Rahmana E. Kartasasmita[a,b], Stefan Laufer[c], Jochen Lehmann[a]

[a] Institute of Pharmacy, University of Bonn, Bonn, Germany
[b] Department of Pharmacy, Bandung Institute of Technology, Bandung, Indonesia
[c] Institute of Pharmacy, University of Tübingen, Tübingen, Germany

NO-Donors (VII [1]): Synthesis and Cyclooxygenase Inhibitory Properties of *N*- and *S*-Nitrooxypivaloyl-cysteine Derivatives of Naproxen – A Novel Type of NO-NSAID

Nitric oxide (NO) has been reported to subserve many of the same mucosal protection mechanisms as prostaglandins and is sufficient for acute gastroprotection and ulcer healing. In fact, NO-donating NSAID hybrid compounds such as the nitrooxybutyl ester of naproxen show reduced ulcerogenic activity while maintaining anti-inflammatory activity. We introduce two prototypes of novel triple-hybrid compounds consisting of cysteine which is known to enhance the activity of organic nitrates and to reduce nitrate tolerance, an NSAID (naproxen), and an organic nitrate (nitrooxypivaloic acid). L-Cysteine ethyl ester first was *N*-acylated in a CH_2Cl_2/H_2O two-phase system using the acid chlorides of naproxen or nitrooxypivaloic acid, respectively, and sodium acetate, or alternatively using the DCC-activated nitrooxy acid in absolute CH_2Cl_2. The *N*-acylated intermediates were subsequently *S*-acylated using the acid chlorides or alternatively the carbonyldiimidazole (CDI)-activated acids again. The two naproxen-cysteine-nitrate hybrid prodrugs were screened *in vitro* for their cyclooxygenase inhibitory properties relative to naproxen. In this screening the *N*-nitrooxyacylcysteine derivative was found to be inactive in the concentration range of 0.1–10 µmol/L against both COX-1 and COX-2, while the *S*-nitrooxyacylcysteine derivative had only weak activity against COX-1.

Keywords: Naproxene; Organic nitrates; Cysteine; Cyclooxygenase inhibition; Inflammation

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Introduction

The application of non-steroidal anti-inflammatory drugs (NSAID's) is limited by their side effects on the gastrointestinal tract (GIT) [2–4]. One approach to improve the safety of NSAIDs is inhibition of COX-2 instead of COX-1. Another one is nitric oxide (NO) donation, as the latter subserves many of the same protective mechanisms in GIT defense as prostaglandins do [4]. NO induces vasodilatation and modulation of vascular factors, and is sufficient for acute gastroprotection and for ulcer healing [5,6]. NO donors enhance collagen deposition at a wound site [7] and even accelerate gastric ulcer healing [8]. NO-donating NSAID hybrid compounds such as the nitrooxybutyl ester of naproxen were found to have reduced ulcerogenic activity compared to the parent drug while maintaining full anti-inflammatory activity [6]. However, organic nitrates generally depress blood pressure and rapidly induce nitrate tolerance. Co-treatment with cysteine or *N*-acetyl-L-cysteine in a group of rats was reported to prevent specific tolerance to glyceryl nitrate [9]. Cysteine activates organic nitrates probably by reducing them to NO and by serving as a NO-store and NO-carrier. Nitrate-cysteine hybrid compounds represent NO-donors nearly free of tolerance and only moderately decrease blood pressure [10]. All this led us to synthesize the first nitrate-cysteine-NSAID triple-hybrids representing potential GIT-safe NSAIDs. The new prodrugs **8** and **9**, consisting of naproxen, cysteine ethyl ester, and nitrooxypivaloic acid may show good anti-inflammatory activity, less GIT-damage, less tolerance, and less decrease in blood pressure in-vivo. This paper presents the synthesis and a preliminary in-vitro screening.

Correspondence: Jochen Lehmann, Institute of Pharmacy, University of Bonn, An der Immenburg 4, D-53121 Bonn, Germany. Phone: +49 (0) 228 735 250, Fax: +49 (0) 228 737 929, e-mail: J.Lehmann@uni-bonn.de.

