ester functions, respectively, and their hydroxyl radical scavenging activity. To correlate $^{\bullet}$OH radical scavenging and COX-1 inhibitory potencies typical acidic COX-1 inhibitors such as diclofenac, acetylsalicylic acid (ASA) and indomethacin were tested, too. We also present structure-activity relationships (SAR) and a proposal for the hydroxyl radical scavenging mechanism.

Chemistry

Compounds **4**, **5**, **7** and **8** were synthesized starting from 3-chloro-2,3-di(4-methoxyphenyl)acrylaldehyde **1** (Scheme 1). The synthesis of **1** was described previously [4]. Compound **1** was converted with ammonium thiocyanate analogous to reference [5] to 4,5-di(4-methoxyphenyl)-isothiazole **2**. Compound **2** was *N*-alkylated with ethyl 2-bromoacetate to yield **3** [6] in moderate yields

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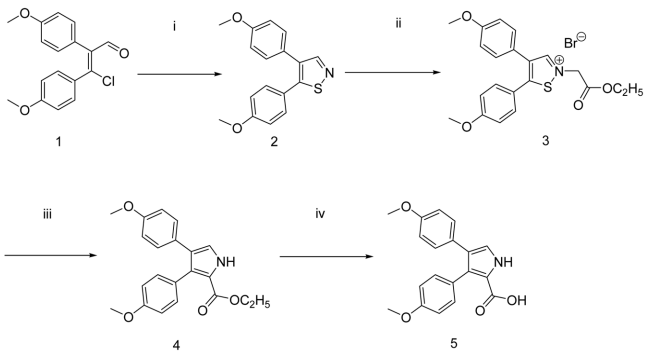
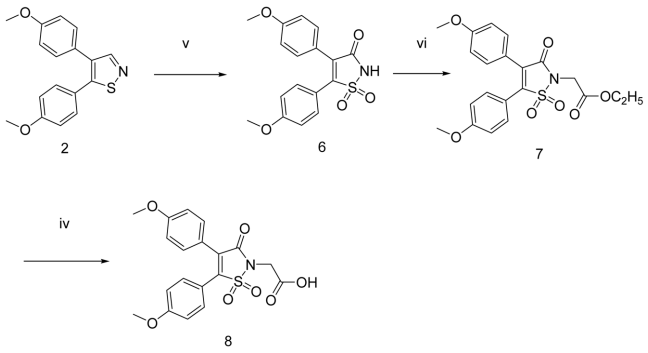
Abbreviations: reactive oxygen species (ROS); 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO); electron paramagnetic resonance (EPR)



Reaction conditions: i) NH_4SCN, acetone, reflux 3.5 h, $NaHCO_3$; ii) $BrCH_2CO_2Et$, EtOH, reflux, 6 h; iii) Et_3N; iv): EtOH, KOH, 18-crown-6, 60°C, 14 h.

Scheme 1. Synthesis route of compounds **1** – **5**.



Reaction conditions: v) H_2O_2, glacial acid, 20 min, 80°C; vi) DMF, NaH, 10 min, $BrCH_2CO_2Et$; iv) EtOH, KOH, 18-crown-6, 60°C, 14 h.

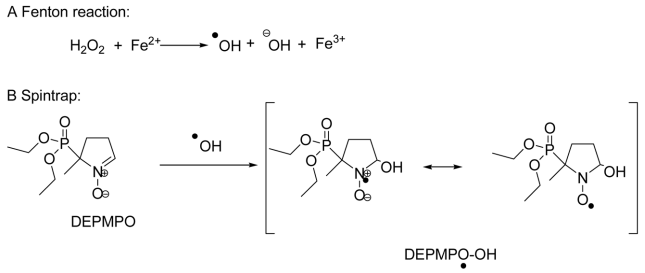
Scheme 2. Synthesis route of compounds **6** – **8**.

