Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 1579–1582

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Inhibition of Glutathione *S*-Transferase in Rat Hepatocytes by a Glycine-Tetrazole Modified *S*-Alkyl–GSH Analogue

Danny Burg,[a,*] Liesbeth Hameetman,[a] Dmitri V. Filippov,[b] Gijs A. van der Marel[b] and Gerard J. Mulder[a]

[a]*Division of Toxicology, Leiden/Amsterdam Center for Drug Research, Leiden University, PO Box 9502, 2300RA, Leiden, The Netherlands*

[b]*Division of Bio-Organic Synthesis, Leiden Institute of Chemistry, Leiden University, PO Box 9502, 2300RA, Leiden, The Netherlands*

Received 26 November 2001; revised 19 February 2002; accepted 12 April 2002

Abstract—Glutathione (GSH) conjugates inhibit enzymes that are involved in drug metabolism and drug resistance, but their cellular uptake is very low. To improve membrane-permeability, we synthesized a novel GSH-conjugate analogue with a tetrazole carboxylate isostere at the glycine position. Introduction of the tetrazole decreases inhibitory potency towards CDNB conjugation by glutathione *S*-transferase. However, the tetrazole derivative inhibited 2-bromoisovalerylurea conjugation in rat liver cytosol, as well as in hepatocytes. © 2002 Elsevier Science Ltd. All rights reserved.

Conjugation to the ubiquitous tripeptide glutathione (GSH, L-γ-glutamyl-L-cysteinyl-glycine) protects cells against many potentially harmful electrophiles. This reaction is catalyzed by glutathione *S*-transferase (GST).[1,2] Overexpression of certain GST-isoenzymes in tumor cells contributes to resistance against cytostatic drugs.[3,4] Thus, effort has been put into the development of GST inhibitors.

GSH-conjugates are often good inhibitors of GST in vitro. For several years we have synthesized analogues of *S*-alkylated GSH, to be used as GST inhibitors. This resulted, for instance, in compound **III**, R-sHep, which is an in vivo inhibitor of rat liver cytosolic GSTs, with preference towards alpha class GSTs.[5–10] Recently we also synthesized a series of novel peptide bond modified GSH-conjugate analogues which inhibited rat and human GST isoenzymes, and were stabilized towards γ-glutamyl transpeptidase, the main enzyme involved in breakdown of GSH-conjugates.[11]

GSH-conjugates are very hydrophilic: binding to the active site in GST is dictated by electrostatic enzyme–substrate interactions.[12] Thus, the terminal acidic groups in GSH-conjugates are important for binding to GST; the glutamyl carboxylic acid is absolutely required for binding, while at the glycine carboxylate position more variation is allowed.[13] Because GSH-conjugates are highly charged, they cannot readily enter cells. To improve cellular uptake, esterification of the terminal carboxylic acid groups is required.[14,15] Intracellularly, these esters are hydrolyzed by esterases, thereby liberating the active inhibitor. Esterification at the glycine moiety sufficiently improves uptake, although di-esterification is even more effective.[16]

Another method to improve the cellular uptake is to replace the carboxylic acid by a biomimetic group. Possible carboxylate isosteres include sulfonamides, sulfonates, phosphates and tetrazoles.[17] Sulfonates and phosphates are even more hydrophilic than the carboxylate and, therefore, cannot be used to improve cellular uptake. The tetrazole is a carboxylate isostere, which has been used frequently within different classes of medicinal agents.[18–20] Being 10-fold more lipophilic than the carboxylate, while having similar acidity,[21] it seemed an attractive option for improving the cellular uptake of GSH analogues. Therefore, we synthesized a novel GSH analogue that contains this tetrazole isostere instead of the glycine carboxylic acid (Fig. 1, Tet-sHep). To test the ability of this compound to inhibit GST, the sulphydryl function was derivatized with an alkyl chain, which interacts with the hydrophobic binding site (H-site) of GST. The 2-heptyl group was used in our previous inhibitor R-sHep (**III**), and provided this compound with selectivity towards alpha class GSTs.