Results and discussion

Chemistry

The synthesis of the target compounds **8** and **9** involves chemoselective *N*- and *S*-acylations. *N*-Acylation of L-cysteine ethyl ester or its hydrochloride salt **1** has been reported [11, 12]. Also the *N*-acylation of L-cysteine ethyl ester with nitrooxypivaloic acid using dicyclohexylcarbodiimide (DCC) as activating reagent has been patented

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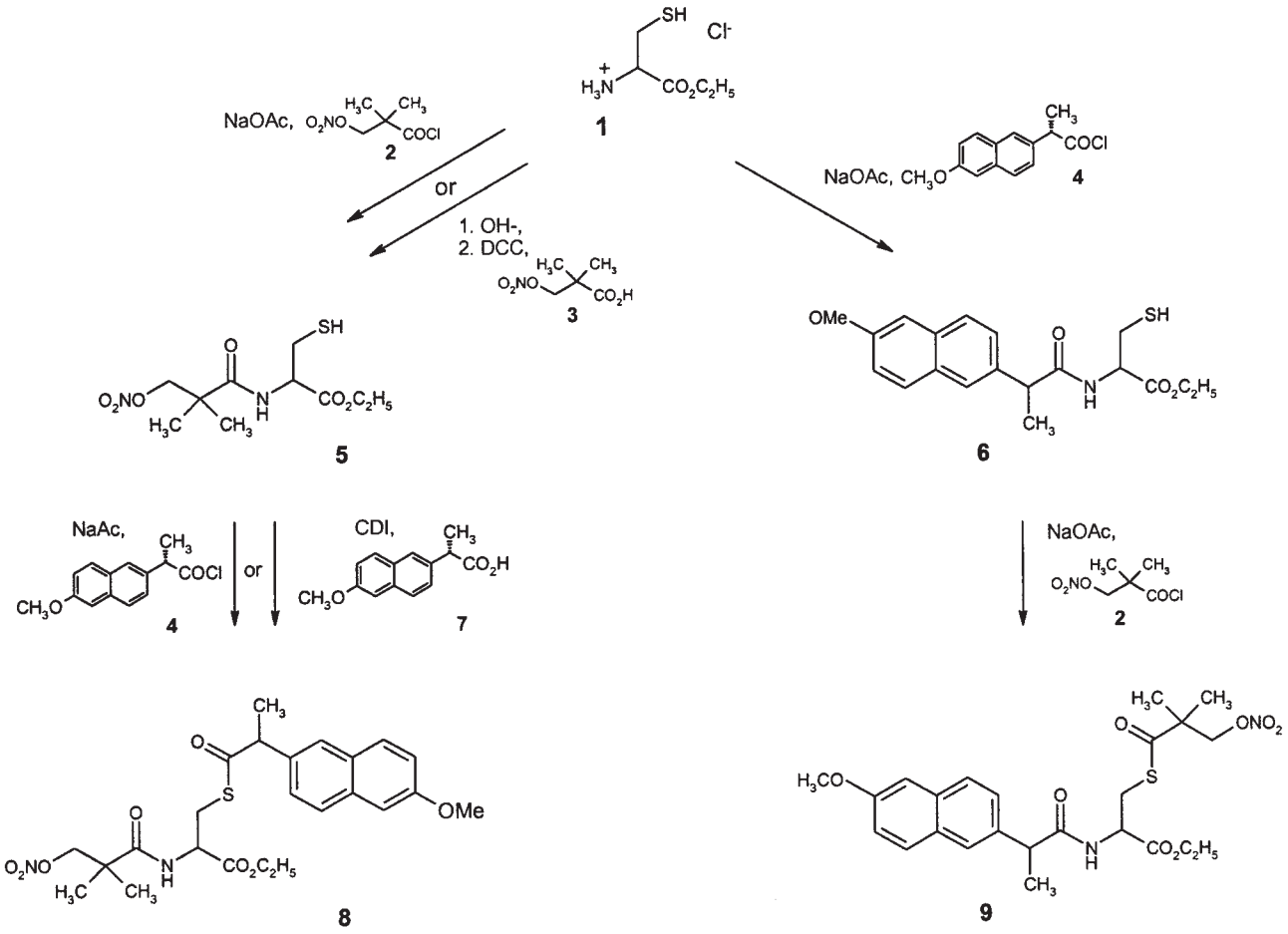