(60%). Treatment of **3** with trimethylamine led to the pyrrole derivative **4** (50%) using a method described by Rolfs *et al*. and Schmidt *et al.* [7, 8]. This compound was previously prepared by Gupton *et al.* [9] using a different method. Compound **4** was saponified using potassium hydroxide according to ref. [10] obtaining the carboxylic acid **5**. ^{1}H-NMR single frequency decoupling (sfd) experiments and nuclear Overhauser effect (NOE) spectroscopy studies of **5** confirmed the chemical shift of NH (11.77 ppm) and OH (12.05 ppm) as well as both aromatic AB systems (7.06 ppm/6.81 ppm and 6.94 ppm/6.72 ppm).

Compound **2** (Scheme 2) was oxidized with hydrogen peroxide [11] to yield the isothiazole-3(2*H*)on-1,1-dioxide derivative **6**. In the next step, *N*-alkylation of **6** to **7** [6] and subsequent saponification [10] results in formation of the carboxylic acid **8**.

Results and discussion

Compounds **4**, **5**, **7** and **8** have been designed in context with diaryl isoselenazoles [4] as dual COX/LOX inhibitors showing additional hydroxyl radical scavenging potencies.



Scheme 3. **A**: Fenton reaction as hydroxyl radical source; **B**: Spin trap reaction of DEPMPO with •OH.

The Fenton reaction was used as hydroxyl radical source and 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) was used as spin trap reagent (Scheme 3, see Experimental). To quantify the impact of the test compounds, the spin-trap method was used [12]. Figure 1 shows the effect of thioctic acid as potent hydroxyl radical scavenger.

Table 1 summarises the impact of the carboxylic acid compared to the corresponding esters. Compounds **5**, **8**, oxalic acid, thioctic acid and even acetic acid showed significant •OH scavenging efficacies in our standard electron paramagnetic resonance (EPR) assay [6, 13, 14]. In each case, the acids are much more potent than the esters. The highest increase in activity was found comparing the ester **4** (10% inhibition at 100 μM) and the corresponding acid **5** (IC_{50}: 14 μM) as well as diethyl oxalate (25% inhibition at 100 μM) and oxalic acid (IC_{50}: 8.7 μM). The esters **4**, **7**, diethyl oxalate and ethyl acetate possessed no or poor activity, and ethyl thioctate (IC_{50}: 57 μM) showed a fivefold reduced potency compared to the free acid (IC_{50}: 12 μM). The relative high potency of ethyl thioctate might be explained by the scavenging potency of the 1,2-dithiolane moiety of the molecule. Stary *et al*. published that α-thioctic acid can be oxidised by singlet oxygen to its sulfoxide derivative [15] – a mechanism which has been also confirmed by Mattern using the Fenton reaction [14].

Based on these results, we investigated the scavenging mechanism of the acids. The first assumption that acids may act as complexing agents for iron ions could be disproved using instead of a $FeSO_4$ solution a fresh prepared $FeSO_4$/EDTA solution in our EPR assay. EDTA forms a very stable complex with the iron ions so the iron ions should not be inactivated by reaction with the acids. Furthermore, the EDTA/Fe^{2+}-complex increases the production of •OH boosting the reduction potency of Fe^{2+}. This leads to intensified DEPMPOOH signals [16].