*Corresponding author. Tel.: +31-71-527-6039; fax: +31-71-527-6292; e-mail: d.burg@lacdr.leidenuniv.nl

0960-894X/02/$ - see front matter
PII: S0960-894X(02)00247-0

The synthesis of R-sHep and Et-R-sHep has previously been described, and is based on the replacement of the cysteinyl-glycine moiety by D-2-aminoadipic acid.[8] The preparation of GSH derivatives GS-sHep and Et-GS-sHep (**I**) is outlined in Scheme 1. Glutathione (reduced form) was conjugated to 2-bromo-heptane, using KI and 12-crown-4 as catalysts for the otherwise slow coupling of the relatively unreactive secondary bromoalkane. After removal of excess bromoalkane by extraction of the aqueous layer with ethyl acetate, the alkylated product, GS-sHep, was collected in 65% yield by crystallization from slightly acidified (pH 3) water. The product was homogeneous on TLC and the identity was confirmed by ^{1}H NMR and LC–MS analysis. The monoethylester was then prepared by reaction with TMSCl in dry ethanol[22] and subsequently purified by Sephadex LH20 gelfiltration. LC–MS analysis confirmed the identity of monoethyl ester **Ib**.

Tet-sHep was constructed by standard peptide synthesis methods from the tetrazoyl-analogue of glycine (Scheme 2), which was synthesized by a method adapted from Duncia et al.[23] First, 3-amino-propionitrile (fumarate salt) was condensed with the, in situ formed, *N*-hydroxysuccinimide active ester of Boc-glycine-OH to obtain **1**. Efficient formation of tetrazole **2** was achieved by treatment with triphenylphosphine (Ph_3P)/diethylazodicarboxylate (DEAD) in the presence of azotrimethylsilane ($TMSN_3$).[20,23] This Mitsunobu-type reaction involves the formation of a strong P–O bond between Ph_3P and the oxygen of the secondary amide. Replacement of the Ph_3O-molecule with the azide anion forms an intermediate iminoyl-azide that tautomerizes to the tetrazole.[23] After Boc removal (→**3**), coupling to Fmoc-Cys(Acm)-OH using BOP/DiPEA yielded dipeptide **4**. By carefully controlling the Fmoc deprotection with DBU, compound **5** was obtained without cleavage of the cyanoethyl protecting group. The fully protected tetrazole analogue of GSH, **6**, was then obtained by condensation with Boc-Glu(OH)-OtBu. After removal of the Acm thiol protective group with I_2 in dry methanol,[24,25] the symmetrical disulfide **7** was reduced using tri-*n*-butylphosphine and subsequently alkylated with 2-bromo-heptane (→**8**).

Removal of the 2-cyanoethyl tetrazole protective group was achieved by treatment with excess DBU.[23]

Tet-sHep (**II**) was then obtained after acidolytic cleavage of the remaining Boc and *t*Bu groups. After purification by LH20 gelfiltration, ^{1}H NMR and LC–MS analysis confirmed the identity of the tetrazoyl derivative. Purity was >90%, as determined by TLC and HPLC (UV detection at 214 and 254 nm).

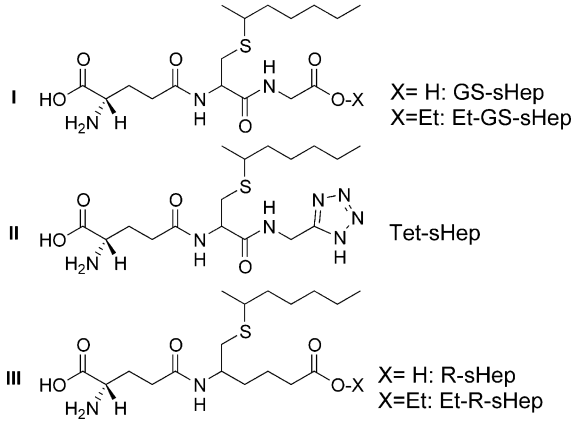
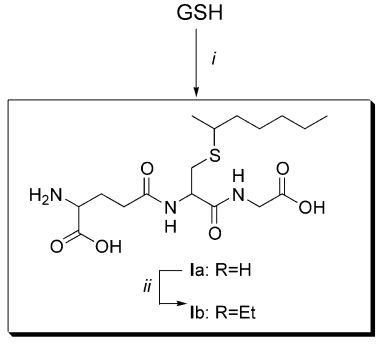
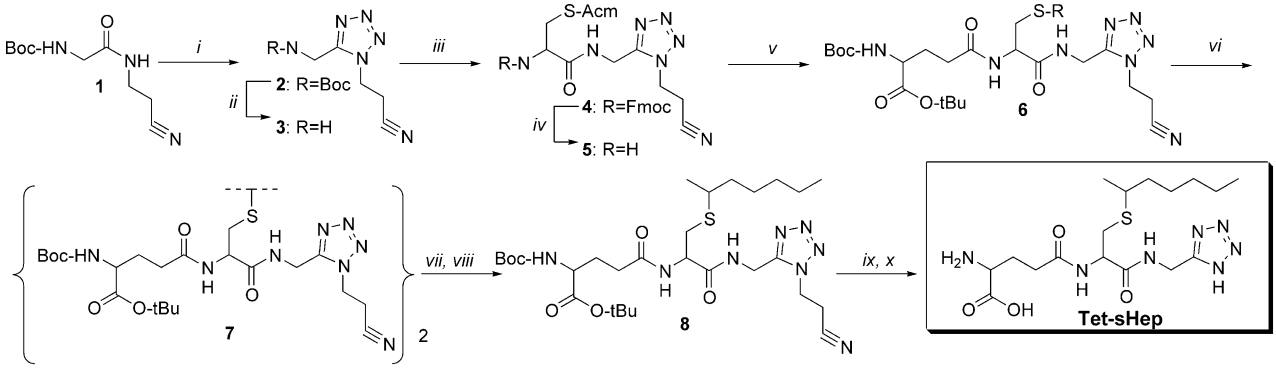


