Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 15 (2005) 1457–1461
Bioorganic & Medicinal Chemistry Letters

Synthesis and evaluation of azalanstat analogues as heme oxygenase inhibitors

Jason Z. Vlahakis,[a] Robert T. Kinobe,[b] Raymond J. Bowers,[a] James F. Brien,[b] Kanji Nakatsu[b] and Walter A. Szarek[a,*]

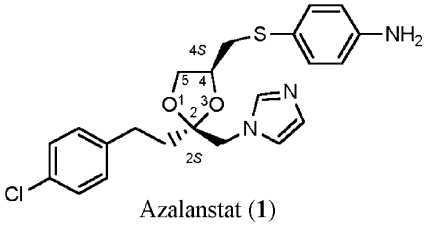
[a]*Departments of Chemistry, Queen's University, Kingston, Canada ON K7L 3N6*
[b]*Pharmacology and Toxicology, Queen's University, Kingston, Canada ON K7L 3N6*

Received 22 November 2004; accepted 29 December 2004
Available online 22 January 2005

Abstract—Several analogues based on the lead structure of azalanstat were synthesized and evaluated as novel inhibitors of heme oxygenase (HO). A number of these compounds, which are structurally distinct from metalloporphyrin HO inhibitors, were found to be selective for the HO-1 isozyme (stress induced), and had substantially less inhibitory activity on HO-2, the constitutive isozyme.

1. Introduction

Carbon monoxide (CO) is formed in mammals primarily through the action of heme oxygenase (HO) on the substrate heme.[1] Approximately 85% of the CO produced by humans under normal physiological conditions is derived from heme,[2] and CO is increasingly becoming recognized as a cellular regulator with actions in brain, blood vessels, and immune systems. HO activity is not attributable to a single enzyme as there are in fact three isozymes. HO-1 is also known as HSP-32 (molecular weight ~32 kD) and is a stress protein induced by a number of stimuli including heat shock, heavy metals, heme, and reactive oxygen species. HO-2 has a molecular weight of 36.5 kD and is constitutive; it is considered to be a member of the glucocorticoid-regulated family of enzymes and is upregulated by glucocorticoids. HO-3 is the least known of the isozymes[3] as its catalytic activity is low, and its physiological function is unknown.



Azalanstat (**1**)

Much of our current information on the role of CO and HO has been garnered by judicious use of drugs, especially HO inhibitors. Almost all of the studies to date have exploited HO inhibitors of the metalloporphyrin class, such as tin protoporphyrin (SnPP).[4,5] Because of the close structural similarity between heme and the metalloporphyrin HO inhibitors, a problem arises with respect to specificity. Heme plays a key role in a number of biologically relevant molecules, where it may be involved in the active site of an enzyme or function as a regulatory prosthetic group. Examples of the former would be cytochromes P-450 and nitric oxide synthase (NOS), and an example of the latter would be soluble guanylyl cyclase (sGC). Although the metalloporphyrins have been subjected to criticism because of their ability to inhibit enzymes other than HO,[6,7] it has been argued that they are useful if they are used cautiously with great attention to concentration.[8] Our laboratory has found that chromium mesoporphyrin was one of the more useful drugs in broken cell preparations as a 10 μM concentration inhibited HO by >90%, with little or no effect on sGC or NOS.[9] Nevertheless, the range of drug concentrations in which selectivity is expressed is limited, a feature which proscribes the utility of the metalloporphyrins in distinguishing amongst a number of enzymes.[10] Thus,

Keywords: Heme oxygenase; Selective inhibitors; Azalanstat; Carbon monoxide; Heme.
* Corresponding author. Tel.: +1 613 533 2643; fax: +1 613 533 6532; e-mail addresses: nakatsuk@post.queensu.ca; szarekw@chem.queensu.ca