Scheme 1

[12]. Applying this procedure to the acylation of L-cysteine ethyl ester with naproxen (**7**) did not yield an *N*-acylation product, but rather an inseparable mixture, containing a high amount of unreacted naproxen. On the other hand, **1** has been *N*-acylated in a CH_2Cl_2/water two-phase system using of 3- and 4-nitrooxymethylbenzoyl chloride and sodium acetate [13]. According to this procedure, **1** was acylated with both acid chlorides of nitrooxypivaloic acid (**2**) and naproxen (**4**), respectively, the latter being prepared as reported [14]. The reaction occurred selectively at the amine group and gave satisfactorily pure **5** and **6**. Due to their sensitivity towards oxidation these *N*-acylated intermediates were subsequently *S*-acylated using the acid chlorides again or alternatively the carbonyldiimidazole (CDI)-activated acid **7**, analogously to a reported procedure [15]. Again, the acylation with acid chlorides gave better results with regard to yield and purity of the crude products. The ^{1}H-NMR spectra show D_2O-exchangeable signals for the thiol groups at 2.41 ppm (**5**) and 2.46 ppm (**6**). All reactions are summarized in Scheme 1.

Biology

The intermediate **6** and the target compounds **8** and **9** were tested for their cyclooxygenase inhibitory properties relative to naproxen. They were tested for COX-1 inhibition in a human platelet cell assay [16] and for COX-2 inhibition in mononuclear cells from whole blood [17]. Naproxen inhibits COX-1 with an IC_{50} of 0.1 μM and COX-2 with an IC_{50} of 9.0 μM. **6** showed no inhibition of COX-2 and only weakly inhibited COX-1 (IC_{50} = 6.0 μM). *N*-3-nitrooxypivaoyl-*S*-(+)-2-(6-methoxy-2-naphthyl)-propanoyl-L-cysteine ethyl ester (**8**) was inactive in the concentration range of 0.1–10 μM against both COX-1 and COX-2, while *N*-(+)-2-(6-methoxy-2-naphthyl)propanoyl-*S*-3-nitrooxypivaloyl-L-cysteine ethyl ester (**9**) exhibited no inhibition of COX-2 in the same concentration range and a low inhibition activity against COX-1 with an IC_{50} of 5.6 μM. These results are in accordance with the prodrug character of **6**, **8**, and **9**. Antiinflammatory activity certainly affords the metabolic liberation of naproxen. In-vivo experiments (rats) are ongoing; the results will be reported later.

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Experimental part

Chemistry

Thin layer chromatography was performed by using precoated silica gel plates (Merck 60 F254) which were detected by short UV light. Melting points were measured on a Gallenkamp melting point apparatus and are uncorrected. NMR spectra were performed on Brucker WH 90 (90 MHz) and Brucker WM 500 (500 MHz), using DMSO-d_6 as solvent and TMS as an internal standard. Chemical shifts (δ) are expressed in ppm downfield from TMS. Elemental analyses were carried out on a Heraeus elemental analyzer, results were within ± 0.4 % of the theoretical values. Column chromatography was performed on 70–230 mesh silica gel from Merck.

3-Nitrooxy-2,2-dimethylpropanoic acid (**3**) and *(+)-2-(6-methoxy-2-naphthyl)propanoic acid chloride* (**4**) were prepared as described previously [12, 14].