Figure 1. Structures of 2-heptyl derivatized GSH analogues.



Scheme 1. Synthesis of GS-sHep (**Ia**) and Et-GS-sHep (**Ib**): (i) 2-bromoheptane (10 equiv), KI (2 equiv), 12-crown-4 (0.5 equiv) in H_2O/EtOH (2:1, v/v), 60 °C, argon atm, 24 h, 65%; (ii) TMSCl, EtOH, 16 h, 85%.



Scheme 2. Synthesis of the tetrazoyl isostere Tet-sHep: (i) **1**, Ph_3P (2.5 equiv), DEAD (2.5 equiv), $TMSN_3$ (2.5 equiv), 0 °C, 16 h, 55%; (ii) TFA/DCM (1:1, v/v), quant; (iii) **3**, Fmoc-Cys(Acm)-OH (1.2 equiv), BOP (1.2 equiv), DiPEA (1 equiv), 0 °C, 30 min, 70%; (iv) DBU (1 equiv), DCM, quant; (v) **5**, Boc-Glu(OH)-OtBu, BOP (1.2 equiv), DiPEA (1 equiv), DMF, 0 °C, 30 min, 55%; (vi) I_2 (2.5 equiv), MeOH, 80%; (vii) (*n*-butyl)$_3$P (1 equiv), *n*PrOH/H_2O (3:1, v/v), 1 h; (viii) 2-bromoheptane (10 equiv), 12-crown-4 (0.5 equiv), KI (1 equiv), 60 °C, 16 h, 70% (two steps); (ix) DBU (7 equiv), DCM, 6 h; (x) TFA/H_2O (98:2, v/v), 90% (two steps).

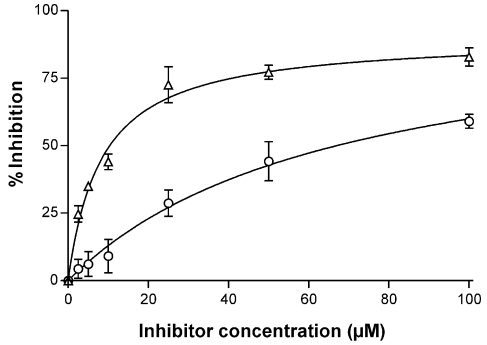


Figure 2. Inhibition of CDNB glutathione conjugation in rat liver cytosol (S100). Cytosol (S100 fraction) was prepared as described previously.[8] Cytosolic GSTs (15 μg total protein/mL) were incubated at 37 °C in 0.1 M potassium phosphate buffer pH 6.5, supplemented with 1 mM GSH, 0.1 mM EDTA and the appropriate amount of inhibitor. Reaction was initiated by addition of CDNB. Formation of GS-DNB was followed by UV absorbance at 340 nm. Initial reaction rates were corrected for non-enzymatic CDNB conjugation. Inhibition is given as percentage (±SD) of the non-inhibited reaction. △: GS-sHep ○: Tet-sHep.