0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.12.075

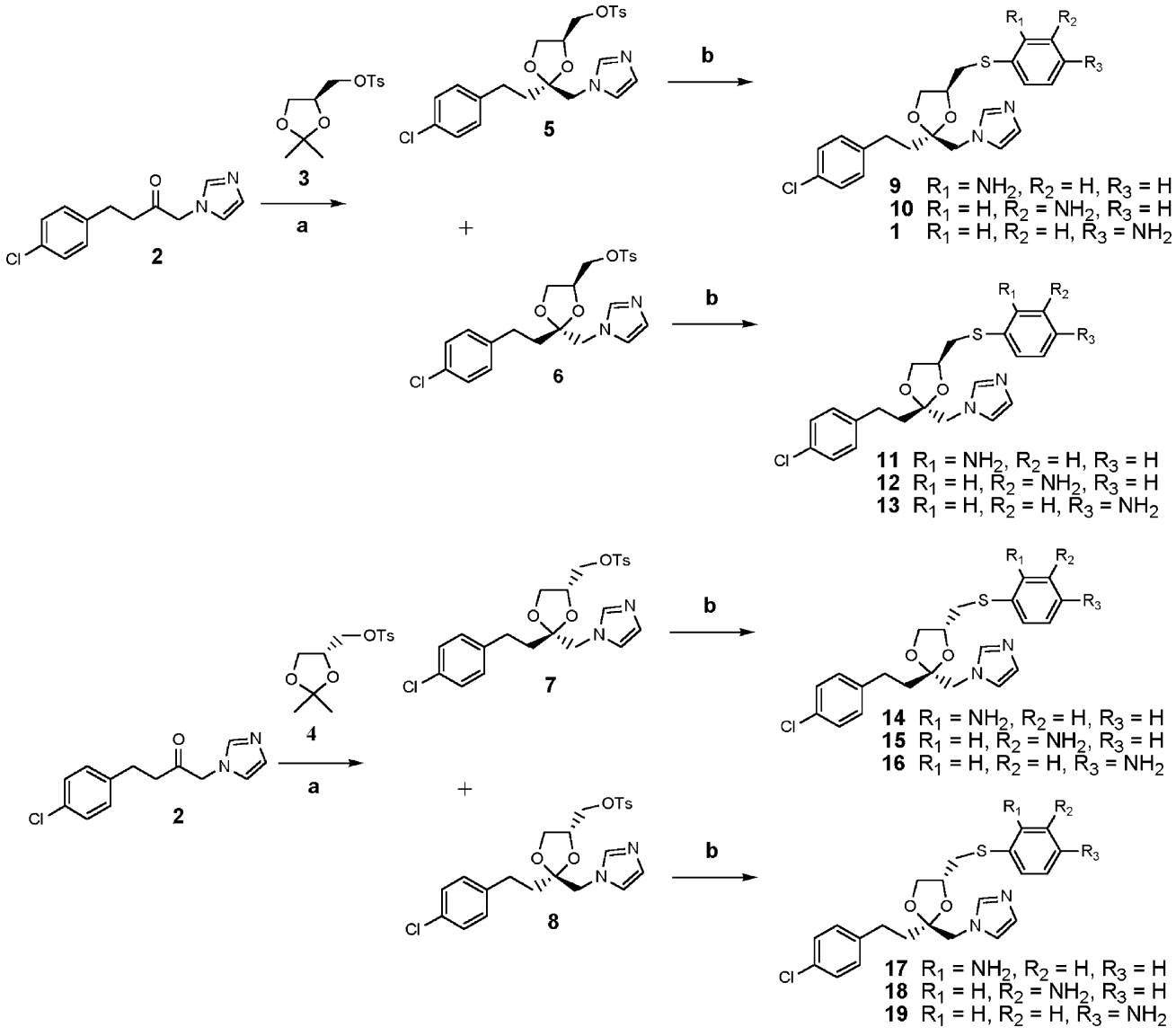
we have embarked on a program to design HO inhibitors that are not based on the porphyrin nucleus with the objective of creating more-selective HO inhibitors. One of our leads, azalanstat, was designed originally at Syntex as an inhibitor of mammalian lanosterol 14α-demethylase (14-DM),[11] and was discovered recently to show activity against HO.[2] Thus, using azalanstat (**1**) as the lead compound and systematically modifying its structure, we have synthesized a series of novel drugs, several of which are selective HO-1 inhibitors.

2. Synthesis

4-(4-Chlorophenyl)-1-(1*H*-imidazol-1-yl)-2-butanone (**2**) was prepared starting from 1-chloro-4-(chloromethyl)-benzene.[12] (4*S*)-(+)-4-*p*-Toluenesulfonyloxymethyl-2,2-dimethyl-1,3-dioxolane (**3**) and (4*R*)-(−)-4-*p*-toluenesulfonyloxymethyl-2,2-dimethyl-1,3-dioxolane (**4**) were prepared from (4*R*)-(−)-4-hydroxymethyl-2,2-dimethyl-1,3-dioxolane and (4*S*)-(+)-4-hydroxymethyl-2,2-dimethyl-1,3-dioxolane, respectively, according to the published tosylation procedure.[13] The diastereomeric tosylates **5**, **6**, **7**, and **8** were prepared from compound **2** by acid-catalyzed transacetalation in toluene using the corresponding tosylate **3** or **4**, according to the procedure reported by Walker et al.,[14] as shown in Scheme 1. In each case, the diastereomeric product mixture was resolved by column chromatography on silica gel using EtOAc as eluent. Azalanstat (**1**) and its analogues (**9**–**19**) were synthesized by a nucleophilic displacement reaction on the appropriate tosylate (**5**, **6**, **7**, or **8**) using the appropriately substituted aminothiophenol in acetone/K_2CO_3 in a manner similar to that reported by Walker et al.,[14] and isolated/characterized as the hydrochloride or dihydrochloride salts.[15,16]

3. Biological evaluation

Azalanstat (**1**) and its structural analogues were screened for evidence of their ability to inhibit HO using



Scheme 1. Reagents and conditions: (a) *p*-TsOH·H_2O, *n*-butanol, toluene, reflux; (b) 2- or 3- or 4-aminothiophenol, K_2CO_3, acetone, reflux.

an in vitro assay for HO in which heme was presented to the enzyme complexed with albumin.[17] HO-1 was obtained from rat spleen[18] and HO-2 was obtained from rat brain[19,20] in the microsomal fractions prepared by differential centrifugation. Several compounds showed good potency as inhibitors of HO activity (Table 1). The ratio of IC_{50} values measured for HO-2 and HO-1 for each compound is shown in the fourth column, and is used as the index of selectivity for HO-1 compared to HO-2.

