H, CH_2), 1.64–1.80 (m, 4 H, CH_2), 1.86–2.00 (m, 2 H, CH_2), 2.53 (br t, 2 H, J = 7.6 Hz, CH_2), 2.63–2.78 (m, 4 H, CH_2), 5.63 (s, 2 H, CH_2N), and 6.84–6.92 (m, 4 H, Ar); IR (KBr) 3600–2400 (br), 1682, 1209, and 1145 cm^{-1}; FABMS calcd for $C_{23}H_{27}{}^{35}ClKN_6O_2$ 493.1521, found 493.1511, $M^+ + K$; FDMS m/e 455 ($M^+ + 1$, ^{35}Cl) and 457 ($M^+ + 1$, ^{37}Cl).

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Registry No. **3a**, 141754-22-5; **3b**, 141754-23-6; **3c**, 141754-24-7; **4a**, 141754-25-8; **4b**, 141754-26-9; **4c**, 141754-27-0; **5a**, 2941-29-9; **5b**, 4513-77-3; **5c**, 7391-45-9; **6a**, 141754-28-1; **6b**, 141754-29-2; **6c**, 141754-30-5; **8a**, 141754-31-6; **8b**, 141754-32-7; **8c**, 141754-33-8; **9a**, 141754-34-9; **9b**, 141754-35-0; **9c**, 141754-36-1; **10a**, 141754-37-2; **10b**, 141754-38-3; **10c**, 141754-39-4; **11**, 79047-41-9; **12**, 137582-52-6; **13a**, 141754-40-7; **13b**, 141754-41-8; **13c**, 141754-42-9; **14a**, 141754-43-0; **14b**, 141754-44-1; **14c**, 141754-45-2; **15a**, 141754-46-3; **15b**, 141754-47-4; **15c**, 141754-48-5; **16a**, 141754-49-6; **16b**, 141754-50-9; **16c**, 141754-51-0; **17a**, 141754-52-1; **17b**, 141754-53-2; **17c**, 141754-54-3.

Metabolism of 5-Hydroxytryptamine by Brain Synaptosomes and Microsomes in the Presence of Cysteine and Glutathione

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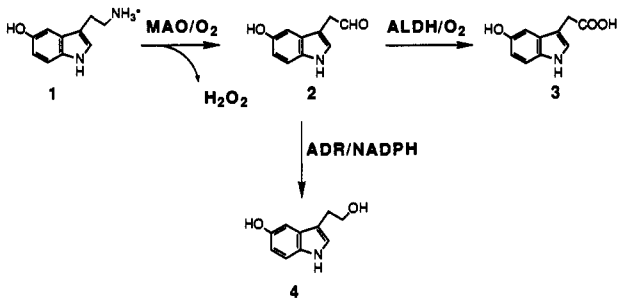
Incubation of 5-hydroxytryptamine (1) with L-cysteine and pig or bovine brain microsomes and an NADPH-generating system or with synaptosomes results in the rapid formation of the (2*R*,4*R*)- and (2*S*,4*R*)-epimers of 2-[(5-hydroxy-1*H*-indol-3-yl)methyl]thiazolidine. Incubation of 1 and glutathione under the same experimental conditions yields the (2*R*,4*R*)- and (2*S*,4*R*)-epimers of α-amino-4-[[(carboxymethyl)amino]carbonyl]-2-[(5-hydroxy-1*H*-indol-3-yl)methyl]-δ-oxo-3-thiazolidinepentanoic acid. These various thiazolidine derivatives are formed by nucleophilic addition of the thiol residues of cysteine or glutathione to the aldehyde group of 5-hydroxyindole-3-acetaldehyde (2), the primary product of the monoamine oxidase-mediated oxidative deamination of 1. The facile reaction of cysteine and glutathione with 2 might represent a mechanism designed to scavenge the biogenic aldehyde and therefore to prevent its alkylation of key intraneuronal protein nucleophiles.

The major catabolic pathway for the indolic neurotransmitter 5-hydroxytryptamine (1; serotonin) in the central nervous system (CNS) derives from the action of monoamine oxidase (MAO; EC 1.4.3.4) which catalyzes the oxidative deamination of the indolamine to give 5-hydroxyindole-3-acetaldehyde (2) (Scheme I).[1] It appears to be generally accepted that 2 is further metabolized primarily by a reaction catalyzed by aldehyde dehydrogenase (ALDH; EC 1.2.1.3) to 5-hydroxyindole-3-acetic acid (3). A minor pathway involves reduction of 2 by aldehyde reductase (ADR; EC 1.1.1.2) to 5-hydroxytryptophol (4). The aldehyde residue of 2 is an electrophilic center which would be expected to undergo facile reactions with cellular nucleophiles. Indeed, suggestions have been made that 2 reacts with nucleophilic residues associated with neuronal macromolecules including membrane proteins,[2-4] although the resulting adducts have not been isolated and characterized. Nevertheless, such reactions in vivo might provide an explanation[3,5] for the low excretion of 3 following administration of 1.[6] However, defense mechanisms exist within neurons to protect against such electrophilic insult. For example, cysteine and glutathione are protective nucleophiles which occur in relatively high concentrations within neurons. Intraneuronal concentrations of glutathione have been estimated to be in the range 0.9–3.4 mM[7,8] whereas cysteine occurs at somewhat lower concentrations (ca. 0.1 mM).[9,10] Accordingly, under metabolic conditions where 2 is not rapidly converted into 3 and 4, it might be anticipated that the aldehyde is scavenged (conjugated) by glutathione and/or cysteine. Recently, Susilo et al.[11,12] reported that incubation of tryptamine with brain homogenates (pig, bovine, rat) resulted in the formation of a new metabolite, (4*R*)-2-(3′-indolylmethyl)-1,3-thiazolidine-4-carboxylic acid.