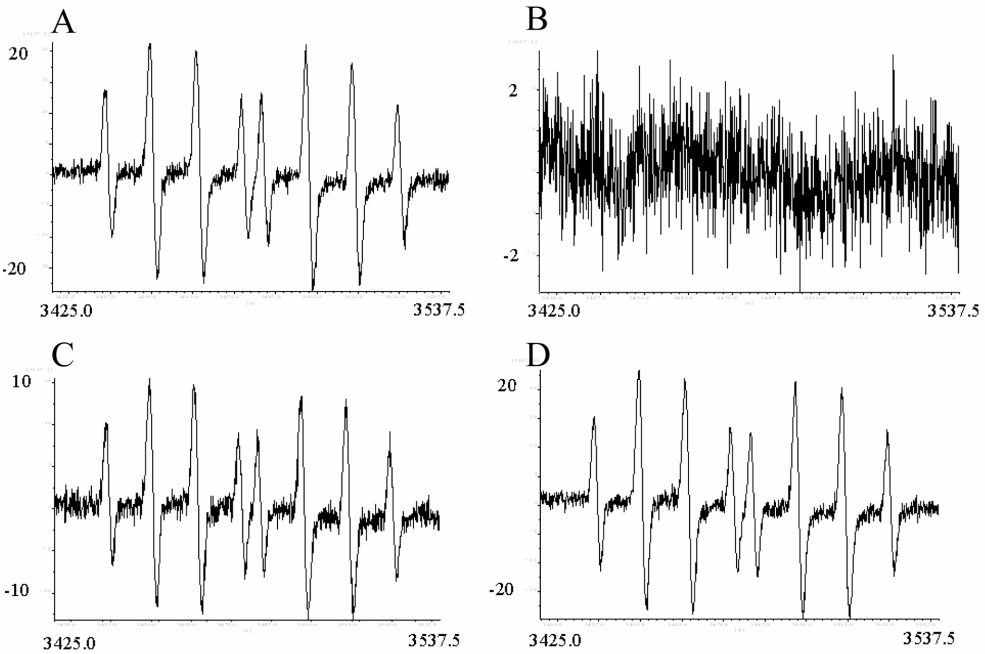
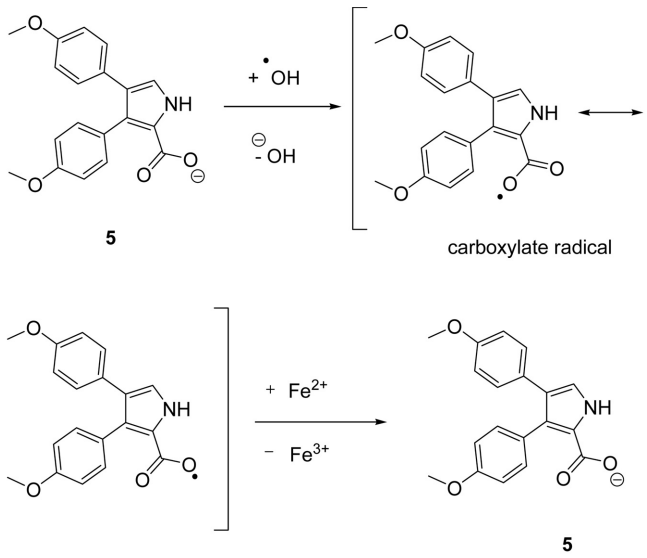


Figure 1. EPR spectra: **A**: acetonitrile control (0% inhibition); **B**: thioctic acid 100 μM (100% inhibition); **C**: thioctic acid 10 μM (~45% inhibition), **D**: thioctic acid 1 μM (~0% inhibition); (for details see Experimental).

According to these results (Table 1), no significant difference of the •OH scavenging efficacy have been found using the modified EPR assay indicating that •OH scavenging efficacy is not due to formation of iron in complexes with the acids.

Additionally, we investigated compounds **5** and **8** using a modified EPR protocol to obtain informations about the likewise scavenging mechanism. The compound concentrations were scaled up by factor 100, the reaction was stopped after 90 s by the addition of saturated sodium thiosulfate solution in order to inactivate hydrogen peroxide. The compounds were extracted by dichloromethane, and mass analysis was performed via FD-MS. The FD-MS spectrum of compound **5** showed only the molecule ion (323.7 Da). The mass analysis of **8** revealed that in addition to the molecule peak of **8** (403.5 Da, 100% relative intensity), two weak signals (100 Da: 9.8% relative intensity, 367 Da: 6.75% relative intensity) could be observed. This is in contrary to the expected OH binding to these molecules. Thus, it is likely that carboxylic acids may inactivate •OH by an alternative mechanism.

In order to elucidate the mechanism, we refer to a couple of publications which identified acids as •OH-scavengers. This is in line for 5,6-dihydroxyindole-2-carboxylic acid (DHICA) which was more powerful than 5,6-dihydroxyindole (DHI) on the Fenton-promoted oxidation of deoxyribose [17].