The data obtained using this series of azalanstat analogues revealed general inhibitory selectivity for HO-1 over HO-2; this selectivity appeared to be dependent on the diastereomeric configuration of the core. Compounds with the (2*R*,4*S*) configuration for the 1,3-dioxolane ring (**11**, **12**, and **13**) were found to be effective against both HO-1 and HO-2 activity, but compounds **11** and **12** showed high selectivity for HO-1. The (2*S*,4*S*) configuration was also found to produce potent analogues (**9**, **10**, and **1**) against HO-1, with compounds **9** and **10** showing high selectivity for HO-1; compound **10** was the most selective inhibitor studied with a selectivity index of 35 (Fig. 1). Nevertheless, the marked differences in three-dimensional structure amongst the compounds with the (2*R*,4*S*)/(2*S*,4*S*) cores appeared to impact only moderately the selectivity of these HO inhibitors; this result is interpreted to mean that the flexible side chains at the C2 and C4 positions of the 1,3-dioxolane ring in these compounds are not exploiting efficiently the three-dimensional space within the active site of the enzyme. The compounds with the (2*R*,4*R*) configuration (**14**, **15**, and **16**) were found to exhibit the lowest potency with respect to both HO-1 and HO-2 activity, with IC_{50} values > 100 μM in all cases except for mild inhibition of HO-1 in the case of compound **16**.

In contrast to the inhibition observed with 14-DM using azalanstat analogues and other related antifungal agents,[11] it is the (2*R*,4*S*) stereoisomer (**13**) not the (2*S*,4*S*) stereoisomer (**1**) that is most potent toward HO. This initial result compelled us to synthesize and evaluate the compounds having all four diastereomeric configurations. For 14-DM inhibition, the (2*R*,4*S*) isomer (**13**) was the least effective of the four diastereomers (**1**, **13**, **16**, and **19**); this difference in sensitivity to the present diastereomers suggests that the synthesis of even more-selective drugs is possible.

Table 1. Inhibitory potency and selectivity of azalanstat (**1**) and various analogues against HO-1 and HO-2 activity

Compound	IC_{50} (μM) rat spleen (HO-1)	IC_{50} (μM) rat brain (HO-2)	Selectivity index IC_{50} (HO-2)/IC_{50} (HO-1)
1	6 ± 1	28 ± 18	4.7
9	5 ± 1	68 ± 5	13.6
10	1.0 ± 0.2	35 ± 6	35.0
11	2.5 ± 0.1	63 ± 3	25.2
12	1.6 ± 0.7	32 ± 9	20.0
13	0.52 ± 0.03	5 ± 3	9.6
14	>100	>100	—
15	>100	>100	—
16	40 ± 8	>100	>2.5
17	>100	>100	—
18	4 ± 2	34 ± 17	8.5
19	27 ± 9	15 ± 4	0.6

Data represent mean IC_{50} values ± standard deviation of replicate experiments.

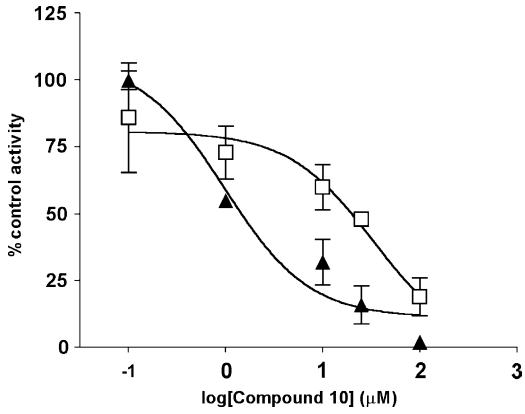


Figure 1. Inhibition of HO-1 and HO-2 activity by compound **10**. Enzyme activity was determined by measuring the quantity of CO produced in 15 min from 50 μM substrate (methemalbumin). All values of activity (ordinate) are expressed as a percentage of the control with no inhibitor present. The values on the abscissa represent the log of the inhibitor concentration. Solid triangles (▲), HO-1 (spleen microsomes) and open squares (□), HO-2 (brain microsomes). Concentrations that inhibited activity by 50% (IC_{50}) were determined by nonlinear regression using GRAPHPAD PRISM version 3.

No striking pattern in selectivity was observed with respect to the position of the amino functionality; nevertheless, the 4-amino compounds (**1**, **13**, **16**, and **19**) all appeared to have higher potency against HO-2 activity than their 2- or 3-amino analogues, an aspect which leads to lower selectivity for HO-1 in three of the four cases (**1**, **13**, and **19**). In fact, compound **19** had the lowest measurable selectivity index and could be taken as the only member of this series with slight selectivity towards HO-2 inhibition. Since the analogues in the (2*S*,4*R*) series (**17**, **18**, and **19**) and the (2*R*,4*R*) series (**14**, **15**, and **16**) proved to have low potency and/or selectivity, further modifications to these structures to create highly potent inhibitors selective for HO-1 should focus on the (2*R*,4*S*) and (2*S*,4*S*) core configurations.