Scheme I



This compound was thought to be formed as a result of the reaction between indole-3-acetaldehyde, the MAO-

* Corresponding author.

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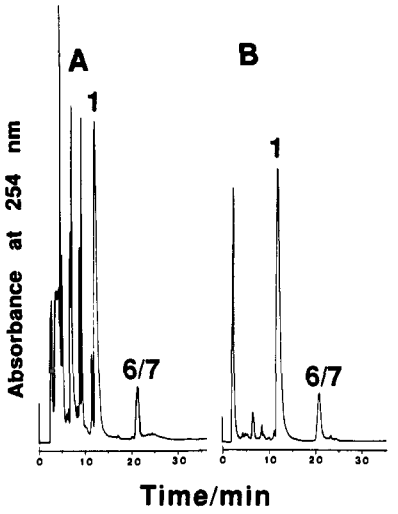

Figure 1. HPLC chromatograms recorded following incubation of 2.0 mM 5-hydroxytryptamine and 10 mM cysteine with (A) microsomal protein (bovine; 3 mL containing ca. 30 mg of protein) in 7.0 mL of a medium containing 5 mM $MgCl_2$, 0.6 mM β-$NADP^+$, 5 mM glucose 6-phosphate, glucose-6-phosphate dehydrogenase (35 units), 0.1 M Tris/HCl, pH 7.8, and 1.5 mM KCN and (B) synaptosomal protein (3 mL containing ca. 30 mg of protein), 100 mM KCl, 10 mM disodium succinate, 75 mM mannitol, 25 mM sucrose, 10 mM dipotassium hydrogen phosphate, 10 mM Tris/HCl, and 0.05 M dipotassium EDTA at pH 7.4 and 37 °C for 1 h. Chromatography employed HPLC method I (injection volume, 800 μL).

catalyzed oxidative deamination product from tryptamine, and cysteine. These authors also incubated 5-HT with a rat brain homogenate and subsequently isolated a compound which, on the basis of its mass spectrum, was proposed to be a thiazolidine derivative formed by a reaction between 2 and cysteine. A complete structure elucidation of this compound was not carried out. In this paper we report the isolation and structure elucidation of new metabolites formed when 1 is incubated with mammalian brain microsomes and synaptosomes in the presence of L-cysteine and glutathione.

Results

An HPLC chromatogram recorded after incubation of 1 and L-cysteine with brain microsomes for 1 h in the presence of an NADPH generating system[13] is shown in Figure 1A. The chromatographic peaks having a retention time (t_R) less than that of 1 (t_R = 13.0 min) are due to various constituents of the incubation medium. During the initial 5 h of the reaction a systematic decrease in the peak of 1 occurs and, correspondingly, a new peak at t_R = 22.0 min appears and grows. Using preparative HPLC the species responsible for this peak were isolated. Spectroscopic analysis (MS, ^1H and ^{13}C NMR) revealed that the peak is due to an approximately equimolar mixture of the (2*R*,4*R*)- and (2*S*,4*R*)-epimers of 2-[(5-hydroxy-1*H*-indol-3-yl)methyl]thiazolidine (**6** and **7**, respectively). Attempts to physically separate **6** and **7** using a variety of HPLC conditions were unsuccessful. After incubation times >5 h the chromatographic peak of 1 continued to decrease but so did that of the mixture of **6** and **7**, indicating further reaction of the latter compounds. The results described above were obtained using bovine brain microsomes. Virtually identical results were obtained when pig brain microsomes were employed. In the absence of added cysteine but otherwise under the conditions described in Figure 1, 1 was converted primarily to **3** along with small yields of **4**. The latter compounds were identified by their HPLC t_R values (**3**, t_R = 19.0 min; **4**, t_R = 20.0 min; HPLC method I), by thermospray mass spectral analyses of solutions of these compounds collected from such HPLC separations, and by UV–visible spectra. When 1 was incubated with microsomes, but without an NADPH-generating system in either the presence or absence of added cysteine, no reactions were observed.

Figure 1B shows a chromatogram obtained after incubation of 1 and cysteine with pig brain synaptosomes. During the initial 3 h of the reaction the chromatographic peak of 1 systematically decreased and that due to epimers **6** and **7** correspondingly increased. ^1H and ^{13}C NMR analysis of the isolated product revealed it to be an approximately equimolar mixture of epimers **6** and **7**. After longer periods of incubation, 1 continued to be consumed but the chromatographic peak corresponding to the mix-