GS-sHep and Tet-sHep were evaluated for their GST-inhibitory properties. We have previously described the inhibition of rat hepatic GST isoenzymes by R-sHep and Et-R-sHep.[15] These compounds are potent inhibitors of alpha-class, and to lesser extent mu-class GSTs in vitro as well as in vivo. GST inhibition by the novel compounds was determined according to Habig et al.[26] Chlorodinitrobenzene (CDNB) was used as substrate because it is a good substrate for almost all GST isoenzymes.

Rat liver cytosol (S100) is a mixture of mainly alpha and mu-class GSTs. GS-sHep is a potent inhibitor of CDNB conjugation by rat liver cytosolic GSTs (Fig. 2). Inhibition reaches a plateau at higher inhibitor concentrations, which indicates that some GST isoenzymes present in S100 are not inhibited. Tet-sHep is a less potent inhibitor of cytosolic GSTs than GS-sHep when CDNB is used as substrate.

The lower inhibition of S100-GSTs by Tet-sHep may result from different isoenzyme selectivity as compared to GS-sHep. Selectivity of the inhibitors was, therefore, evaluated with purified alpha and mu class GST isoenzymes (Table 1). Both compounds show the same inhibition pattern: GST2–2 (alpha), 3–3 and 4–4 (both mu-class) were inhibited to the same extent, while GST1–1 is inhibited less. For all isoenzymes, Tet-sHep is a less efficient inhibitor than GS-sHep.

Table 1. Inhibition of isolated rat GST isoenzymes

	% GST inhibition (±SD)			
	GST1–1	GST2–2	GST3–3	GST4–4
GS-sHep	53±8	98±5	95±4	91±10
Tet-sHep	16±7	53±12	65±6	59±20

Individual purified isoenzymes were supplied by Prof. Dr. P. van Bladeren. Each isoenzyme (20–50 nM) was incubated in 0.1 M potassium phosphate buffer pH 6.5, containing 0.1 mM EDTA, 1 mM GSH and 50 μM inhibitor at 37 °C. Reaction was initiated by addition of 1 mM CDNB in ethanol (maximal EtOH concentration = 2%). Formation of GS-DNB was followed by UV absorbance at 340 nm. Initial reaction rates were corrected for non-enzymatic conjugation. Values are given as percentage inhibition (±SD) compared to the non-inhibited reaction rate.

Evaluation of isoenzyme selectivity of the inhibitors in intact hepatocytes is not possible using CDNB. We therefore used 2-bromoisovalerylurea (BIU), which has been used in the rat in vivo to study GSH conjugation.[27] The *S*-enantiomer (S-BIU) is preferentially conjugated by alpha class GST1–1 and 2–2, while mu class GST3–3 and 4–4 are stereoselective towards R-BIU.[28] Background (non-GST mediated) GSH conjugation of this substrate is very low (less than 5%). Formation of the GSH-conjugates (*R*)-IU-G and (*S*)-IU-G, products of S-BIU and R-BIU respectively, was followed by HPLC with electrochemical detection as previously described.[15]

In contrast to their effects on CDNB conjugation, GS-sHep and Tet-sHep inhibited GST Mu mediated conjugation of (*R*)-BIU in rat liver cytosol equally well (Fig. 3). This discrepancy in inhibition patterns when using various substrates has been noted before and may result from different binding modes of the acceptor substrates.[15]

Because both compounds inhibited GST activity in cytosol equally well, we also investigated GST inhibition in intact freshly isolated rat hepatocytes (Fig. 4). GS-sHep (**Ia**) and Et-*R*-sHep (**III**b) were used as 'reference' compounds. As expected, **Ia** was a poor inhibitor, presumably due to its low cellular uptake. Et-*R*-sHep showed preference towards alpha-class GSTs, because it only inhibited (*S*)-BIU conjugation. Et-GS-sHep was also a better inhibitor of alpha than of mu class GSTs. Interestingly, selectivity of Tet-sHep shifted slightly towards mu-class GSTs. Tet-sHep is a less efficient GST inhibitor in hepatocytes than Et-*R*-sHep.