4. Conclusion

We have synthesized several compounds (**9**, **10**, **11**, and **12**) that express good selectivity as inhibitors of HO-1 relative to HO-2. As HO-1 is the isozyme that has attracted the most research interest to date, these drugs are anticipated to become useful tools in elucidating the physiological roles of HO-1 in mammalian and other biological systems.

Acknowledgements

This work was supported by a grant-in-aid from the Canadian Institutes of Health Research, MOP 64305.

References and notes

1. Maines, M. D. *Ann. Rev. Pharmacol. Toxicol.* **1997**, *37*, 517.
2. Vreman, H. J.; Wong, R. J.; Stevenson, D. K. In *Carbon Monoxide and Cardiovascular Function*; Wang, R., Ed.; CRC: Boca Raton, 2002; Chapter 15, p 273.
3. McCoubrey, W. K., Jr.; Huang, T. J.; Maines, M. D. *Eur. J. Biochem.* **1997**, *247*, 725.
4. Johnson, R. A.; Colombari, E.; Colombari, D. S.; Lavesa, M.; Talman, W. T.; Nasjletti, A. *Hypertension* **1997**, *30*, 962.
5. Tulis, D. A.; Durante, W.; Peyton, K. J.; Evans, A. J.; Schafer, A. I. *Atherosclerosis* **2001**, *155*, 113.
6. Luo, D.; Vincent, S. R. *Eur. J. Pharmacol.* **1994**, *267*, 263.
7. Meffert, M. K.; Haley, J. E.; Schumann, E. M.; Schulman, H.; Madison, D. V. *Neuron* **1994**, *13*, 1225.
8. Zakhary, R.; Poss, K. D.; Jaffrey, S. R.; Ferris, C. D.; Tonegawa, S.; Snyder, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 14848.
9. Appleton, S. D.; Chretien, M. L.; McLaughlin, B. E.; Vreman, H. J.; Stevenson, D. K.; Brien, J. F.; Nakatsu, K.; Maurice, D. H.; Marks, G. S. *Drug Metab. Dispos.* **1999**, *27*, 1214.
10. Vreman, H. J.; Cipkala, D. A.; Stevenson, D. K. *Can. J. Physiol. Pharmacol.* **1996**, *74*, 278.
11. Walker, K. A. M.; Kertesz, D. J.; Rotstein, D. M.; Swinney, D. C.; Berry, P. W.; So, O.-Y.; Webb, A. S.; Watson, D. M.; Mak, A. Y.; Burton, P. M.; Mills-Dunlap, B.; Chiou, M. Y.; Tokes, L. G.; Kurz, L. J.; Kern, J. R.; Chan, K. W.; Salari, A.; Mendizabal, G. R. *J. Med. Chem.* **1993**, *36*, 2235.
12. Walker, K. A. M.; Braemer, A. C.; Hitt, S.; Jones, R. E.; Matthews, T. R. *J. Med. Chem.* **1978**, *21*, 840.
13. Baldwin, J. J.; Raab, A. W.; Mensler, K.; Arison, B. H.; McClure, D. E. *J. Org. Chem.* **1978**, *43*, 4876.
14. Walker, K. A. M.; Burton, P. M.; Swinney, D. C. European Patent EP 0 492 474 B1, 1997.
15. Representative procedure for the displacement of tosylates using thiophenol-containing nucleophiles: **(2*S*,4*S*)-2-[2-(4-Chlorophenyl)ethyl]-2-[(1*H*-imidazol-1-yl)methyl]-4-[{(3-aminophenyl)thio}methyl]-1,3-dioxolane dihydrochloride (10)**. Under a nitrogen atmosphere, a mixture of **5** (200 mg, 0.42 mmol), 3-aminothiophenol (113 mg, 0.90 mmol, 2.1 equiv) and K_2CO_3 (116 mg, 0.84 mmol, 4 equiv) in acetone (8 mL) was heated at reflux temperature with stirring for 5 h. The solids were removed by filtration, and washed with hot acetone and then with hot EtOAc. The filtrate was concentrated, and the residue ($R_f \cong 0.2$ in EtOAc) purified by flash chromatography on silica gel (EtOAc) to give 180 mg (0.42 mmol, 100%) of the free base as a pale yellow oil. To a solution of the oil in hot 2-propanol (2 mL) was added a solution of 37% aqueous HCl (82 mg, 0.84 mmol, 2 equiv) in 2-propanol (1 mL). The mixture was concentrated and dried under high vacuum. The residue was dissolved in hot 2-propanol (5 mL), the solution cooled in the freezer, and then Et_2O was slowly added. The solid was removed by filtration and washed with Et_2O. High-vacuum drying left 103 mg (53%) of **10** as a beige solid: mp 189–200 °C (decomp); R_f = 0.10 (EtOAc); $[\alpha]_D^{22}$ +9.1 (*c* 1.76, CD_3OD); ^1H NMR (400 MHz, CD_3OD): δ 1.96–2.10 (m, 2H), 2.75 (t, J = 8.4 Hz, 2H), 2.92–3.03 (m, 2H), 3.39 (t, J = 7.8 Hz, 1H), 4.18 (dd, J = 8.4, 6.4 Hz, 1H), 4.39–4.46 (m, 1H), 4.50–4.63 (m, 2H), 7.19–7.30 (m, 5H), 7.40–7.51 (m, 3H), 7.58–7.62 (m, 2H), 8.95 (s, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ 30.0, 36.3, 38.5, 54.1, 70.5, 76.8, 110.0, 120.4, 121.6, 123.8, 125.3, 129.6, 129.8, 131.0, 131.8, 132.9, 133.2, 137.9, 140.3, 141.3; HRMS (ES) $[M+H]^+$ Calcd for $C_{22}H_{25}ClN_3O_2S$: 430.1350. Found: 430.1362. Anal. Calcd for $C_{22}H_{26}Cl_3N_3O_2S$: C, 52.54; H, 5.21; N, 8.36. Found: C, 52.28; H, 5.80; N, 8.27.