3-Nitrooxy-2,2-dimethylpropanoic acid chloride (**2**)

To a mixture of thionyl chloride (4.1 mL, 57.2 mmol, $d = 1.64$) and chloroform (10 mL), cooled with an ice bath, were added 3-nitrooxy-2,2-dimethylpropanoic acid (**3**, 6.53 g, 40 mmol) and two drops of absolute dimethylformamide. The mixture was heated slowly under stirring up to 50 °C and was kept at this temperature until evolution of gas had finished. After cooling, dry dichloromethane (100 mL) and active carbon (40 mg) were added, the mixture was stirred for a few minutes, filtered, and the filtrate was evaporated under reduced pressure. The product was used for further reaction without purification. 7.26 g (100 %), yellowish oil.

N-3-Nitrooxy-2,2-dimethylpropanoyl-L-cysteine ethyl ester (**5**)

To a solution of L-cysteine ethyl ester hydrochloride (**1**, 5.12 g, 27.5 mmol) and sodium acetate (4.10 g, 50.0 mmol) in water (50 mL) were added 25 mL dichloromethane. This mixture was then stirred strongly under argon, a solution of **2** (4.54 g, 25.0 mmol) in dichloromethane (25 mL) was added dropwise, and stirring was maintained for 1.5 hours. The organic phase was separated, washed with 50 mL water, 50 mL 5 % sodium bicarbonate solution, and 50 mL water again (twice), dried over anhydrous magnesium sulphate, evaporated under reduced pressure, and the remaining yellowish oil was dried in vacuo and subsequently used for further reaction. Yield 5.45 g (74 %). ^{1}H-NMR (DMSO-D_6, 500 MHz, δ ppm): 1.17 (t, $J = 7.1$ Hz, 3 H, –O–CH_2–CH_3), 1.20 (2 × s, 6 H, $(CH_3)_2$C<), 2.41 (t, $J = 8.4$ Hz, 1 H, –*SH*), 2.75–2.92 (m, 2 H, >CH–*CH_2*–SH), 4.08 (q, $J = 7.1$ Hz, 2 H, –O–*CH_2*–CH_3), 4.30–4.35 (m, 1 H, >*CH*–CH_2–SH), 4.58 (s, 2 H, –*CH_2*–ONO_2), 8.03 (d, $J = 7.7$ Hz, 1 H, –C(=O)–*NH*–).

N-3-Nitrooxy-2,2-dimethylpropanoyl-S-(+)-2-(6-methoxy-2-naphthyl)propanoyl-L-cysteine ethyl ester (**8**)

Procedure 1: To a solution of **5** (1.62 g, 5.5 mmol) in dichloromethane (30 mL) was added under ice cooling and argon atmosphere a solution of **4** (1.24 g, 5.0 mmol) and triethylamine (0.51 g, 5.0 mmol) in dry dichloromethane (30 mL). The mixture was stirred overnight at room temperature. Then, the mixture was washed with 25 mL 1N hydrochloric acid solution, with 25 mL saturated sodium bicarbonate solution and 25 mL water (twice), dried over anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude oily product was dissolved and stirred in ethanol (20 mL). To the solution was added slowly ≈ 20 mL of water from a dropping funnel. The precipitated white solid was separated by suction and dried in a desiccator under reduced pressure. Yield 1.87 g (74 %).

Procedure 2: A solution of naproxen (**7**, 1.73 g, 7.5 mmol) in 30 mL absolute dimethylformamide (DMF) was cooled to –10 to –15 °C, treated portionwise with carbonyldiimidazole (CDI) (1.28 g, 7.5 mmol) under argon atmosphere, and stirred for 2 hours. To the mixture was slowly added a solution of **5** (2.94 g, 10 mmol) in 20 mL absolute DMF. The mixture was then stirred for additional 2–3 hours at –10 °C. After the addition of 50 mL ethyl acetate, the mixture was washed three times with 30 mL of a saturated solution of sodium chloride, dried over Na_2SO_4 and evaporated under reduced pressure. The crude product was purified by flash chromatography (ethyl acetate : *n*-hexane 1 : 1). Yield 0.29 g (7.6 %), white solid.