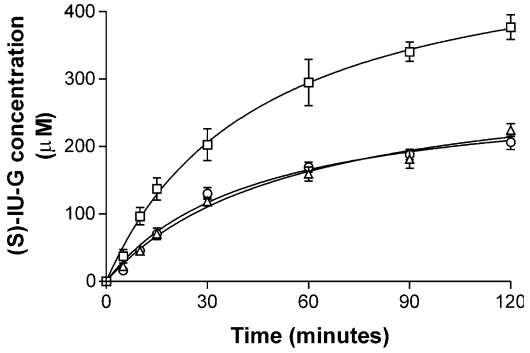


Figure 3. Inhibition of (*R*)-BIU conjugation in rat liver cytosol. (*R*)-BIU (250 μM, final volume 1 mL) was added to a solution of cytosol (10 mg protein/mL) in potassium phosphate buffer pH 7.4, supplemented with 1 mM GSH and 50 μM inhibitor. At appropriate timepoints a sample (100 μL) was taken and added to 300 μL ice-cold HPLC buffer, containing 60% methanol. Samples were analyzed by HPLC on an Inertsil ODS 2 column [(2 × 10 cm) × 0.3 cm, Chrompack, The Netherlands]. HPLC buffer consisted of 0.1 M $NaNO_3$, 0.01 M KBr, 0.01 M citric acid, 0.1 mM EDTA, and 0.1 mM 1-decanesulfonic acid. Thioethers were quantified by online bromine-generation and subsequent electrochemical detection as previously described.[15] □: No Inhibitor, ○: GS-sHep, △: Tet-sHep. Shown are mean values, ±SD, determined in three separate experiments.

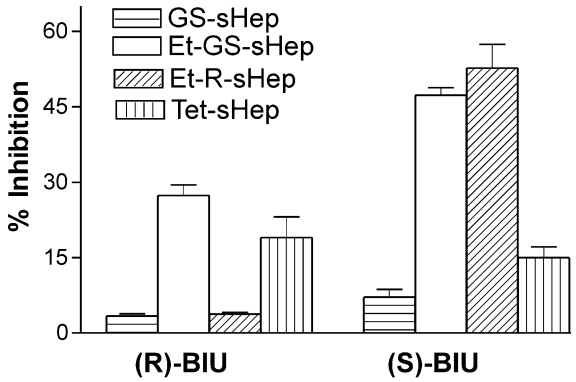


Figure 4. Inhibition of GST activity in rat hepatocytes. Fresh hepatocytes were isolated by collegenase perfusion, as described by Nagelkerke et al.[29] Hepatocytes (8×10^6 cells/ mL) in Hanks/HEPES buffer pH 7.4, containing 1% BSA, were preincubated with L-methionine at 37 °C for 30 min to stimulate GSH synthesis. Inhibitor and (*R*)- or (*S*)-BIU (250 μM) dissolved in DMSO (max. DMSO concentration 0.5%) were added, after which the suspension was incubated at 37 °C for 3 h. A sample (250 μL) was taken and added to 500 μL ice-cold HPLC buffer, containing 60% methanol. Precipitated protein was removed by centrifugation and the supernatant subsequently analyzed by HPLC as previously described.[15] Cell viability was always above 85%, as determined by trypan blue exclusion.

In conclusion: we synthesized a novel *S*-alkyl-GSH analogue with a tetrazole as glycine carboxylic acid isostere. This compound was a less efficient inhibitor of rat liver cytosolic GST isoenzymes than its parent GSH analogue when CDNB was used as substrate. Using (*R*)-BIU, both compounds were equipotent. In intact hepatocytes, the tetrazole inhibited GST activity less than the esterified GSH analogue; because these compounds were equally inhibitory in cytosol, this suggests that Tet-sHep may be less taken up by cells. The neutral ester moiety is more lipophilic than the charged tetrazole. The introduction of a tetrazole moiety into the GSH backbone will improve the cellular permeability.

Acknowledgements

This work was financially supported by the Dutch Cancer Society, grant RUL 97–1407. We thank Prof. Dr. Peter van Bladeren for the generous supply of purified rat GST isoenzymes.