16. Characterization of the new compounds synthesized following the procedure in Note 15 as outlined in Scheme 1:
1 Dihydrochloride: beige solid in 73% yield from **5**: mp 72–180 °C (decomp); R_f = 0.19 (EtOAc); $[\alpha]_D^{23}$ +11.7 (*c* 0.70, CH_3OH); ^1H NMR (400 MHz, D_2O): δ 2.01–2.09 (m, 2H), 2.68–2.74 (m, 2H), 2.95 (dd, J = 14.2, 4.6 Hz, 1H), 3.23–3.27 (m, 1H), 3.99–4.03 (m, 1H), 4.25–4.30 (m, 1H), 4.43–4.52 (m, 1H), 7.20 (d, J = 8.0 Hz, 2H), 7.29 (d, J = 8.4 Hz, 2H), 7.36 (d, J = 8.4 Hz, 2H), 7.43–7.46 (m, 4H), 8.69 (s, 1H); ^{13}C NMR (100 MHz, D_2O): δ 28.6, 35.3, 36.8, 53.2, 69.4, 75.6, 109.1, 119.4, 123.6, 123.9, 128.7, 129.7, 130.1, 130.7, 131.4, 135.9, 136.1, 140.1; HRMS (ES) $[M+H]^+$ Calcd for $C_{22}H_{25}ClN_3O_2S$: 430.1350. Found: 430.1356. Anal. Calcd for $C_{22}H_{26}Cl_3N_3O_2S$: C, 52.54; H, 5.21; N, 8.36. Found: C, 51.89; H, 5.61; N, 7.94.
9 Dihydrochloride: beige solid in 99% yield from **5**; mp 80–175 °C (decomp); R_f = 0.19 (EtOAc); $[\alpha]_D^{22}$ +28.7 (*c* 0.77, D_2O); ^1H NMR (500 MHz, D_2O): δ 2.03–2.13 (m, 2H), 2.56–2.61 (m, 1H), 2.73–2.76 (m, 2H), 2.82–2.85 (m, 1H), 3.15–3.18 (m, 1H), 4.13–4.15 (m, 1H), 4.26–4.34 (m, 1H), 4.48 (s, 2H), 7.22–7.30 (m, 4H), 7.35–7.40 (m, 3H), 7.41–7.46 (m, 1H), 7.48 (s, 1H), 7.56–7.60 (m, 1H), 8.63 (s, 1H); ^{13}C NMR (100 MHz, D_2O): δ 28.6, 36.8, 36.9, 53.2, 69.6, 76.4, 109.1, 119.4, 121.8, 123.9, 125.3, 127.0, 128.7, 130.1, 130.4, 131.5, 135.5, 136.0, 136.9, 140.1; HRMS (ES) $[M+H]^+$ Calcd for $C_{22}H_{25}ClN_3O_2S$: 430.1350. Found: 430.1359. Anal. Calcd for $C_{22}H_{26}Cl_3N_3O_2S$: C, 52.54; H, 5.21; N, 8.36. Found: C, 52.76; H, 5.24; N, 7.81.
11 Dihydrochloride: off-white solid in 72% yield from **6**: mp 125–165 °C (decomp); R_f = 0.11 (EtOAc); $[\alpha]_D^{22}$ +5.6 (*c* 2.15, D_2O); ^1H NMR (400 MHz, D_2O): δ 1.88 (t, J = 8.0 Hz, 2H), 2.56 (t, J = 8.0 Hz, 2H), 2.92–3.06 (m, 2H), 3.63 (t, J = 8.0 Hz, 1H), 3.76–3.82 (m, 1H), 4.03 (t, J = 7.4 Hz, 1H), 7.03 (d, J = 8.0 Hz, 2H), 7.21–7.39 (m, 5H), 7.43 (s, 2H), 7.55 (d, J = 7.6 Hz, 1H), 8.73 (s, 1H); ^{13}C NMR (100 MHz, D_2O): δ 28.3, 37.3, 37.5, 53.7, 69.9, 77.1, 109.2, 119.8, 122.0, 123.6, 125.9, 127.3, 128.8, 130.2, 130.3, 131.5, 135.2, 136.0, 136.3, 140.1; HRMS (ES) $[M+H]^+$ Calcd for $C_{22}H_{25}ClN_3O_2S$: 430.1350. Found: 430.1336. Anal. Calcd for $C_{22}H_{26}Cl_3N_3O_2S$: C, 52.54; H, 5.21; N, 8.36. Found: C, 52.92; H, 5.19; N, 8.18.
12 Hydrochloride: yellowish solid in 81% yield from **6**: mp 165–166 °C; R_f = 0.09 (EtOAc); $[\alpha]_D^{22}$ −14.1 (*c* 2.41, CD_3OD); ^1H NMR (400 MHz, CD_3OD): δ 1.97 (t, J = 8.4 Hz, 2H), 2.67–2.81 (m, 2H), 3.01 (dd, J = 14.0, 6.8 Hz, 1H), 3.16 (dd, J = 14.0, 5.2 Hz, 1H), 3.56–3.63 (m, 1H), 3.68 (t, J = 8.2 Hz, 1H), 4.02 (dd, J = 8.2, 5.8 Hz, 1H), 4.42 (s, 2H), 6.59–6.62 (m, 1H), 6.67–6.70 (m, 1H), 6.75 (t, J = 1.8 Hz, 1H), 7.05 (t, J = 8.0 Hz, 1H), 7.18 (d, J = 8.4 Hz, 2H), 7.27 (d, J = 8.4 Hz, 2H), 7.47 (t, J = 1.4 Hz, 1H), 7.56 (t, J = 1.6 Hz, 1H), 8.83 (s, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ 29.8, 36.4, 39.2, 54.7, 70.9, 77.9, 109.9, 115.3, 117.7, 120.7, 120.9, 124.9, 130.8, 131.0, 132.8, 137.1, 137.8, 141.4, 148.6; HRMS (ES) $[M+H]^+$ Calcd for $C_{22}H_{25}ClN_3O_2S$: 430.1350. Found: 430.1358. Anal. Calcd for $C_{22}H_{25}Cl_2N_3O_2S$: C, 56.65; H, 5.40; N, 9.01. Found: C, 56.53; H, 5.21; N, 8.68.
13 Hydrochloride: beige solid in 41% yield from **6**: mp 161–163 °C; R_f = 0.06 (EtOAc); $[\alpha]_D^{23}$ −11.6 (*c* 1.90, CD_3OD); ^1H NMR (400 MHz, CD_3OD): δ 1.92–1.99 (m, 2H), 2.66–2.78 (m, 2H), 2.82 (dd, J = 13.6, 7.2 Hz, 1H), 3.00 (dd, J = 13.6, 5.2 Hz, 1H), 3.51–3.56 (m, 1H), 3.60 (t, J = 8.2 Hz, 1H), 4.00 (dd, J = 8.2, 5.8 Hz, 1H), 4.39 (s, 2H), 6.68 (d, J = 6.4 Hz, 2H), 7.17 (d, J = 2.0 Hz, 2H),