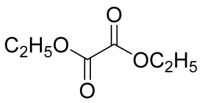
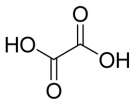
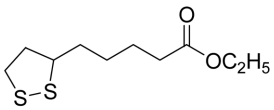
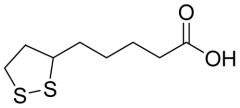
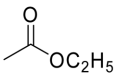
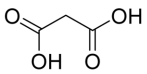
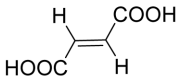
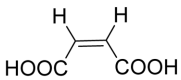


Scheme 4. Proposed •Oh scavenging mechanism of **5**; conditions: 100 μM compound **5**, 200 μM DEPMPO, 25 μM H_2O_2 and 50 μM $FeSO_4$ in 200 μM H_2O.

Under the pH conditions used (~10.4), the formed carboxylate ions may react with hydroxyl radicals producing carboxylate radicals which subsequently can be reduced by excessive Fe^{2+} ions (Scheme 4) which were added in a twofold excess.

Despite the inactivation of hydroxyl radicals, there is no correlation of the COX-1 inhibiting efficacies as sug-

Table 1. Hydroxyl radical scavenging potencies.

Compound	•OH radical assay[a] IC_{50} (μM)	•OH radical assay[a] EDTA, IC_{50} (μM)	COX-1 IC_{50} (μM)
4	10%*	n.t.	0.075
5	14	11	10.8
7	0%*	n.t.	0.11
8	71%*	79%*	2.4
	25%*	n.t.	n.t.
	8.7	7.4	n.t.
	57	55	n.t.
	12	10	26% [10 μM]
	0%*	n.t.	n.t.
	54%*	n.t.	n.t.
	83%*	n.t.	n.t.
	8.6 (89%*)	90%*	n.t.
	88%*	88%*	n.t.
	88%*	93%*	n.t.
diclofenac	85	n.t.	0.001
ASA	27	n.t.	0.43
indomethacin	13	n.t.	0.004

[a] Values are means of three determinations.
* Inhibition at a concentration of 100 μM.
n. t.: not tested.

gested by our test results of the compounds (Table 1): diclofenac and indomethacin behaved both as potent COX-1 inhibitors (IC_{50} COX-1: 0.001 vs. 0.004 μM) but indomethacin inhibited the hydroxyl radicals over sixfold more potent than diclofenac (IC_{50} OH radical assay: 13 μM vs. IC_{50} OH radical assay: 85 μM), almost as potent as thioctate (IC_{50} OH radical assay: 12 μM). Interestingly, thioctate provided only a weak COX-1 inhibitors activity (26% inhibition at a concentration of 10 μM). The weak COX-1 inhibitor ASA (IC_{50} COX-1: 0.43 μM) showed a good ROS inhibiting activity (IC_{50} OH radical assay: 27 μM).

Compound **5** (IC_{50} OH radical assay: 14 μM) displayed a good ROS inhibiting activity equipotent to indomethacin but showed a weak activity in our COX-1 assay (IC_{50} COX-1: 10.8 μM). In contrast, compound **7** exhibited a potent COX-1 inhibitory activity (IC_{50} COX-1: 0.1 μM) without showing ROS scavenging activity (0% at a concentration of 100 μM). Noticeable is the fact, that with regard to the COX-1 inhibitory activities, the esters **4** and **7** (IC_{50} COX-1: 0.075 vs. IC_{50} COX-1: 0.11 μM) are much more potent than the corresponding free acids **5** and **8** (IC_{50} COX-1: 10.8 vs. IC_{50} COX-1: 2.4 μM).

In conclusion, the hydroxyl radical scavenging potency of compounds bearing a carboxylic acid moiety has taken into account testing potential new NSAIDs but the impact of it to the overall inflammatory potency depends on the complete chemical structure of the drugs.

The authors have declared no conflict of interest.

