Highly Effective Mechanism-Based Inactivation of Dopamine β-Hydroxylase by a Novel Ketene Thioacetal

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Dopamine β-hydroxylase (DBH, E.C. 1.14.17.1), a copper-dependent monooxygenase, catalyzes the conversion of dopamine to the sympathetic neurotransmitter norepinephrine.[4] Potent, selective inhibitors may find uses in the treatment of diseases resulting from an imbalance in dopamine and norepinephrine levels.[5,6] Recently, attention has focused on an understanding of the mechanism of enzyme action and subsequent rational design of inhibitors.[7]

We wish to describe the design and synthesis of 2-(1,3-dithiolan-2-ylidene)ethanamine (**1a**), a highly potent representative of a new class of sulfur-containing, mechanism-based inhibitors of DBH, which differs from all previous substrates in not having an aromatic ring.

Recent studies of oxygenation catalyzed by DBH suggest that the initial event is an electron transfer to an as yet unidentified copper-bound oxygen species, and in this sense DBH functions in a manner analogous to the iron dependent cytochrome P-450 and lipoxygenase enzymes.[8] Lipoxygenase can be inhibited with a substrate analogue capable of forming a stable radical cation upon removal of one electron by enzyme bound Fe(III) species.[8] In the case of DBH, inhibition can be achieved by providing stabilized radical cations derived from 2-arylallylamines.[9] We reasoned that since 2-(2-thienyl)allylamine was so well recognized,[6] another planar, five-membered sulfur-containing ring capable of stabilizing a radical cation may lead to even better activity. Accordingly, we targeted compound **1a** (Table I) because one-electron oxidation should lead to a highly stabilized radical cation.[10] The dithiole ring system is well-known for its ability for forming donor–acceptor complexes.[11]

Preparation of compound **1a** was straightforward, and experimental details for this as well as several analogues (Table I) designed to probe structure-activity relationships are found in the Supplementary Material.[12] DBH was isolated and purified from bovine adrenal.[13] The enzyme activity was assayed, and the time-dependent inhibition experiments were performed as described previously.[6] The competitive inhibition experiments were performed under standard assay conditions[6] except that the

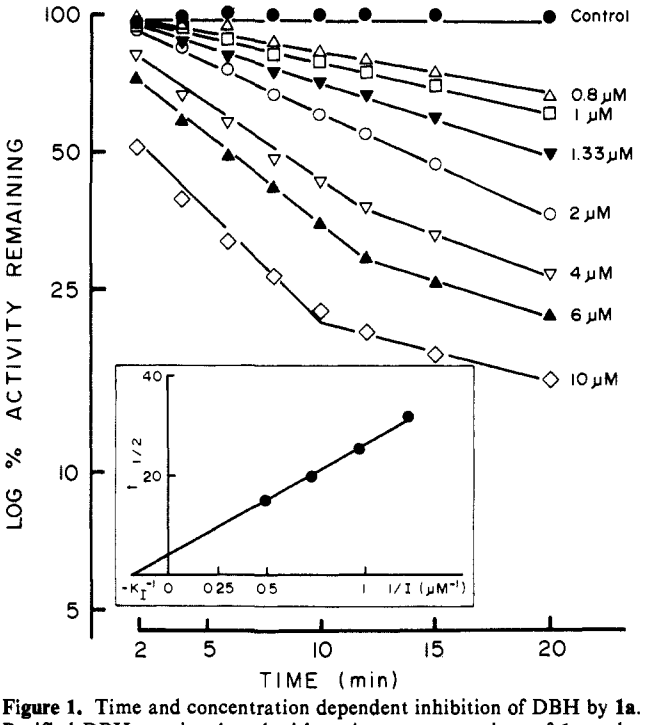
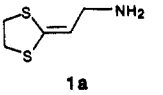
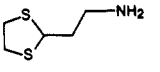
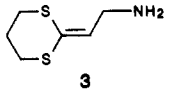
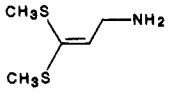
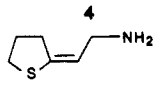
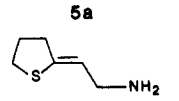


Figure 1. Time and concentration dependent inhibition of DBH by **1a**. Purified DBH was incubated with various concentrations of **1a** under conditions described in the text. At indicated times, aliquots were withdrawn and tested for remaining enzyme activity with use of tyramine as a substrate. The inset shows a Kitz and Wilson plot $t_{1/2}$ versus $[I]^{-1}$ from which the kinetic parameters K_I and k_{inact} for **1a** can be calculated.[15]

Table I. Kinetic Constants for DBH Inhibitors

compound	k_{inact} (min^{-1})	K_I (μM)	k_{inact}/K_I (M^{-1} min^{-1})
 **1a**	0.15	5	30000
 **2**		11[a]	
 **3**	0.63	570	1,105
 **4**	no time-dependent inhibition up to 5 mM		
 **5a**	0.15	280	571
 **5b**	no time-dependent inhibition up to 5 mM		

[a] Competitive inhibitor; K_I calculated versus tyramine as substrate (see text).