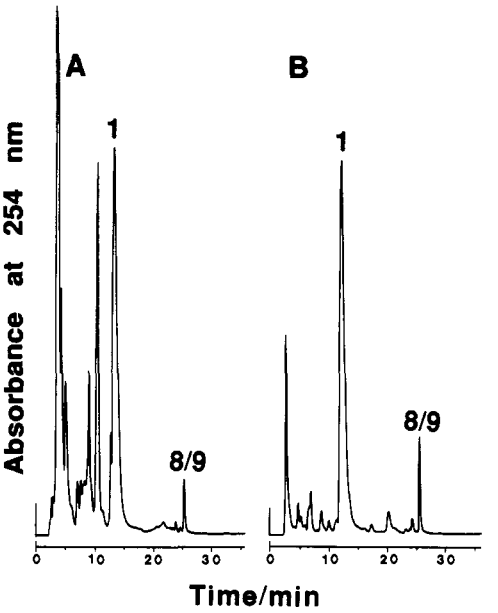

Figure 2. HPLC chromatograms recorded following incubation of 2.0 mM 5-hydroxytryptamine and 10 mM glutathione with (A) microsomal protein (bovine; 3 mL containing ca. 30 mg of protein) in 7.0 mL of a medium containing 5 mM $MgCl_2$, 0.6 mM β-$NADP^+$, 5 mM glucose 6-phosphate, glucose-6-phosphate dehydrogenase (35 units), 0.1 M Tris/HCl, pH 7.8, and 1.5 mM KCN and (B) synaptosomal protein (3.0 mL containing ca. 30 mg of protein) and 7.0 mL of a medium consisting of 100 mM KCl, 10 mM disodium succinate, 75 mM mannitol, 25 mM sucrose, 10 mM dipotassium hydrogen phosphate, 10 mM Tris/HCl, and 0.05 M dipotassium EDTA at pH 7.4 and 37 °C. In A the incubation time was 1 h; in B the incubation time was 5 h. Chromatography employed HPLC method I (injection volume, 800 μL).

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ture of **6** and **7** also decreased and many additional peaks appeared. No attempt was made to identify the compounds responsible for the latter peaks although they probably represent products of further reactions of epimers **6** and **7**. In the absence of added cysteine, incubation of **1** with brain synaptosomes resulted in formation of **3** and **4**.

A chromatogram recorded after the incubation of **1** and glutathione in the presence of brain microsomes and an NADPH-generating system for 1 h is shown in Figure 2A. Throughout the first 15 h of the reaction the HPLC peak of **1** decreased and a single new chromatographic peak (t_R = 25.0 min) correspondingly appeared and grew. On the basis of ^1H and ^{13}C NMR spectroscopy of the isolated product, it was concluded that the latter peak corresponds to an approximately equimolar mixture of the (2*R*,4*R*)- and (2*S*,4*R*)-epimers of α-amino-4-[[(carboxymethyl)amino]-carbonyl]-2-[(5-hydroxy-1*H*-indol-3-yl)methyl]-δ-oxo-3-thiazolidinepentanoic acid (**8** and **9**, respectively). After longer periods of incubation the concentrations of **8** and **9** declined and, on the basis of HPLC analysis, a major product of the decomposition of the latter epimers was **3**. When **1** and glutathione were incubated with synaptosomes the concentrations of **8**/**9** increased (Figure 2B) for >30 h, but in the latter stages of the reaction a small amount of **3** appeared. Although a variety of different chromatographic conditions was employed, it was not possible to separate epimers **8** and **9**. The synaptosome-mediated transformation of **1** to **3** and **4**, in the absence of added cysteine and glutathione, and to **6**/**7** or **8**/**9**, in the presence of the latter thiols, respectively, occurred without an added NADPH-generating system.

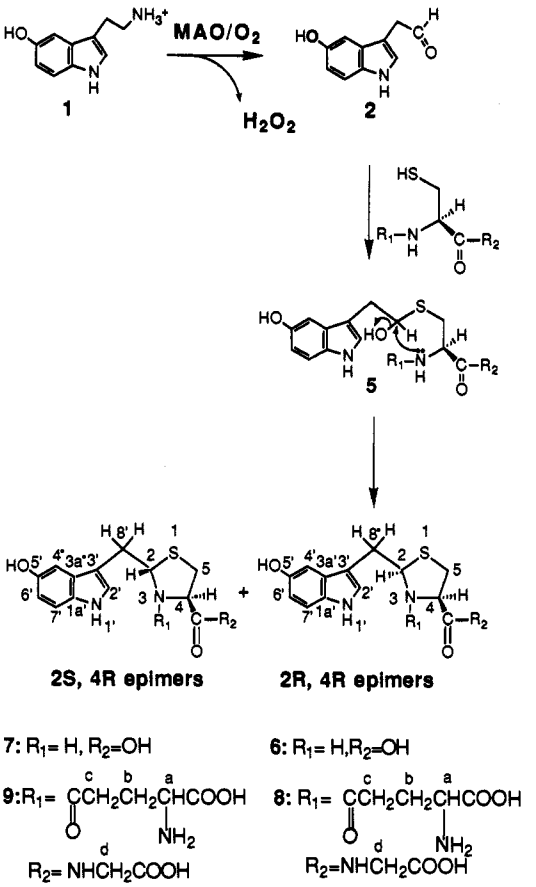
Reaction Pathways

In the absence of added cysteine or glutathione, **1** is metabolized in the presence of either pig or bovine brain microsomes and an NADPH-generating system or brain synaptosomes predominantly to **3** along with some **4**. However, in the presence of added cysteine or glutathione, epimers **6** and **7**, or **8** and **9**, respectively, replace **3** and **4** as the metabolites. This appears to be accomplished as a result of nucleophilic addition of the thiol residue of cysteine or glutathione to the aldehydic group of **2** to yield the intermediate thioether **5**. Intramolecular nucleophilic substitution by the amino residue in **5** then leads to the thioazolidine derivatives **6**–**9** as conceptualized in Scheme II. The carbonyl group in **2** is a prochiral center and hence nucleophilic addition of the sulfhydryl residue of L-cysteine or glutathione can occur from either the *re* or the *si* face, thus forming the epimeric mixture of the resulting thiazolidine derivatives at C(2). Support for the pathway shown in Scheme II was provided by reacting the free aldehyde **2**, synthesized according to the method of Nilsson and Tottmar[2] (see Experimental Section), with L-cysteine in aqueous solution. HPLC analysis of the resulting product solution showed a single chromatographic peak (t_R = 22 min). HPLC method II was used to isolate and purify the product. After freeze-drying the ^1H NMR spectrum of the resulting product in D_2O displayed two sets of signals in a 1:1 ratio. These signals were superimposable with those obtained with the product formed by incubating 5-HT and L-cysteine with microsomes or synaptosomes, i.e., **6**/**7**. Similarly, the UV and mass spectra of the product mixture formed from reaction of **2** and L-cysteine were identical to those observed for **6**/**7**.