Experimental

Enzyme assay

COX-1 inhibition was determined by using platelets isolated from bovine blood, incubated with the test substance, and stimulated by the Ca ionophor A23187. The inhibition of COX-1 was assessed by quantitative HPLC determination of the formation of 12-hydroxyheptadecatrienoic acid [18].

EPR spin trapping assay

Standard EPR assay

Assay for hydroxyl radical scavenging: DEPMPO was purchased from Calbiochem (San Diego, CA, USA). The test solution was prepared by adding 2 μL of a solution of the test substance in acetonitrile (concentration 10, 1 or 0.1 mM) or acetonitrile, as zero value, to 192 ml demineralized water in a 1.5 ml micro-centrifuge tube. DEPMPO solution (2 μL; 20 mM), 2 μL $FeSO_4$ solution (5 mM) and 2 μL hydrogen peroxide solution (2.5 mM) were put on different points on the wall of the tube. The reaction was started by vortexing the tube. After 10 s, a sample was gathered with a 100 μL ringcap from Hirschmann1 Laborgeräte and closed with wax. After exactly 90 s, the EPR experiment was started. Instrumental parameters were as follows: receiver gain 3.99×10^5, modulation amplitude 3.0 G, time constant 0.64 ms, conversion time 81.92 ms, attenuation 4 ms. Spectra were analysed with the WinEPR1 (Version 1.0) software. The raw data were integrated, the background noise was subtracted and the heights of the second peaks were analysed. Each concentration was tested three times. Thioctic acid in test concentrations of 100, 10 and 1 μM was used as standard substance in every test series. For an amount of 30 spectra totalling six zero values as controls were analysed.

Modified EPR assay

The difference of the modified EPR assay was the usage of 2 μL fresh prepared $FeSO_4$ (5 mM)/EDTA (5 mM) solution instead of the $FeSO_4$ solution.

Scaled up $^{\bullet}$OH scavenging assay

All reagent amounts of the standard assay, except for the not required DEPMPO solution were multiplicated by factor 100. The following amounts were added to 19.4 ml water: 0.2 ml compound solution (100 μM), 0.2 ml $FeSO_4$ solution (50 μM) and 0.2 ml hydrogen peroxide (25 μM). The reaction was stopped after 90 s with 2 ml saturated sodium thiosulfate solution. The preparation was extracted with dichloromethane (3×20 ml). The organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was analysed by FD-MS.

Chemicals, materials and spectroscopy

The EPR spectra were acquired on a Bruker EMX EPR spectrometer (Bruker Bioscience, USA). Melting points were determined with a Büchi apparatus according to Dr. Tottoli and are uncorrected (Büchi Labortechnik, Flawil, Switzerland). ^{1}H-NMR spectra (300 MHz) were recorded on a Bruker AC 300 spectrometer (Bruker). Column chromatography was performed with Merck silica gel 60 (0.063 – 0.200 mm; Merck, Germany). The progress of the reactions was monitored by thinlayer chromatography (TLC) performed with Merck silica gel 60 F_{245} plates. All reagents and solvents were obtained from commercial sources and used as received. Reagents were purchased from Sigma-Aldrich Chemie Steinheim, Germany, or Acros, Nidderau, Germany.

Chemistry

*4,5-Di(4-methoxyphenyl)-isothiazole **2***

Compound **2** was prepared in a manner analogous to reference [5]. A mixture of 3-chloro-acrylaldehyde **1** (33.34 mmol) and ammonium thiocyanate (100 mmol) in acetone (80 ml) was heated under reflux for 3.5 h (caution HCN formation!). After cooling to rt, the mixture was poured into a solution of saturated aqueous sodium hydrogen carbonate (100 ml) and extracted three times with ether. The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. After column chromatography (silica gel, ethyl acetate/petroleum ether 3 : 7) 60% of the isothiazole **2** was obtained as white solid; mp. 81°C, ^{1}H-NMR: DMSO-d_6, 300 MHz, δ [ppm] = 8.61 (s, 1H, CHN), 7.245 (d, 8.69 Hz, 4H, Ar-H), 6.95 (t, 8.95 Hz, 4H, Ar-H), 3.76 (s, 3H, OCH_3), 3.75 (s, 3H, OCH_3); FD-MS *m/z*: 297.6 [M^+]. Anal. calcd. for $C_{17}H_{15}NO_2S$ (%): C 68.66, H 5.08, N 4.71, S 10.78. Found (%): C 69.01, H 5.00, N 4.64, S 10.62.