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(4) (a) Villafranca, J. J. *Copper Proteins*; Spiro, T. G., Ed.; Wiley: New York, 1981; pp 264–289. (b) Rosenberg, R. C.; Lovenberg, W. *Essays Neurochem. Neuropharm.* **1980**, *4*, 163.
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(10) We also prepared the ring unsaturated derivative 2-(1,3-dithio-2-ylidene)ethanamine tosylate (**1b**) (Supplementary Material); however, a control experiment revealed that **1b** was not stable under inactivation assay conditions. Compound **1a** was found to be stable.
(11) For a leading reference, see: Krief, A. *Tetrahedron* **1986**, *42*, 1209.
(12) All new compounds are fully supported by ^1H NMR, mass spectrometry, and elemental analysis, or high-resolution mass spectrometry.
(13) Aunis, D.; Miras-Portugal, M. T.; Mandel, P. J. *Neurochemistry* **1975**, *24*, 425.

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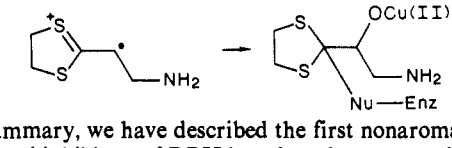
concentration of the substrate tyramine was varied in the range 0.25–5 mM at each concentration of inhibitor. The rate of turnover of saturated analogue **2** was again performed under standard assay conditions except that 640 μg of DBH and 10.5 μM $CuSO_4$ were required to obtain a measurable rate of O_2 consumption with use of a Clark oxygen electrode at 40 mM of **2**.

Figure 1 shows the psuedo-first-order decay on incubation of DBH with **1a** at concentrations in the range 0.1–10 μM. Inset is the Kitz and Wilson plot[14] from which the kinetic parameters, k_{inact} and K_I, can be estimated.[15] In addition, compounds **3** and **5a** also produced the expected pseudo-first-order loss of enzymatic activity (see Table I) and showed linear inactivation plots. In order to produce time-dependent inhibition, O_2, Cu^{2+}, and ascorbate were required and tyramine protected from inhibition by **1a**. Extensive dialysis did not result in reversal of the inhibition. These facts suggest that the inhibition is an enzyme-activated phenomenon. Fully saturated derivative **2** was not a time-dependent inhibitor but did exhibit good competitive inhibition (K_i = 11 μM versus tyramine), indicating that the ylidene moiety is essential for time-dependent inactivation but not for active site recognition. Compound **2** was also a poor substrate with a rate of turnover at 40 mM of about 6% of tyramine. Dithiane **3** inactivated DBH at a much lower rate than dithiolane **1a**. This indicates the importance of mimicking the planarity of an aromatic ring and is reinforced by the fact that the open chain analogue **4** was not an inhibitor. Of the two monosulfur analogues of **1a**, only isomer **5a**, with aminomethyl trans to sulfur, is a time-dependent inhibitor. The other isomer, **5b**, is neither time-dependent nor competitive. This result suggests that the spatial arrangement of the NH_2 relative to S is very important for recognition and indicates that the heterocyclic ring is not simply mimicking the planar aromatic ring of tyramine but implies some positive interaction between one of the S atoms and the active site.

One possible mechanism of inhibition by **1a**, analogous to those proposed for other unsaturated inhibitors,[9] proceeds by abstraction of an electron from **1a** by HOO-Cu(II) to form a resonance stabilized radical cation whose unpaired electron should combine with the unpaired electron of O=Cu(III) (eq 1). The resulting sulfur-stabilized cation can undergo attack by enzyme nucleophile. Consistent with this mechanism is the observation that prolonged dialysis of inactivated enzyme did not lead to recovery of activity.



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In summary, we have described the first nonaromatic mechanism-based inhibitors of DBH based on the concept that a planar substrate analogue capable of forming a very stable radical cation should result in enzyme inactivation. Compound **1a** appears to have the largest value of k_{inact}/K_I for any mechanism-based inhibitor of DBH yet reported. Further work is ongoing to define the active site of the enzyme and to determine the potential therapeutic utility of the described inhibitors.

