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UNSATURATED THIOACETIC ACIDS AS NOVEL MECHANISM - BASED INHIBITORS OF PEPTIDYLGLYCINE α-HYDROXYLATING MONOOXYGENASE

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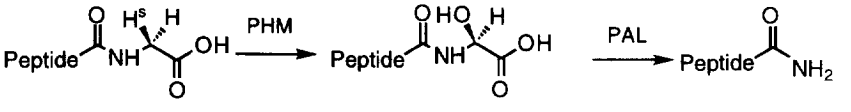
Abstract: Several unsaturated thioacetic acids were synthesized as potential mechanism-based inhibitors of peptidylglycine α-hydroxylating monooxygenase (PHM) prepared from horse serum. *Trans*-styrylthioacetic acid produced potent time-dependent inhibition of PHM. Potential mechanisms are proposed to explain PHM inactivation by unsaturated thioacetic acids.

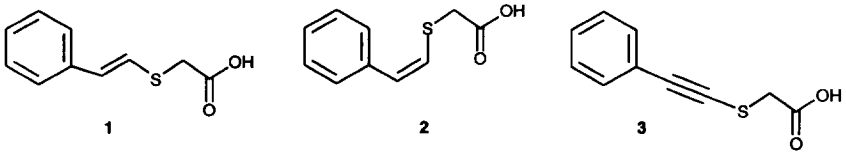
The last post-translational modification step involved in the bioactivation of many neuropeptide pro-hormones is carboxy-terminal amidation from their glycine-extended precursors[1]. The reaction is catalyzed by a bifunctional enzyme activity referred to as peptidylglycine α-amidating monooxygenase (PAM). In a first step, peptidylglycine α-hydroxylating monooxygenase (PHM, EC 1.14.17.3) catalyzes stereospecific removal of the pro-S glycine hydrogen followed by hydroxylation of the α-carbon. PHM activity is copper-dependent and requires ascorbate and molecular oxygen; in this respect, PHM is similar to dopamine β-hydroxylase (EC 1.14.17.1), another monooxygenase[1]. The resulting peptidyl-α-hydroxyglycine intermediate is stable under physiological conditions and requires cleavage by a second enzyme, peptidyl-α-hydroxyglycine α-amidating lyase (PAL, EC 4.3.2.5) to generate α-amidated product peptide and glyoxylate[1,2] (**Scheme I**). Whereas selective inhibitors of either PAM activity could be useful tools in neuroendocrinology, few examples have been described in the literature[3]. Here, we report the mechanism-based inactivation of PHM by α-unsaturated thioacetic acid derivatives.

Scheme I



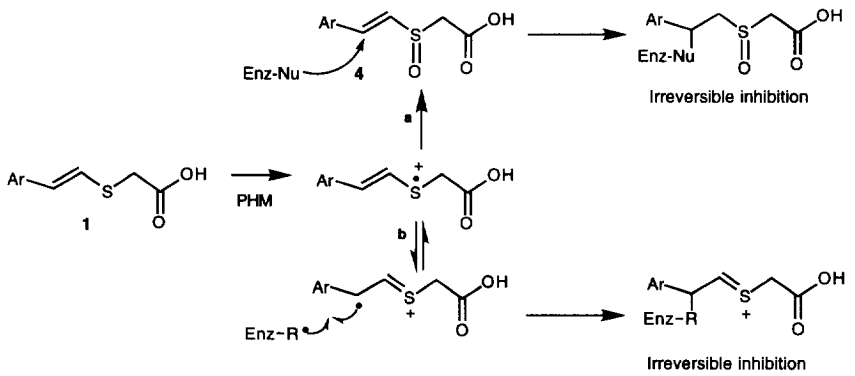
The design and synthesis[4] of the potential inhibitors **1-3** was based on the finding that sulfides are excellent substrates of PHM and are converted to sulfoxides[5]. We envisioned that conjugated unsaturated sulfoxides might be generated from compounds **1-3**. Such unsaturated sulfoxides are potential Michael-acceptors[6] and may alkylate a nucleophilic residue of PHM, resulting in enzyme inactivation (**Scheme IIa**). Furthermore, there is a strong probability that, as in the case of dopamine β-hydroxylase- and cytochrome P_{450}-catalyzed reactions, sulfoxidation proceeds through the intermediary of a sulfonium radical generated by initial abstraction of a single electron from the sulfur atom[5,7]. Considering the especially high resonance stabilization of the thioallylic-type radical[8] S=C-$\dot{C}$-Ph, an alternative mechanism can be proposed: after delocalization, a more stable benzylic

radical can be produced according to **Scheme IIb**. This resonance-stabilized radical may react with a radical residue of PHM and produce enzyme inactivation.