*Ethyl 4,5-di(4-methoxyphenyl)isothiazoliumbromid-2-acetat **3***

Compound **3** was prepared in a manner analogous to reference [6]. A mixture of **2** (10 mmol) and ethyl 2-bromoacetate (30 mmol) in 50 ml dry ethanol was heated under reflux for 6 h. The preparation was concentrated under reduced pressure. The remaining brown oil was dissolved in dichloromethane. In the next step, ether was added to the solution and compound **3** crystallized in green needles (yield: 60%). The crystallization process has to be repeated until the product is clean; mp. 132°C, ^{1}H-NMR: DMSO-d_6, 300 MHz, δ [ppm] = 9.46 (s, 1H, CHN), 7.42 (d, 8.74 Hz, 2H, Ar-H), 7.29 (d, 8.71 Hz, 2H, Ar-H), 7.09 (d, 8.8 Hz, 2H, Ar-H), 7.04 (d, 8.75 Hz, 2H, Ar-H), 5.69 (s, 2H, NCH_2-COO), 4.38 (q, 7.11 Hz, 7.11 Hz, 7.14 Hz, 2H, OCH_2), 3.82 (s, 3H, OCH_3), 3.78 (s, 3H, OCH_3), 1.28 (t, 7.11 Hz, 7.11 Hz, 3H, CH_3).

*Ethyl 3,4-di(4-methoxyphenyl)-1H-pyrrol-2-carboxylate **4***

Compound **4** was prepared in a manner analogous to reference [7, 8]. Compound **3** was suspended in 5 ml trimethylamine and heated until it dissolved. The preparation was quenched with water. The precipitate was washed with ice cold ether and recrystallized in ethanol to obtain compound **4** in yields of 50% as fine white needles; mp. 136°C, ^{1}H-NMR: DMSO-d_6, 300 MHz, δ [ppm] = 11.88 (s, 1H, NH), 7.14 (d, 3.03 Hz, 1H, CHN), 7.06 (d, 8.51 Hz, 2H, Ar-H), 6.97 (d, 8.55 Hz, 2H, Ar-H), 6.83 (d, 8.57 Hz, 2H, Ar-H), 6.73 (d, 8.65 Hz, 2H, Ar-H), 4.06 (q, 7.05 Hz, 7.05 Hz, 7.05 Hz, 2H, OCH_2), 3.74 (s, 3H, OCH_3), 3.67 (s, 3H, OCH_3), 1.08 (t, 7.04 Hz, 7.04 Hz, 3H, CH_3); FD-MS *m/z*: 351.2 [M^+]. Anal. calcd. for $C_{21}H_{21}NO_4$ (%): C 71.78, H 6.02, N 3.99. Found (%): C 72.82, H 5.79, N 3.93.

*General procedure for the synthesis of the acetic acid derivatives **5** and **8***

Compounds **4** or **7** (3 mmol) and 1 ml of 18-crown-6 were dissolved in ethanol (6 ml). Potassium hydroxide solution (6 ml; 20% w/w) was added and the mixture was heated (60°C) for 14 h. The solution was cooled down on an ice bath and acidified with diluted chlorine acid (2 mol/L). The preparation was extracted with dichloromethane (3 × 20 ml) and the combined organic layers were concentrated under reduced pressure to obtain **5** or **8** in yields of 80%. The crystallization process has to be repeated until the product was clean.