7.19 (d, J = 2.0 Hz, 2H), 7.27 (d, J = 8.4 Hz, 2H), 7.46 (t, J = 1.4 Hz, 1H), 7.53 (t, J = 1.4 Hz, 1H), 8.79 (s, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ 29.8, 39.3, 38.2 (in DMSO-d_6), 54.7, 71.2, 78.3, 109.8, 117.3, 120.9, 123.0, 124.9, 129.5, 131.0, 132.8, 135.2, 137.8, 141.4, 148.2; HRMS (ES) $[M+H]^+$ Calcd for $C_{22}H_{25}ClN_3O_2S$: 430.1350. Found: 430.1361. Anal. Calcd for $C_{22}H_{25}Cl_2N_3O_2S$: C, 56.65; H, 5.40; N, 9.01. Found: C, 57.00; H, 5.19; N, 9.17.

14 Dihydrochloride: white solid in 87% yield from **7**: mp 90–160 °C (decomp); R_f = 0.14 (EtOAc); $[\alpha]_D^{22}$ −27.0 (c 2.15, D_2O); ^{1}H NMR (400 MHz, D_2O): δ 1.85–1.97 (m, 2H), 2.52 (dd, J = 14.0, 7.6 Hz, 1H), 2.60 (t, J = 7.8 Hz, 2H), 2.75 (dd, J = 14.0, 4.4 Hz, 1H), 3.07 (t, J = 8.0 Hz, 1H), 3.99 (t, J = 7.2 Hz, 1H), 4.12–4.18 (m, 1H), 4.37 (s, 2H), 7.11 (d, J = 8.0 Hz, 2H), 7.19–7.25 (m, 4H), 7.29 (d, J = 8.0 Hz, 1H), 7.36 (t, J = 7.6 Hz, 1H), 7.42 (s, 1H), 7.49 (d, J = 7.6 Hz, 1H), 8.57 (s, 1H); ^{13}C NMR (100 MHz, D_2O): δ 28.6, 36.9, 37.0, 53.2, 69.7, 76.4, 109.1, 119.5, 122.0, 123.8, 125.5, 127.1, 128.7, 130.1, 130.4, 131.5, 135.6, 136.0, 136.5, 140.1; HRMS (ES) $[M+H]^+$ Calcd for $C_{22}H_{25}ClN_3O_2S$: 430.1350. Found: 430.1336. Anal. Calcd for $C_{22}H_{26}Cl_3N_3O_2S$: C, 52.54; H, 5.21; N, 8.36. Found: C, 53.04; H, 5.16; N, 8.11.