Structure Elucidation

Molecular models reveal that in **6** free rotation about the C(3′)–C(8′) single bond should bring the carboxyl group

Scheme II



at C(4) into close proximity to C(2′)-H and C(4′H)-H and, therefore, a downfield shift of the NMR resonances for these protons would be expected. In the case of epimer **7** the carboxyl group at C(4) cannot closely approach either C(2′)-H or C(4′H)-H so that the resonances for these protons should appear upfield relative to those in **6**. The ^1H NMR spectrum of the product isolated following incubation of **1** and L-cysteine in the presence of brain microsomes and synaptosomes showed two sets of signals of equal intensity. Similarly, the ^{13}C spectrum of this product also showed the presence of several closely-spaced peaks of equal intensity. Accordingly, it was concluded that the product consists of an equimolar mixture of two isomers. These two isomers could not be physically separated, but the ^1H NMR resonances corresponding to epimers **6** and **7** could be readily distinguished, particularly with the aid of ^1H,^1H-correlated spectroscopy (COSY) techniques. The spectral assignments for **6** and **7** are reported in the Experimental Section. However, in agreement with predictions derived from molecular models one isomer, **6**, exhibited a small downfield shift of the C(4′)-H (δ 7.109) and C(2′)-H (δ 7.338) resonances compared to isomer **7** (C(4′)-H, δ = 7.096, C(2′)-H, δ = 7.314). Molecular models also indicate that in **6** C(2)-H and C(4)-H are cis whereas in **7** these protons are trans. Using delayed COSY experiments (Δ = 0.5 s; pulse width 45°)[14] a very weak coupling was observed between C(2)-H (δ 5.231) and C(4)-H (δ 4.610) in the spectrum of **6**. Molecular models indicate that in **6** these protons lie in the same plane. In the case of isomer **7** no such long-range coupling was observed between C(2)-H (δ 5.132) and C(4)-H (δ 4.459). Such weak,

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long-range couplings between C(2)-H and C(4)-H across nitrogen in thiazolidine or related ring systems have, to the authors' knowledge, not been previously reported. Thus, in the absence of such literature precedence, it is only possible to tentatively conclude that 6 has the cis conformation with respect to C(2)-H and C(4)-H and, correspondingly, that 7 has the anti conformation. Delayed COSY experiments showed no long-range couplings between C(2)-H and the C(5)-H protons in either 6 or 7.

The thiazolidine derivatives 8 and 9 formed when 1 and glutathione were incubated with brain microsomes and synaptosomes could not be physically separated. However, the ^{1}H NMR spectrum of the mixture clearly exhibited two sets of resonances of equal intensity which could be readily assigned to the individual isomers. That a mixture of isomers was present was also apparent from the ^{13}C NMR spectrum where many resonances appeared as very closely spaced double peaks of equal intensity. Similar to the behavior observed for 6, delayed COSY experiments on the mixture of 8 and 9 showed a weak, long-range coupling between the ^{1}H NMR resonance of C(2)-H (δ 5.251) and C(4)-H (δ 4.820) for 8, suggesting the cis conformation between these protons. No such long-range coupling was observed for 9, indicating that this isomer might have an anti conformation with respect to these protons.

Conclusions

Incubations of 1 and L-cysteine with mammalian brain microsomes and synaptosomes result in the facile formation of the thiazolidine epimers 6 and 7. Under the experimental conditions employed, concentrations of 6/7 reach a maximal level after ca. 5 h in the reaction mediated by microsomes and after ca. 3 h in the reaction mediated by synaptosomes. The levels of 6 and 7 then decrease although only very small amounts of 3 and 4 appear in the latter stages of the reactions, indicating these compounds do not represent the major products of further reactions of 6 and 7. Using a rat brain homogenate it has been noted[12] that a metabolite having the same molar mass as 6 and 7 reached a maximal concentration after about 30 min and then decreased slightly during the next 3.5 h whereas 3 increased continuously. These observations have been interpreted to indicate that the thiazolidine is cleaved by a carbon–sulfur lyase, present mainly in cytosolic fraction, to give cysteine and 3. This reaction does not occur to an appreciable extent with the subcellular brain particles employed in the present study.

Thiazolidine derivatives 8 and 9 are formed rapidly when 1 and glutathione are incubated with brain microsomes and an NADPH-generating system or with synaptosomes. However, the structurally more complex epimers 8 and 9 are more stable than 6 and 7 with the result that they accumulate for much longer periods of time. Some decomposition of 8 and 9 occurred after 15 h in the presence of microsomes to yield 3.

The above results support the conclusion that not only L-cysteine[12] but also glutathione are probably involved in the metabolism of 1 by reacting with the very reactive biogenic aldehyde 2 to form the corresponding thiazolidine derivatives by the routes shown in Scheme II. This pathway might represent a mechanism to scavenge the biogenic aldehyde and therefore to prevent its alkylation of key intraneuronal protein nucleophiles.

Experimental Section

Pig and bovine brains were obtained from a local slaughterhouse within 2 h after death and were immediately homogenized. 5-Hydroxytryptamine hydrochloride, 5-hydroxyindole-3-acetic acid, 5-hydroxytryptophol, L-cysteine, glutathione, succinic acid (disodium salt), potassium chloride, mannitol, sucrose, Tris/HCl, ethylenediaminetetraacetic acid (EDTA, dipotassium salt), magnesium chloride hexahydrate, nicotinamide adenine dinucleotide phosphate ($NADP^+$), glucose 6-phosphate, glucose-6-phosphate dehydrogenase (type XXIII, EC 1.1.1.49), and potassium cyanide were obtained from Sigma (St. Louis, MO) and were used without additional purification.