*3,4-Di(4-methoxyphenyl)-1H-pyrrol-2-carboxylic acid **5***

Mp. 184°C, ^{1}H-NMR: DMSO-d_6, 300 MHz, δ [ppm] = 12.05 (s, 1H, COOH), 11.77 (s, 1H, NH), 7.06 (m, 3H, CHN, Ar-H), 6.94 (d, 7.8 Hz, 2H, Ar-H), 6.81 (d, 7.5 Hz, 2H, Ar-H), 6.72 (d, 7.6 Hz, 2H, Ar-H), 3.73 (s, 3H, OCH_3), 3.66 (s, 3H, OCH_3); FD-MS *m/z*: 323.3 [M^+]. Anal. calcd. for $C_{19}H_{17}NO_4$ (%): C 70.58, H 5.30, N 4.33. Found (%): C 69.42, H 5.01, N 4.20.

*2-(4,5-Di(4-methoxyphenyl)-isothiazole-3(2H)on-1,1-dioxide-2-yl)acetic acid **8***

Mp. 202°C, ^{1}H-NMR: $CDCl_3$, 300 MHz, δ [ppm] = 7.51 (d, 8.91 Hz, 2H, Ar-H), 7.38 (d, 8.85 Hz, 2H, Ar-H), 6.92 – 6.85 (m, 4H, Ar-H), 6.42 (bs, 1H, COOH), 4.48 (s, 2H, NCH_2), 3.83 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3); FD-MS *m/z*: 403.4 [M^+].

*4,5-Di(4-methoxyphenyl)-isothiazole-3(2H)-on-1,1-dioxide **6***

Compound **2** (20 mmol) was dissolved in glacial acetic acid and heated to 80°C. In a period of two minutes, 20 ml hydrogen peroxide (30% v/v) was added dropwise. The preparation was stirred for 20 min at 80°C. After this period, the solution was cooled down. Product **6** precipitates as yellow solid. The precipitate was filtered and washed with an acidified and saturated sodium thiosulfate solution (freshly produced and filtered). The product was purified by recrystallization in diluted acetic acid. Mp. 311°C, ^{1}H-NMR: DMSO-d_6, 300 MHz, δ [ppm] = 7.39 (d, 8.9 Hz, 2H, Ar-H), 7.3 (d, 8.85 Hz, 2H, Ar-H), 7.01 (d, 8.9 Hz, 2H, Ar-H), 6.95 (d, 8.85 Hz, 2H, Ar-H), 3.77 (s, 3H, OCH_3), 3.76 (s, 3H, OCH_3); FD-MS *m/z*: 345.4 [M^+].

*Ethyl-2-(4,5-di(4-methoxyphenyl)-isothiazole-3(2H)-on-1,1-dioxide-2-yl)-acetate **7***

Compound **6** (5 mmol) was dissolved in 20 ml absolute DMF, and 15 mmol of sodium hydride was added slowly. The mixture became brown and was stirred for 10 min. After this period, 7.5 mmol of ethyl 2-bromoacetate was added and the composition was stirred until the reaction was completed (TLC control). The preparation was concentrated under reduced pressure and purified by column chromatography (silica gel, ethyl acetate/petroleum ether 1 : 2) to yield 45% of compound **7**; m.w. 431.4; mp. 124°C, ^{1}H-NMR: $CDCl_3$, 300 MHz, δ [ppm] = 7.52 (d, 8.99 Hz, 2H, Ar-H), 7.38 (d, 8.95 Hz, 2H, Ar-H), 6.89 (d, 8.99 Hz, 2H, Ar-H), 6.88 (d, 8.96 Hz, 2H, Ar-H), 4.42 (s, 2H, NCH_2), 4.27 (q, 7.11 Hz, 7.11 Hz, 7.09 Hz, 2H, OCH_2), 3.83 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 1.31 (t, 7.13 Hz, 7.13 Hz, 3H, CH_3); FD-MS *m/z*: 431.4 [M^+]. Anal. calcd. for $C_{21}H_{21}NO_7S$ (%): C 58.46, H 4.91, N 3.25, S 7,43. Found (%): C 58.37, H 5.06, N 2.99, S 7.34.

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