15 Dihydrochloride: beige solid in 87% yield from **7**: mp 191–200 °C (decomp); R_f = 0.11 (EtOAc); $[\alpha]_D^{22}$ −9.5 (c 1.68, CD_3OD); ^{1}H NMR (400 MHz, CD_3OD): δ 1.93–2.06 (m, 2H), 2.75 (t, J = 8.4 Hz, 2H), 2.93–3.03 (m, 2H), 3.39 (t, J = 7.8 Hz, 1H), 4.18 (dd, J = 8.0, 6.4 Hz, 1H), 4.39–4.46 (m, 1H), 4.57 (q, J = 15.2 Hz, 2H), 7.19–7.29 (m, 5H), 7.43 (d, J = 8.0 Hz, 2H), 7.49 (t, J = 8.0 Hz, 1H), 7.56–7.62 (m, 2H), 8.95 (s, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ 30.0, 36.3, 38.5, 54.1, 70.5, 76.8, 110.0, 120.4, 121.7, 123.9, 125.3, 129.5, 129.9, 131.0, 131.8, 132.9, 133.0, 137.9, 140.3, 141.3; HRMS (ES) $[M+H]^+$ Calcd for $C_{22}H_{25}ClN_3O_2S$: 430.1350. Found: 430.1348. Anal. Calcd for $C_{22}H_{26}Cl_3N_3O_2S$: C, 52.54; H, 5.21; N, 8.36. Found: C, 52.61; H, 4.95; N, 8.22.

16 Dihydrochloride: white solid in 80% yield from **7**: mp 75–185 °C (decomp); R_f = 0.06 (EtOAc); $[\alpha]_D^{22}$ −11.3 (c 1.95, CD_3OD); ^{1}H NMR (400 MHz, CD_3OD): δ 1.93–2.06 (m, 2H), 2.74 (t, J = 8.4 Hz, 2H), 2.89 (dd, J = 13.6, 6.0 Hz, 1H), 2.97 (dd, J = 13.8, 5.8 Hz, 1H), 3.35 (dd, J = 8.4, 7.6 Hz, 1H), 4.16 (dd, J = 8.4, 6.4 Hz, 1H), 4.34–4.41 (m, 1H), 4.56 (q, J = 13.3 Hz, 2H), 7.21 (d, J = 8.4 Hz, 2H), 7.27 (d, J = 8.4 Hz, 2H), 7.37 (d, J = 8.8 Hz, 2H), 7.49 (d, J = 8.4 Hz, 2H), 7.57–7.61 (m, 2H), 8.95 (s, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ 30.0, 36.5, 38.5, 54.1, 70.5, 76.9, 110.0, 120.3, 124.8, 125.4, 129.6, 130.5, 131.0, 131.4, 132.9, 137.9, 138.6, 141.3; HRMS (ES) $[M+H]^+$ Calcd for $C_{22}H_{25}ClN_3O_2S$: 430.1350. Found: 430.1335. Anal. Calcd for $C_{22}H_{26}Cl_3N_3O_2S$: C, 52.54; H, 5.21; N, 8.36. Found: C, 52.86; H, 5.17; N, 8.11.

17 Hydrochloride: beige solid in 99% yield from **8**: mp 78–110 °C (decomp); R_f = 0.14 (EtOAc); $[\alpha]_D^{21}$ −3.4 (c 1.74, D_2O); ^{1}H NMR (400 MHz, D_2O): δ 1.77 (t, J = 8.0 Hz, 2H), 2.50 (t, J = 8.0 Hz, 2H), 2.71 (d, J = 5.6 Hz, 2H), 3.42 (t, J = 8.0 Hz, 1H), 3.52–3.59 (m, 1H), 3.88 (t, J = 7.0 Hz, 1H), 4.24 (s, 2H), 6.69 (t, J = 7.4 Hz, 1H), 6.87 (d, J = 8.0 Hz, 1H), 6.98 (d, J = 8.4 Hz, 2H), 7.09–7.14 (m, 3H), 7.20 (d, J = 7.6 Hz, 1H), 7.27 (s, 1H), 7.36 (s, 1H), 8.61 (s, 1H); ^{13}C NMR (100 MHz, D_2O): δ 28.5, 37.0, 37.5, 53.7, 69.9, 76.8, 108.9, 117.3, 118.8, 119.8, 120.7, 123.4, 128.7, 130.2, 130.5, 131.5, 135.6, 135.9, 140.1, 146.3; HRMS (ES) $[M+H]^+$ Calcd for $C_{22}H_{25}ClN_3O_2S$: 430.1350. Found: 430.1337.