Preparation of Synaptosomes and Microsomes. Synaptosomes and microsomes were prepared according to the procedures described by Gray and Whittaker[15] and Bradford.[16] Fresh brains were kept in ice-cold isolation medium (0.32 M sucrose, 10 mM Tris/HCl, 1.0 mM potassium EDTA, pH 7.4) and chopped into small pieces with scissors. Blood and other debris were washed off the brain tissue by adding more isolation medium and decanting the supernatant. This washing procedure was repeated three or four times. The chopped tissue was homogenized with a low-shear continuous tissue homogenizer (Yamato, Inc., Northbrook, IL) having a clearance of 0.005 in between the Teflon pestle and the glass cylinder. The homogenizer was operated at 1700–1770 rpm and 12 passes were used to homogenize the tissue. The resulting crude homogenate (10% in the isolation medium) was centrifuged at 1000g for 10 min and the supernatant carefully decanted. The resulting pellet, which consisted principally of crude nuclei and debris, was discarded. The supernatant was then centrifuged at 17000g for 60 min to obtain the crude mitochondrial pellet. The supernatant from this centrifugation was further centrifuged at 10^5g for 120 min and the microsomes, which settled at the bottom, were collected; the supernatant was discarded. The microsomes obtained in this fashion were resuspended in isolation medium (ca. 10 mg/mL). The crude mitochondrial pellet, reconstituted in isolation medium (0.10 g/mL), was carefully layered on 0.8 M sucrose which, in turn, was layered on 1.2 M sucrose and centrifuged at 10^5g for 60 min using a swinging-bucket rotor (Beckman). The mitochondria formed a pellet while the contaminating myelin floated at the top of the 0.8 M sucrose. Synaptosomes were suspended in a diffuse layer at the interphase between the 0.8 and 1.2 M sucrose. The myelin layer was carefully removed and the synaptosomes were gently sucked from the interphase[16] and diluted with water by a factor of 2.2 in order to adjust the concentration of the sucrose solution to approximately 0.4 M. The resulting solution was centrifuged at 17000g for 20 min. The final synaptosomal pellet was suspended in isolation medium to give ca. 10 mg/mL.

High-Performance Liquid Chromatography (HPLC). HPLC employed a Bio-Rad instrument equipped with dual Model 1300 pumps, an Apple IIe controller, a Rheodyne 7125 loop injector, and an ISCO Model 226 or Bio-Rad Model 1305A UV detector (254 nm).

In order to monitor the rate of oxidative deamination of 5-HT by MAO, HPLC method I was employed. For this method a reversed-phase column (Brownlee Laboratories, Santa Clara, CA; RP-18, 5 μm particle size, 25 × 0.7 cm) was used which was protected by a short guard column (Brownlee; RP-18, 5 μm, OD-GU, 5 × 0.5 cm). Two mobile-phase solvents were employed. Solvent A contained 0.7% NH_4OH and 1.0% acetonitrile (MeCN) in water which was adjusted to pH 3.5 with concentrated formic acid (HCOOH). Solvent B was 0.7% NH_4OH, 40% MeCN in water adjusted to pH 4.5 with HCOOH. The following gradient was employed: 0–4 min, 100% solvent A, flow rate 2–5 mL/min; 4–11 min, linear gradient to 10% solvent B; 11–20 min, linear gradient to 30% solvent B and a corresponding linear increase of flow rate to 3.0 mL/min; 20–32 min, linear gradient to 65% solvent B; 32–35 min, linear gradient to 100% solvent B; 35–38 min, 100% solvent B. The mobile phase was then linearly returned to 100% solvent A over the next 7 min and the column equilibrated for 5 min (3 mL/min) before the next sample was injected. Typically, 1.0-mL sample injections were employed.

Preparative HPLC, method II, employed a reversed-phase column (J. T. Baker; Bakerbond C_{18}, 25 × 2.12 cm, 10 μm). Two mobile-phase systems were employed. Solvent C was 5% MeCN

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in water adjusted to pH 2.1 with trifluoroacetic acid (TFA). Solvent D was 40% MeCN in water adjusted to pH 2.1 with TFA. The following gradient was employed: 0–5 min, 100% solvent C with a linear increase of flow rate from 4 to 5 mL/min; 5–10 min, linear increase to 12% solvent D and flow rate to 5.5 mL/min; 10–22 min, linear gradient to 25% solvent D and flow rate to 6 mL/min; 22–35 min, linear gradient to 35% solvent D; 35–40 min, 35% solvent D; 40–50 min, linear gradient to 65% solvent D; 50–55 min, 65% solvent D. The mobile phase was then linearly returned to 100% solvent C over 5 min (6.0 mL/min) and the column equilibrated for 5 min before another sample was injected. Typically, 10-mL sample injections were employed.

Spectroscopy. Low- and high-resolution fast atom bombardment mass spectrometry (FAB-MS) was carried out with a VG Instruments (Manchester, U.K.) Model ZAB-E spectrometer. Thermospray mass spectral data were obtained with a Kratos MS 25/RFA instrument equipped with a thermospray source. The source was maintained at 300–305 °C and the thermospray capillary tip at 195 °C. The mobile phase was 0.1 M ammonium acetate in water at a flow rate of 0.9 mL/min. All thermospray MS results were obtained by injecting aliquots (0.2–2.0 mL) of fractions, collected from conventional HPLC separations, directly into the thermospray source using a loop injector (Rheodyne, Model 7125).