All analytical and spectral data of the products obtained by both procedures are identical:

Mp 74–77 °C, ^{1}H-NMR (DMSO-D_6, 500 MHz, δ ppm): 1.07, 1.09 (2 x s, 6 H, $(CH_3)_2$C<), 1.12 (t, $J = 7.1$ Hz, 3 H, –O–CH_2–*CH_3*), 1.49 (d, $J = 7.3$ Hz, 3 H, >CH–*CH_3*), 3.10 and 3.32 (2 x dd, $J = 13.7/9.6$ Hz, $J = 13.7/5.0$ Hz, 2 H, >CH–*CH_2*–S–), 3.86 (s, 3 H, –O–*CH_3*), 4.03 (m, 2 H, –O–*CH_2*–CH_3), 4.11 (q, $J = 7.3$ Hz, 1 H, >*CH*–CH_3), 4.27–4.33 (m, 1 H, >*CH*–CH_2–S–), 4.58 (2 × d, 2 H, $J = 10.1$ Hz, –*CH_2*–ONO_2), 7.15 (dd, $J = 9.1/2.5$ Hz, 1 H, aromat. H), 7.28 (d, $J = 2.5$ Hz, 1 H, aromat. H), 7.36 (dd, $J = 8.5/1.9$ Hz, 1 H, aromat. H), 7.73 (d, $J = 1.3$ Hz, 1 H, aromat. H), 7.76 (d, $J = 8.5$ Hz, 1 H, aromat. H), 7.79 (d, $J = 9.1$ Hz, 1 H, aromat. H), 8.03 (d, 1 H, –C(=O)–*NH*–), ^{13}C-NMR (DMSO-D_6, δ ppm): 13.98 (–O–CH_2–*CH_3*), 18.20 (>CH–*CH_3*), 22.05 and 22.11 (*CH_3*)$_2$C<), 29.55 (>CH–*CH_2*–S–), 41.32 ((*CH_3*)$_2$*C*<), 51.70 (>*CH*–CH_2–S–), 53.18 (>*CH*–CH_3), 55.32 (–O–CH_3), 60.98 (–O–*CH_2*–CH_3), 78.28 (–*CH_2*–ONO_2), 105.89 C_t, 118.6 C_t, 126.60 C_t, 126.61 C_t, 127.14 C_t, 128.51 C_q, 129.35 C_t, 133.70 C_q, 134.72 C_q, 157.49 C_q, 170.10 (–C(=*O*)–O–), 173.96 (–*C(=O)*–NH–), 200.53 (–*C(=O)*–S–), anal. ($C_{24}H_{30}N_2O_8S$) C, H, N.

N-(+)-2-(6-methoxy-2-naphthyl)propanoyl-L-cysteine ethyl ester (**6**)

To a solution of L-cysteine ethyl ester hydrochloride (**1**, 2.04 g, 11.0 mmol) and sodium acetate (1.64 g, 20.0 mmol) in water (20 mL) was added dichloromethane (15 mL). This mixture was stirred vigorously under argon atmosphere and a solution of **4** (2.49 g, 10,0 mmol) in dichloromethane (15 mL) was added dropwise. Stirring was maintained for 1.5 hours. The organic phase was separated, washed with 20 mL water, 20 mL 5 % sodium bicarbonate solution (twice) and 20 mL water (twice), dried over $MgSO_4$ and evaporated under reduced pressure to dryness. Yield 2.66 g (74 %), white solid, mp 104–105 °C, ^{1}H-NMR (DMSO-D_6, 90 MHz, δ ppm): 1.08 (t, 3 H, –O–CH_2–*CH_3*), 1.42 (d, 3 H, >CH–*CH_3*), 2.46 (s, 1 H, –*SH*), 3.31–3.58 (m, 2 H, >CH–*CH_2*–SH), 3.85 (s, 3 H, –O–*CH_3*), 3.85–4.15 (m, 3 H, –O–*CH_2*–CH_3 and >*CH*–CH_3), 4.27–4.54 (m, 1 H, >*CH*–CH_2–S–), 7.04–7.81 (m, 6 H, aromatic), 8.39 (d, 1 H, –C(=O)–*NH*–), anal. ($C_{19}H_{23}NO_4S$) C, H, N.