Incubation of PHM partially purified from horse serum[9] with *trans*-styrylthioacetic acid (**1**), in the presence of the cofactors ascorbate and Cu^{II}, resulted in a time-dependent loss of enzyme activity which followed first-order kinetics. Loss of activity was related to the concentration of inhibitor. By plotting the time of half-inactivation ($t_{1/2}$) as a function of the reciprocal of the inhibitor concentration ($1/I$) according to the method of Kitz and Wilson[10], a straight line was obtained. This line did not pass through the origin but intercepted the positive y axis, demonstrating a saturation effect which involves the enzyme active-site in the inhibitory process. Kinetic constants for the time-dependent inhibition of PHM by **1**, extrapolated from such a Kitz and Wilson replot, were found to be $K_I = 0.1$ mM and $k_{inact} = 0.2$ min^{-1}.

Scheme II



Further studies showed that incubation of the enzyme with 200 μM **1** for 30 min, followed by 20-fold dilution and addition of a saturating concentration of substrate[11] did not give any recovery of activity, suggesting a covalent linkage of the inhibitor to the enzyme active-site. Moreover, **1** did not produce PHM inactivation if incubated with the enzyme in the absence of either Cu^{II} or ascorbate, in agreement with a mechanism-based type of enzyme inhibition. In order to discriminate between pathways **a** and **b** (**schemes II**), we synthesized[4] **4**, the potential sulfoxidation product of **1**. Compound **4** did not produce any reversible or time-dependent inhibition of PHM, even when tested at a concentration of 3 mM. This result would favour pathway **b** as responsible for the inactivation. Stereochemistry seems to be an important feature in the inactivation of PHM by **1** since the *cis*-isomer **2** did not produce significant time-dependent inhibition. The fact that **3**, which also produced PHM inactivation ($K_I = 0.19$ mM; $k_{inact} = 0.06$ min^{-1}), was less potent than **2**, in spite of a higher reactivity of the expected intermediate[12], may further confirm that geometry is an important factor[3c, 13].

In conclusion, we have shown for the first time that unsaturated thioacetic acids can behave as mechanism-based inhibitors of PHM. All biological data reported in this paper are in agreement with an intermediate along the sulfoxidation pathway (**scheme IIb**) which is responsible for enzyme inactivation, similarly to what was concluded for PAM inactivation by olefins[5].
More detailed studies are in progress to elucidate the precise mechanisms of PHM inactivation by **1-3** and other related compounds.

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(4) a) *trans*-styrylthioacetic acid **1** was synthesized as following: a mixture of mercaptoacetic methyl ester (13 mmoles), styrene oxide (10 mmoles) and triethylamine (14 mmoles) in methanol (20 mL) was heated under reflux for 3 hours. Usual workup and chromatography on silica gel afforded the two alcohols 1-phenyl- and 2-phenyl-2-hydroxyethyl thioacetic acid methyl ester in 23% and 57% yields, respectively. After addition of mesylchloride (5.5 mmoles) to a mixture of the secondary alcohol (5 mmoles) and DBU (15 mmoles) in dichloromethane (25 mL) at O°C, the resulting mixture was stirred at RT for 24 hours. Usual workup and chromatography on silica gel afforded *trans*-styrylthioacetic acid methyl ester in 12% yield. Subsequent saponification by LiOH (1 eq.) in a mixture of dioxane: water; 1:1 at RT for 2 hours, acidification and extraction give **1** in 62% yield (mp: 68-70°C).
b) Synthesis of *cis*-styrylthioacetic acid **2** was adapted from Reddy, M.V.R.; Reddy, S. *Synthesis* **1984**, 322-323: a mixture of mercaptoacetic acid (10 mmoles), phenylacetylene (10 mmoles) and sodium hydroxyde (20 mmoles) in methanol (10 mL) was heated under reflux for 24 hours. Then, the mixture was acidified and extracted with dichloromethane, and recrystallized in diethylether: pentane to afford **2** in 84% yield (mp: 84-85°C).
c) Synthesis of the acetylenic sulfide **3** was adapted from Raap, R.; Micetich, R.G. *Can J. Chem.* **1968**, *46*, 1057-1063 as modified by Lalezari, I. *Synthesis* **1984**, 660-661: to a cooled (-78°C) solution of 4-phenyl-1,2,3-thiodiazole (10 mmoles) in tetrahydrofuran (50 mL), n-butyllithium (11 mmoles) was