^1H and ^{13}C NMR spectra were recorded on a Varian 300XL spectrometer. The complete chemical names of **6–9** are given elsewhere in the paper. However, assignments of proton resonances employ the numbering system shown in Scheme II. UV–visible spectra were recorded on a Hewlett-Packard 8452A diode-array spectrophotometer.

Reactions of 1 with Cysteine and Glutathione in the Presence of Synaptosomes. 5-Hydroxytryptamine hydrochloride (4.2 mg, 2 mM) was incubated with synaptosomal protein (30 mg, 3.0 mL) in 7.0 mL of a medium consisting of succinic acid (disodium salt; 27.02 mg, 10 mM), KCl (100 mM), mannitol (75 mM), sucrose (25 mM), dipotassium hydrogen phosphate (10 mM), Tris/HCl (10 mM), EDTA (dipotassium salt, 0.05 mM), and either L-cysteine (12.12 mg, 10 mM) or glutathione (30.7 mg, 10 mM) at 37 °C with stirring. In some experiments the above medium was changed such that 100 mM NaCl replaced the 100 mM KCl. This change caused no effects on the reactions observed. The reactions were monitored by periodically withdrawing a sample followed by analysis by HPLC (method I). The cysteine adducts **6/7** were formed in maximum yield after 5 h. The glutathione adducts **8/9** were formed in maximal yield after ca. 20 h of reaction.

Reactions of 1 with Cysteine and Glutathione in the Presence of Microsomes. 5-Hydroxytryptamine hydrochloride (4.25 mg, 2.0 mM) was incubated at 37 °C with microsomal protein (ca. 30 mg, 3.0 mL), $MgCl_2$-$6H_2O$ (10.16 mg, 5.0 mM), β-$NADP^+$ (4.59 mg, 0.6 mM), glucose 6-phosphate (47 μL, 5 mM), glucose-6-phosphate dehydrogenase (35 units, 14 μL), and cysteine (12.12 mg, 10 mM) or glutathione (30.7 mg, 10 mM) in 7.0 mL of a medium consisting of 0.1 M Tris/HCl, pH 7.8, 1.5 mM KCN. The reaction solutions were stirred with a Teflon-coated magnetic stirring bar. The course of these reactions was monitored by periodically removing a sample followed by analysis by HPLC method I. The maximum yields of cysteine adducts **6/7** were obtained after 5 h of reaction. The maximum yields of glutathione adducts **8/9** were obtained after 20 h.

Product Isolation and Characterization. The products formed as a result of incubation of **1** with L-cysteine or glutathione in the presence of brain microsomes or synaptosomes were isolated using HPLC method II. The products of several reactions were combined, frozen, and freeze-dried.

Adducts Formed in the Presence of L-Cysteine. Under all HPLC conditions employed using reversed-phase columns only a single chromatographic peak was observed corresponding to the products formed when **1** was incubated with L-cysteine in the presence of brain microsomes and synaptosomes. In pH 7.4 phosphate buffer the mixture of epimers **6** and **7** exhibited a UV spectrum [λ_{max}, nm (log ϵ_{max}, M^{-1} cm^{-1})] of 296 (sh) (3.59) and 276 (3.64). This spectrum was very similar to that of **1** [294 (sh) (3.73), 276 (3.82)]. FAB-MS (negative ion; triethylamine matrix) showed a pseudomolecular ion ($M - H^-$) at m/e = 277.0665 (6.5; $C_{13}H_{13}N_2O_3S$; calcd m/e = 277.0647). ^{13}C NMR (D_2O) data was as follows: δ 30.714 (C-8′), 31.548 (C-5), 62.066 (C-4), 68.063 and 68.495 (C-2), 105.172 and 105.281 (C-6′), 110.401 and 110.443 (C-3′), 114.753 (C-4′), 115.652 (C-7′), 128.517 and 128.558 (C-2′), 129.689 (C-3a′), 134.085 and 134.129 (C-1a′), 151.656 and 151.676 (C-5′), 180.442 (C=O). Thus, in accord with the fact that isomers **6** and **7** are formed as an equimolar mixture, several resonances in the ^{13}C NMR spectrum appeared as closely-spaced double peaks of equal intensity. The ^1H NMR spectrum of **6/7** in D_2O (300 MHz) was quite complex. However, largely on the basis of ^1H,^1H-correlated spectroscopy (COSY) experiments it was clear that the spectrum contained two sets of signals of equal intensity, also indicating that the isolated sample consisted of an approximately equimolar mixture of epimers **6** and **7**. For reasons which have been discussed earlier, it has been tentatively concluded that in **6** the C(2)-H and C(4)-H protons are cis and in **7** they are trans.

(2*R*,4*R*)-2-[(5-Hydroxy-1*H*-indol-3-yl)methyl]thiazolidine (6): ^1H NMR (D_2O) δ 7.381 (d, $J_{6',7'}$ = 8.7 Hz, C(7′)-H), 7.338 (s, 1 H, C(2′)-H), 7.109 (d, $J_{4',6'}$ = 2.4 Hz, 1 H, C(4′)-H), 6.838 (dd, $J_{4',6'}$ = 2.4 Hz, $J_{6',7'}$ = 8.7 Hz, 1 H, C(6′)-H), 5.231 (dd, J = 5.7, 9.0 Hz, 1 H, C(2)-H), 4.610 (t, J = 6.6 Hz, 1 H, C(4)-H), 3.486, 3.467 (dd, J = 4.5, 18.0 Hz, 1 H and dd, J = 7.2, 18.0 Hz, 1 H, C(8′)-H_2), 3.369, 3.292 (dd, J = 6.9, 14.5 Hz, 1 H and dd, J = 9.0, 14.5 Hz, 1 H, C(5)-H_2).