N-(+)-2-(6-methoxy-2-naphthyl)propanoyl-S-3-nitrooxy-2,2-dimethylpropanoyl-L-cysteine ethyl ester (**9**)

To a solution of **6** (1.99 g, 5.5 mmol) in dichloromethane (30 mL) was added under ice cooling and argon atmosphere a solution of 3-nitrooxy-2,2-dimethylpropanoic acid chloride (**2**, 0.91 g,

5.0 mmol) and triethylamine (0.51 g, 5.0 mmol) in dry dichloromethane (30 mL). The mixture was stirred overnight at room temperature, washed with 25 mL 1N hydrochloric acid solution, 25 mL saturated sodium bicarbonate solution and 25 mL water (twice), dried over $MgSO_4$ and evaporated under reduced pressure. The crude oily product was dissolved and stirred in ethanol (20 mL). To the solution was added the same volume of water from a dropping funnel. The precipitated white solid was separated and dried in a desiccator under reduced pressure. Yield 1.96 g (77 %), mp 50–51 °C, ^{1}H-NMR (DMSO-D_6, 500 MHz, δ ppm): 1.06 (t, *J* = 7.1 Hz, 3 H, –O–CH_2–*CH_3*), 1.18, 1.23 (2 × s, 6 H, (*CH_3*)$_2$C<), 1.40 (d, *J* = 7.1 Hz, 3 H, >CH–*CH_3*), 3.12 and 3.35 (2 × dd, *J* = 13.6/8.5 Hz, *J* = 13.6/5.4 Hz, 2 H, >CH–*CH_2*–S–), 3.79 (q, *J* = 7.1 Hz, 1 H, >*CH*–CH_3), 3.85 (s, 3 H, –O–*CH_3*), 4.00 (q, *J* = 7.1 Hz, 2 H, –O–*CH_2*–CH_3), 4.39–4.45 (m, 1 H, >*CH*–CH_2–S–), 4.58 (2 × d, *J* = 2 × 10.0 Hz, 2 H, –*CH_2*–ONO_2), 7.13 (dd, *J* = 9.0/2.7 Hz, 1 H, aromat. H), 7.26 (d, *J* = 2.5 Hz, 1 H, aromat. H), 7.41 (dd, *J* = 8.5/1.6 Hz, 1 H, aromat. H), 7.69 (s, 1 H, aromat. H), 7.73 (d, *J* = 8.5 Hz, 1 H, aromat. H), 7.75 (d, *J* = 9.1 Hz, 1 H, aromat. H), 8.48 (d, 1 H, –C(=O)–*NH*–), ^{13}C-NMR (DMSO-D_6, δ ppm): 13.94 (–O–CH_2–*CH_3*), 18.61 (>CH–*CH_3*), 22.07 and 22.25 ((*CH_3*)$_2$C<), 29.68 (>CH–*CH_2*–S–), 48.79 ((*CH_3*)$_2$*C*<), 51.28 (>*CH*–CH_2–S–), 44.76 (>*CH*–CH_3), 55.28 (–O–*CH_3*), 60.99 (–O–*CH_2*–CH_3), 77.47 (–*CH_2*–ONO_2), 105.85 C_t, 118.65 C_t, 125.52 C_t, 126.62 C_t, 126.65 C_t, 128.50 C_q, 129.19 C_t, 133.30 C_q, 136.94 C_q, 157.16 C_q, 170.06 (–*C(=O)*–O–), 173.67 (–*C(=O)*–NH–), 202.3 (–*C(=O)*–S, anal. ($C_{24}H_{30}N_2O_8S$) C, H, N.

Biology

COX-1 inhibition was determined in platelets isolated from fresh human blood according to a reported method [16]. Platelets were incubated in the presence and absence of inhibitors for 10 minutes in IKP buffer. Prostaglandin E_2 as a key intermediate was determined using an ELISA assay method. COX-2 inhibition was determined in mononuclear cells isolated from human fresh blood according to Laufer et al. [17]. COX-2 was induced over 5 hours by LPS. PGE2 determination again was assayed with an ELISA method.

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