Acknowledgment. We thank Dr. Michael Whalon and Robert Barbuch of the Analytical Chemistry Department for aid with spectral interpretations and Dr. Perry Gordon of the Drug Metabolism Department for aid with the stability study of **1a** and **1b**. In addition, we thank Professor Michael Johnston for helpful discussions.

Supplementary Material Available: Experimental procedures for compounds **1a**, **1b**, **2**, **3**, **4**, **5a**, and **5b**, Schemes I–IV, which outline the syntheses of the compounds, and a brief discussion of the syntheses (18 pages). Ordering information is given on any current masthead page.

(14) Kitz, R.; Wilson, T. B. *J. Biol. Chem.* **1962**, *237*, 3245.

(15) Owing to the nonlinearity of the curves at 4 μM **1a** and above and the uncertainty whether the curvature is biphasic or triphasic, these data were not included when the estimates of the kinetic parameters given in Table I were calculated from the Kitz and Wilson plot.

A Revision of the Generally Accepted Hypothesis for the Biosynthesis of the Tropane Moiety of Cocaine

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It has been established[2] that the administration of [5-^{14}C]-ornithine (**1**) to *Erythroxylum coca* plants affords radioactive cocaine which is labeled equally at C-1 and C-5 of its tropane moiety. It was also shown that [1-^{14}C]acetate preferentially labeled C-3 (48%) and C-9 (38%).[3] These results are consistent with the generally accepted biosynthesis of cocaine which is illustrated in Scheme I. Ornithine is decarboxylated to yield putrescine (**2**) which is methylated to afford *N*-methylputrescine (**3**). Oxidation of the primary amino group of **3** affords 4-(methylamino)butanal (**4**), the cyclic form of which is the 1-methyl-Δ^1-pyrrolinium salt **7**. Condensation with acetoacetate (**8**) (possibly as the thioester with coenzyme A) affords **6**. Oxidation then yields a new iminium salt **5** which then undergoes another aldol condensation to yield the bicyclic tropane ring system **9**. This compound, 2-carbomethoxy-3-tropinone, is a direct precursor of cocaine.[4] Subsequent steps are presumably reduction to methyl ecgonine (**10**) and then benzoylation to yield cocaine (**11**).[5]

This hypothesis has now been further examined by feeding [2-^{13}C,^{14}C,^{15}N]-1-methyl-Δ^1-pyrrolinium chloride (**7**, X = Cl)[7] to *Erythroxylum coca* plants, by the leaf painting method.[2] Radioactive cocaine was isolated from the plants, harvested 3 weeks after the initial feeding. However, the specific incorporation (0.1%) was not high enough for us to observe a ^{13}C–^{15}N coupling in the signals for C-1 or C-5 in the ^{13}C NMR spectrum of the labeled cocaine. A chemical degradation[2] of the labeled cocaine indicated that negligible radioactivity was present at the C-1 position (activities of the degradation products are recorded in the Supplementary Material). This surprising result led us to carry out a second feeding, this time administering the diethyl acetal of [1-^{13}C,^{14}C,^{15}N]-4-(methylamino)butanal (**12**).[7] It was considered that this acetal would undergo hydrolysis to **4** in the acidic plant tissues.[8] In order to monitor whether cocaine was being synthesized at the time of feeding this acetal, the *N*-acetylcysteamine thioester of [4-^{3}H]benzoic acid was administered to the same coca plants 2 weeks after the initial feeding of **12**. After a total feeding time of 24 days labeled cocaine was isolated with specific incorporations of ^{14}C and ^{3}H of 0.32% and 8.5%, respectively.[9] Examination of the ^{13}C NMR spectrum of this labeled cocaine and natural cocaine, using exactly the same concentrations and instrument parameters, revealed an enhancement (27%) of the signal resulting from C-5. By using a narrow spectral window, the signal at C-5 (61.58 ppm from TMS)[10] showed an upfield satellite resulting from ^{13}C adjacent

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(5) The benzoyl moiety of cocaine is derived from phenylalanine,[3] and the *N*-acetylcysteamine thioester of [*carbonyl*-^{13}C,^{14}C]benzoic acid is an excellent (10.5% specific inc.) precursor of cocaine.[6]

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(7) Leete, E.; Kim, S. H.; Rana, J. *Phytochemistry* **1988**, *27*, 401.

(8) The pH of the aqueous solution squeezed from coca leaves was 4.6–4.8.

(9) Hydrolysis of this labeled cocaine (^{14}C: 2.30 × 10^5 dpm/mmol, ^{3}H: 8.29 × 10^5 dpm/mmol) afforded ecgonine (^{14}C: 2.30 × 10^5 dpm/mmol) and benzoic acid (^{3}H: 8.51 × 10^5 dpm/mmol).

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