(2*S*,4*R*)-2-[(5-Hydroxy-1*H*-indol-3-yl)methyl]thiazolidine (7): ^1H NMR (D_2O) δ 7.381 (d, $J_{6',7'}$ = 8.7 Hz, 1 H, C(7′)-H), 7.314 (s, 1 H, C(2′)-H), 7.096 (d, $J_{4',6'}$ = 2.4 Hz, 1 H, C(4′)-H), 6.838 (dd, $J_{4',6'}$ = 2.4 Hz, $J_{6',7'}$ = 8.7 Hz, 1 H, C(6′)-H), 5.132 (t, J = 7.2 Hz, 1 H, C(2)-H), 4.459 (t, J = 6.9 Hz, 1 H, C(4)-H), 3.517, 3.393 (dd, J = 7.2, 12.1 Hz, 1 H and dd, J = 5.4, 12.1 Hz, 1 H, C(8′)-H_2), 3.486, 3.307 (dd, J = 3.9, 9.9 Hz, 1 H, and dd, J = 4.8, 9.9 Hz, 1 H, C(5)-H_2).

Synthesis of 6/7 from 5-Hydroxyindole-3-acetaldehyde (2). The bisulfite adduct of **2** was prepared using a modification of the procedure of Nilsson and Tottmar.[2] A solution of **1**·HCl (4.24 mg, 2 mM) in 10 mL of 100 mM KCl containing sodium succinate (27.02 mg, 10 mM) and sodium metabisulfite (7.6 mg, 4 mM) adjusted to pH 7.4 with aqueous KOH was prepared. To this solution was added 2 mL of a suspension of rat brain synaptosomal protein (10 mg/mL) in isolation medium prepared as described previously.[15,16] This solution/suspension was incubated at 37 °C for 4 h. The resulting reaction mixture was centrifuged at 17000*g* for 20 min. The supernatant was collected and filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA). HPLC analysis of the filtrate (HPLC method I) showed a peak at t_R = 9 min in addition to that of unreacted **1** (t_R = 12 min). The component eluted under the peak at t_R = 9 min exhibited a UV spectrum with λ_{max} = 294 (sh) and 278 nm (HPLC mobile phase, pH 4.5). Thermospray MS on this solution showed m/e = 193 ($MH^+ - SO_2$, 100), 176 ($MH^+ - SO_3H$, 65). Thus, the compound eluted at t_R = 9 min was the bisulfite adduct of **2** (molar mass 257 g). The free aldehyde was prepared from the supernatant (ca. 10 mL) by first decomposing the bisulfite adduct with sodium pyrophosphate buffer (6 mL, 50 mM, pH 8.8). The resulting solution was extracted four times with 15-mL aliquots of diethyl ether. The combined extracts were washed with deionized water (10 mL × 2). The ether was then evaporated with a stream of dry N_2. The resulting free aldehyde **2** was obtained as a viscous, colorless liquid. This sample of **2** was shaken with 5 mL of a solution of L-cysteine (6.06 mg, 10 mM) in water with N_2 gas bubbling through the solution for 15–20 min. HPLC analysis (method I) of the resulting product solution showed a single chromatographic peak at t_R = 22 min, i.e., identical to that of **6/7**. Using HPLC method II followed by freeze-drying a solid sample was obtained. The ^1H NMR spectrum of this solid in D_2O displayed two sets of signals having equal intensities which were identical to those observed for **6/7** prepared by incubating **1** with brain microsomes and synaptosomes.

Adducts Formed between 2 and Glutathione. The thiazolidine adducts formed upon incubation of **1** and glutathione in the presence of microsomes or synaptosomes could not be separated from each other under any of the chromatographic conditions employed. In pH 7.4 phosphate buffer the spectrum of the mixture of epimers **8** and **9** showed λ_{max} (log ϵ_{max}, M^{-1} cm^{-1}) of 296 (sh) (3.59) and 278 nm (3.64). FAB-MS (positive ion; thioglycerol/glycerol matrix) showed no pseudomolecular ion but a major fragment ion at m/e = 336. This corresponds to the molar

mass of 8 or 9 (444) minus a glutamic acid fragment (130). ^{13}C NMR (D_2O) data was as follows: δ 30.474 (C-8'), 31.394 (C-5), 35.131 (C-b), 35.487 (C-c), 44.254 and 44.314 (C-d), 64.954 (C-a), 65.638 and 65.940 (C-4), 68.660 and 69.106 (C-2), 105.180 and 105.276 (C-6'), 110.394 and 110.440 (C-3'), 114.743 (C-4'), 115.639 (C-7'), 128.479 and 128.571 (C-2'), 129.686 (C-3a'), 134.087 and 134.124 (C-1a'), 151.666 (C-5'), 170.667 (C=0), 175.841 (C=0), 180.444 (C=0), 181.246 (C=0). Thus, a total of 20 carbon resonances were observed in accord with the proposed structure of 8/9. However, several resonances appeared as closely-spaced double peaks of equal intensity indicative of an equimolar mixture of 8 and 9. The $^1H,^1H$-COSY NMR spectrum also clearly showed two sets of signals of equal intensity. For reasons discussed earlier, it has been tentatively concluded that in 8 the C(2)-H and C(4)-H protons are cis whereas in 9 they are trans.