18 Hydrochloride: beige solid in 95% yield from **8**: mp 163–164 °C; R_f = 0.09 (EtOAc); $[\alpha]_D^{21}$ +12.5 (c 2.08, CD_3OD); ^{1}H NMR (400 MHz, CD_3OD): δ 1.97 (t, J = 8.6 Hz, 2H), 2.68–2.81 (m, 2H), 3.01 (dd, J = 13.8, 6.6 Hz, 1H), 3.15 (dd, J = 14.0, 5.2 Hz, 1H), 3.58–3.65 (m, 1H), 3.68 (t, J = 8.2 Hz, 1H), 4.02 (dd, J = 8.0, 5.6 Hz, 1H), 4.42 (s, 2H), 6.61 (ddd, J = 8.0, 2.4, 0.8 Hz, 1H), 6.69 (ddd, J = 8.0, 1.7, 0.9 Hz, 1H), 6.76 (t, J = 1.8 Hz, 1H), 7.05 (t, J = 8.0 Hz, 1H), 7.17 (d, J = 8.8 Hz, 2H), 7.26 (d, J = 8.4 Hz, 2H), 7.46 (t, J = 1.4 Hz, 1H), 7.54 (t, J = 1.6 Hz, 1H), 8.82 (s, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ 29.8, 36.4, 39.3, 54.7, 71.0, 78.0, 109.9, 115.2, 117.7, 120.7, 121.0, 124.9, 129.5, 130.8, 131.0, 132.8, 137.1, 137.8, 141.4, 148.9; HRMS (ES) $[M+H]^+$ Calcd for $C_{22}H_{25}ClN_3O_2S$: 430.1350. Found: 430.1335. Anal. Calcd for $C_{22}H_{25}Cl_2N_3O_2S$: C, 56.65; H, 5.40; N, 9.01. Found: C, 56.82; H, 5.31; N, 8.89.

19 Hydrochloride: white solid in 62% yield from **8**: mp 163–164 °C; R_f = 0.06 (EtOAc); $[\alpha]_D^{21}$ +11.7 (c 2.23, CD_3OD); ^{1}H NMR (400 MHz, CD_3OD): δ 1.95 (t, J = 8.4 Hz, 2H), 2.66–2.76 (m, 2H), 2.82 (dd, J = 13.8, 7.0 Hz, 1H), 3.00 (dd, J = 13.8, 5.4 Hz, 1H), 3.50–3.57 (m, 1H), 3.60 (t, J = 8.2 Hz, 1H), 4.01 (dd, J = 7.8, 5.8 Hz, 1H), 4.40 (s, 2H), 6.70 (d, J = 8.4 Hz, 2H), 7.17 (d, J = 8.4 Hz, 2H), 7.19 (d, J = 8.4 Hz, 2H), 7.26 (d, J = 8.0 Hz, 2H), 7.47 (s, 1H), 7.53 (s, 1H), 8.81 (s, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ 29.8, 39.2, 38.2 (in DMSO-d_6), 54.7, 71.2, 78.3, 109.8, 116.5, 117.3, 121.0, 123.0, 124.9, 129.5, 131.0, 132.8, 135.2, 137.8, 141.4, 148.2; HRMS (ES) $[M+H]^+$ Calcd for $C_{22}H_{25}ClN_3O_2S$: 430.1350. Found: 430.1334. Anal. Calcd for $C_{22}H_{25}Cl_2N_3O_2S$: C, 56.65; H, 5.40; N, 9.01. Found: C, 56.85; H, 5.26; N, 8.86.

17. In vitro HO activity assay: HO activity in rat spleen and brain microsomal fractions was determined by the quantitation of CO formed from the degradation of methemalbumin (heme complexed with albumin).[21,22] Spleen and brain (Sprague–Dawley rats) microsomal fractions were prepared according to the procedure outlined by Appleton et al.[9] Protein concentration of microsomal fractions was determined by a modification of the Biuret method.[22] Incubations for HO activity analysis were done under conditions for which the rate of CO formation (pmol CO min^{-1} mg $protein^{-1}$) was linear with respect to time and microsomal protein concentration. Briefly, reaction mixtures (150 μL) consisting of 100 mM phosphate buffer (pH 7.4), 50 μM methemalbumin and 1 mg/mL protein were pre-incubated with the inhibitors at final concentrations ranging from 0.1 to 100 μM for 10 min at 37 °C. Reactions were initiated by adding NADPH at a final concentration of 1 mM and incubations were performed for an additional 15 min at 37 °C. Reactions were stopped by instantly freezing the reaction mixture on dry ice, and CO formation was monitored by gas chromatography according to the method described by Vreman and Stevenson.[21]
18. Xia, Z. W.; Cui, W. J.; Zhang, X. H.; Shen, Q. X.; Wang, J.; Li, Y. Z.; Chen, S. N.; Yu, S. C. *World J. Gastroenterol.* **2002**, *6*, 1123.
19. Trakshel, G. M.; Kutty, R. K.; Maines, M. D. *Arch. Biochem. Biophys.* **1988**, *260*, 732.
20. Maines, M. D. *FASEB J.* **1988**, *10*, 2557.
21. Vreman, H. J.; Stevenson, D. K. *Anal. Biochem.* **1988**, *168*, 31.
22. Cook, M. N.; Nakatsu, K.; Marks, G. S.; McLaughlin, B. E.; Vreman, H. J.; Stevenson, D. K. *Can. J. Physiol. Pharmacol.* **1995**, *73*, 515.