added. The mixture was stirred at -60°C during 20 min and warmed to 0°C. Then, a solution of bromoacetic acid methyl ester (11 mmoles) in tetrahydrofuran (10 mL) was added and the resulting mixture was stirred during 4 hours. After usual workup and flash chromatography on silica gel, phenylethynylthioacetic acid methyl ester was obtained in 82% yield. Subsequent saponification by LiOH (1 eq.) in a mixture of dioxane: water; 1:1 at RT for 12 h, acidification and extraction afforded **3** (mp: 85°C).

d) The sulfoxide **4** was obtained quantitatively by oxidation with sodium periodate (0.34 mmoles) of the sulfide **1** (0.32 mmoles) in a water: methanol: DME; 1:1:1 mixture at O°C overnight. The water phase was acidified with HCl and **4** was extracted with dichloromethane.

All these compounds gave spectroscopic data (FT-IR, ^{1}H NMR, MS) and elemental analysis (C,H,N) in agreement with the assigned structure. ^{1}H NMR ($CDCl_3$), 360 MHz, δ (ppm), TSP as reference, J (Hz). **1**: 7.3 (m, 5H), 6.74 (d, 1H, J=15), 6.63 (d, 1H, J=15), 3.55 (s, 2H); **2**: 7.4 (m, 4H), 7.35 (d, 1H), 6.55 (d, 1H, J=10), 6.35 (d, 1H, J=10), 3.55 (s, 2H); **3**: 7.72 (m, 2H), 7.60 (m, 3H), 3.95 (s, 2H); **4**: 7.75 (m, 2H), 7.55 (m, 3H), 7.45 (d, 1H, J=14), 7.25 (d, 1H, J=14), 4.10 (d, 1H, JAB=10), 4.02 (d, 1H, JAB=10).

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(9) The enzyme was purified essentially as described by Tajima, M.; Iida, T.; Yoshida, S.; Komatsu, K.; Namba, R.; Yanagi, M.; Noguchi, M.; Okamoto, H. *J. Biol. Chem.* **1990**, *265*, 9602-9605, but using heparin Sepharose and Affigel 10-Tyr-Trp-Gly affinity chromatography. Imidazole (1 M, pH 8.5) was used in the final elution step[5]. Activity was assayed by a modification of the method of Katopodis and May[5], with Tyr-Val-Gly as the substrate. The formation of Tyr-Val-Gly-OH was followed by HPLC and spectrophotometric detection at 280 nm. No lyase was present in our purified preparation. The specific activity of this purified preparation was 250 nmol x h^{-1} x mg^{-1}. Time-dependent inactivation of PHM was essentially assayed according to ref. 5.

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(11) The concentration of substrate (Tyr-Val-Gly) used in this experiment was 1mM, i.e. approximately 10 times the K_M value.

(12) Similarly to pathway b (**scheme II**), the intermediate which may produce enzyme inactivation could be Ph-$\overset{\bullet}{C}$=C=$\overset{+}{S}$- but such a radical would be much less stable than in the case of Ph-$\overset{\bullet}{C}$H-CH=$\overset{+}{S}$-; therefore synthesis of the sulfoxidation product of **3** could be favoured (in analogy to pathway **a**).

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