(2*R*,4*R*)-α-Amino-4-[[(carboxymethyl)amino]carbonyl]-2-[(5-hydroxy-1*H*-indol-3-yl)methyl]-δ-oxo-3-thiazolidinepentanoic acid (8): ^1H NMR (D_2O) δ 7.376 (d, $J_{6',7'}$ = 8.4 Hz, 1 H, C(7')-H), 7.325 (s, 1 H, C(2')-H), 7.109 (d, $J_{4',6'}$ = 2.4 Hz, 1 H, C(4')-H), 6.834 (dd, $J_{4',6'}$ = 2.4 Hz, $J_{6',7'}$ = 8.4 Hz, 1 H, C(6')-H), 5.251 (dd, J = 5.7, 9.0 Hz, 1 H, C(2)-H), 4.820 (t, J = 7.5 Hz, 1 H, C(4)-H), 4.027 (t, J = 8.1 Hz, 1 H, C(a)-H), 3.997 (s, 2 H, C(d)-H_2), 3.836-3.781 (m, 2 H, C(c)-H_2), 3.763-3.630 (m, 2 H, C(b)-H_2), 3.513, 3.497 (dd, J = 5.7, 9.9 Hz, 1 H, and dd, J = 4.2, 9.9 Hz, 1 H, C(8')-H_2), 3.380, 3.304 (dd, J = 6.0, 12.3 Hz, 1 H, and dd, J = 7.5, 12.3 Hz, 1 H, C(5)-H_2).

(2*S*,4*R*)-α-Amino-4-[[(carboxymethyl)amino]carbonyl]-2-[(5-hydroxy-1*H*-indol-3-yl)methyl]-δ-oxo-3-thiazolidinepentanoic acid (9): ^1H NMR (D_2O) δ 7.376 (d, $J_{6',7'}$ = 8.4 Hz, 1 H, C(7')-H), 7.309 (s, 1 H, C(2')-H), 7.089 (d, $J_{4',6'}$ = 2.4 Hz, 1 H, C(4')-H), 6.834 (dd, $J_{4',6'}$ = 2.4 Hz, $J_{6',7'}$ = 8.4 Hz, 1 H, C(6')-H), 5.181 (t, J = 7.2 Hz, 1 H, C(2)-H), 4.685 (t, J = 7.8 Hz, 1 H, C(4)-H), 4.027 (t, J = 8.1 Hz, 1 H, C(a)-H), 3.997 (s, 2 H, C(d)-H_2), 3.836-3.781 (m, 2 H, C(c)-H_2), 3.763-3.630 (m, 2 H, C(b)-H_2), 3.548, 3.544 (dd, J = 5.7, 18.9 Hz, 1 H and dd, J = 8.1, 18.9 Hz, 1 H, C(8')-H_2), 3.409, 3.330 (dd, J = 7.5, 12.6 Hz, 1 H, and dd, J = 5.4, 12.6 Hz, 1 H, C(5)-H_2).

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Supplementary Material Available: Delayed COSY ^1H NMR spectrum of the mixture of epimers 6 and 7 (1 page). Ordering information is given on any current masthead page.

Inhibitors of Blood Platelet cAMP Phosphodiesterase. 2. Structure-Activity Relationships Associated with 1,3-Dihydro-2*H*-imidazo[4,5-*b*]quinolin-2-ones Substituted with Functionalized Side Chains[1,2]

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A series of 1,3-dihydro-2*H*-imidazo[4,5-*b*]quinolin-2-one derivatives, substituted at the 7-position with functionalized side chains, was synthesized and evaluated as inhibitors of human blood platelet cAMP phosphodiesterase (PDE) as well as ADP- and collagen-induced platelet aggregation, in vitro. Structural modifications focused on variation of the side-chain terminus, side-chain length, and side-chain connecting atom. Functionality incorporated at the side-chain terminus included carboxylic acid, ester and amide, alcohol, acetate, nitrile, tetrazole, and phenyl sulfone moieties. cAMP PDE inhibitory potency varied and was dependent upon the side-chain terminus and its relationship with the heterocyclic nucleus. Methylation at N-1 or N-3 of the heterocycle diminished cAMP PDE inhibitory potency. Several representatives of this structural class demonstrated potent inhibition of ADP- and collagen-induced blood platelet aggregation and were half-maximally effective at low nanomolar concentrations. Amides **13d**, **13f**, **13h**, **13k**, **13m**, and **13w** are substantially more potent than relatively simply substituted compounds. However, platelet inhibitory properties did not always correlate with cAMP PDE inhibition across the series, probably due to variations in membrane permeability. Several compounds inhibited platelet aggregation measured ex vivo following oral administration to rats. Ester **11b**, acid **12b**, amide **13d**, and sulfone **29c** protected against thrombus formation in two different animal models following oral dosing and were found to be superior to anagrelide (2) and BMY 20844 (5). However, ester **11b** and acid **12b** demonstrated a unique pharmacological profile since they did not significantly affect hemodynamic parameters in dogs at doses 100-fold higher than that required for complete prevention of experimentally induced vessel occlusion in a dog model of thrombosis.

Introduction

Clinical trials with inhibitors of blood platelet aggregation have established therapeutic benefit in a number of pathological conditions that have a thrombotic or thromboembolic component.[3-13] However, these studies have also revealed deficiencies associated with currently available drugs which offer the clinician imprecise and limited control over platelet function and are characterized by a high incidence of side effects.[5,14] Inhibitors of blood platelet cAMP phosphodiesterase (PDE) have been shown to prevent platelet aggregation in response to most physiologically-relevant stimuli (i.e. they demonstrate broad-spectrum activity) and have been explored both

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(2) For part 1, see: Meanwell, N. A.; Roth, H. R.; Smith, E. C. R.; Wedding, D. L.; Wright, J. J. K.; Fleming, J. S.; Gillespie, E. 1,3-Dihydro-2*H*-imidazo[4,5-*b*]quinolin-2-ones—Inhibitors of Blood Platelet cAMP Phosphodiesterase and Induced Aggregation. *J. Med. Chem.* 1991, *34*, 2906-2916.

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