References and Notes

1. van Bladeren, P. J. *Chem. Biol. Interact*. **2000**, *129*, 61.
2. Hayes, J. D.; Pulford, D. J. *Crit. Rev. Biochem. Mol. Biol*. **1995**, *30*, 445.
3. Tew, K. D. *Cancer Res*. **1994**, *54*, 4313.
4. Tew, K. D.; Dutta, S.; Schultz, M. *Adv. Drug Deliv. Rev*. **1997**, *26*, 91.
5. Adang, A. E.; Brussee, J.; Meyer, D. J.; Coles, B.; Ketterer, B.; van der Gen, A.; Mulder, G. J. *Biochem. J*. **1988**, *255*, 721.
6. Adang, A. E.; Brussee, J.; van der Gen, A.; Mulder, G. J. *J. Biol. Chem*. **1991**, *266*, 830.
7. Adang, A. E.; Moree, W. J.; Brussee, J.; Mulder, G. J.; van der Gen, A. *Biochem. J*. **1991**, *278*, 63.
8. Ouwerkerk, M. S.; van Boom, J. H.; Dreef, T. M.; Ploemen, J. H.; Meyer, D. J.; Mulder, G. J. *Biochem. J*. **1995**, *308*, 283.
9. Ouwerkerk, M. S.; van Boom, J. H.; Mulder, G. J. *J. Pharmacol. Exp. Ther*. **1996**, *276*, 923.
10. Ouwerkerk, M. S.; Mulder, G. J. *Chem. Biol. Interact*. **1998**, *111–112*, 163.
11. Burg, D.; Filippov, D. V.; Hermanns, R.; van der Marel, G. A.; van Boom, J. H.; Mulder, G. J. *Bioorg. Med. Chem*. **2001**, *10*, 195.
12. Koehler, R. T.; Villar, H. O.; Bauer, K. E.; Higgins, D. L. *Proteins* **1997**, *28*, 202.
13. Adang, A. E.; Brussee, J.; van der Gen, A.; Mulder, G. J. *Biochem. J*. **1990**, *269*, 47.
14. Anderson, M. E.; Powrie, F.; Puri, R. N.; Meister, A. *Arch. Biochem. Biophys*. **1985**, *239*, 538.
15. Ouwerkerk, M. S.; Tirona, R. G.; Ripping, R. A.; Ploemen, J. H.; van Bladeren, P. J.; Pang, K. S.; van Boom, J. H.; Mulder, G. J. *Drug Metab. Dispos*. **1997**, *25*, 1137.
16. Levy, E. J.; Andrews, P. A.; Meister, A. *Proc. Natl. Acad. Sci. U.S.A*. **1993**, *90*, 9171.
17. Patani, G. A.; laVoie, E. J. *Chem. Rev*. **1996**, *96*, 3147.
18. Itoh, F.; Yukishige, K.; Wajima, M.; Ootsu, K.; Akimoto, H. *Chem. Pharm. Bull*. **1995**, *43*, 230.
19. Schoep, D. D.; Lunn, W. H. W.; Salhoff, C. R.; McDonald, J. W. *Eur. J. Pharmacol*. **1994**, *270*, 67.
20. DeLombaert, S.; Blanchard, L.; Stamford, L. B.; Tan, J.; Wallace, E. M.; Satoh, Y.; Fitt, J.; Hoyer, D.; Simonsbergen, D.; Moliterni, J.; Marcopoulos, N.; Savage, P.; Chou, M.; Trapani, A. J.; Jeng, A. Y. *J. Med. Chem*. **2000**, *43*, 488.
21. Hansch, C.; Leo, L. Exploring QSAR. In *Exploring QSAR. Fundamentals and Applications in Chemistry and Biology;* Heller, S. R., Cons. Ed.; Am. Chem. Soc.: Washington, 1995; Chapter 13.
22. Brook, M. A.; Chan, T. H. *Synthesis* **1983**, 201.
23. Duncia, J. V.; Pierce, M. E.; Santella, J. B. *J. Org. Chem*. **1991**, *56*, 2395.
24. Calcagni, A.; Dupre, S.; Lucente, G.; Luisi, G.; Pinnen, F.; Rossi, D. *Int. J. Pept. Protein Res*. **1995**, *46*, 434.
25. Calcagni, A.; Dupre, S.; Lucente, G.; Luisi, G.; Pinnen, F.; Rossi, D.; Spirito, A. *Arch. Pharm. Weinheim* **1996**, *329*, 498.
26. Habig, W. H.; Pabst, M. J.; Jakoby, W. B. *J. Biol. Chem*. **1974**, *249*, 7130.
27. te Koppele, J. M.; Dogterom, P.; Vermeulen, N. P. E.; Meijer, D. K. F.; van der Gen, A.; Mulder, G. J. *J. Pharmacol. Exp. Ther*. **1986**, *239*, 905.
28. te Koppele, J. M.; Coles, B.; Ketterer, B.; Mulder, G. J. *Biochem. J*. **1988**, *252*, 137.
29. Nagelkerke, J. F.; Barto, K. P.; van Berkel, T. J. C. *J. Biol. Chem*. **1983**, *258*, 12221.