# Irreversible Inhibition of Mammalian and Insect Peptidylglycine α-Hydroxylating Monooxygenases (PHMs), Peptide Amidating Enzymes, by *N*-Formyl Amides

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A series of *N*-formyl amides were synthesized by condensation of *N,N*-bis(trimethylsilyl)formamide with acid chlorides (59–90% yields), or by reaction of hydroxyglycyl peptides with 90% hydrogen peroxide (45% yield); a number of *N*-formyl amides which bear a phenyl substituent are mechanism-based irreversible inactivators of peptidylglycine α-hydroxylating monooxygenases purified from pig pituitary and from honeybee heads.

Neuropeptides bearing a *C*-terminal amide functionality control a large variety of biological processes in animals[1,2] and may have important functions in plants.[3] Their formation occurs by two stage enzymatic processing of glycine-extended precursors **1**; the first step is hydroxylation of the glycine α-carbon with retention of configuration in a process dependent on oxygen, copper, and ascorbate, whereas the second step is a stereospecific lysis to glyoxylate (**3**) and the amide product **2** (Scheme 1).[2,4,5] The latter reaction also proceeds spontaneously without enzyme catalysis under basic conditions. The overall transformation is accomplished by either a single large bifunctional protein, peptidylglycine α-amidating monooxygenase (PAM), or by two separate enzymes derived from the same structural gene, peptidylglycine α-hydroxylating monooxygenase (PHM) (EC 1.14.17.3) and peptidyl-α-hydroxyglycine α-amidating lyase (PAL) (EC 4.3.2).[2] Mechanism-based irreversible inactivators of PHM which would interfere with production of peptide hormones possess potential as biological tools, as therapeutic drugs, or as toxins for invertebrate pests. For example, inhibitors of insect PHM could prevent formation of insect neuropeptides, such as pheromone biosynthesis activating neuropeptide (PBAN), and could act as insect control agents.[6] Previous investigations have shown that phenylbutenoic acids[7] and peptides terminating in D-vinylglycine[8,9] are suicide inhibitors of PHM. The present study reports the synthesis of several *N*-formyl amides and demonstrates that they are mechanism-based irreversible inactivators of PHM from pig pituitary and from honeybee (*Apis mellifera*) heads.

Since PHM has broad substrate specificity[1,2] and will accept substrate analogues wherein the *pro R* hydrogen of the *C*-terminal glycine residue is replaced by a non-bulky group (*e.g.* methyl, vinyl),[8–10] introduction of a hydroperoxy group at that site could potentially afford useful mechanistic probes. Condensation of *N*-acetyl-D-phenylalanyl-L-phenylalanine-amide (Ac-D-Phe-L-Phe–$NH_2$) (**2a**) with glyoxylic acid (**3**) in refluxing acetone gives the corresponding hydroxyglycine derivative **4a** (1:1 mixture of stereoisomers),[11] whose hydroxy group can be exchanged for other heteroatom nucleophiles (*e.g.* thiols) under acidic conditions.[12] However, reaction of **4a** with 90% hydrogen peroxide in glacial acetic acid containing sulfuric acid generates the peptidic *N*-formyl amide **5a** in 45% yield after HPLC purification.† Presumably **5a** forms by decarboxylation of the intermediate with concomitant cleavage of the peroxy bond. Incubation of this compound with PHM isolated from pig pituitary[8] gives time dependent irreversible loss of activity (apparent $K_i$ = 400 μmol $dm^{-3}$), using transformation of D-Phe-L-Phe-[1,2-$^{14}$C]Gly (**1b**) to amide **2b** and **3** as an assay procedure.[5] No inhibition could be observed without the required cofactors, copper(II) ion and ascorbic acid, thereby indicating that the inactivation is mechanism-based and requires transformation of **5a** by PHM.‡ Incubation of PHM with unlabelled **1b** and **5a** protects the enzyme in proportion to the amount of added substrate, which demonstrates that both substrate and inhibitor **5a** are competing for the same active site of PHM. Finally, inactivation of PHM with **5a** followed by repetitive concentration/dilution steps on a membrane ultrafiltration device gives no recovery of activity and shows that the inhibition is irreversible.

Several non-peptidic *N*-formyl amides were synthesized to probe structural requirements for recognition by PHM and to minimize potential *in vivo* degradation which commonly occurs with peptides. Reaction of *N,N*-bis(trimethylsilyl)-formamide (**7**) with various acid chlorides **6** generates *N*-formyl amides **8** (Scheme 2, Table 1).[13] Results of tests with PHM purified from honeybee heads[9] show that *aliphatic N*-formyl amides do not significantly inhibit *C*-terminal amide

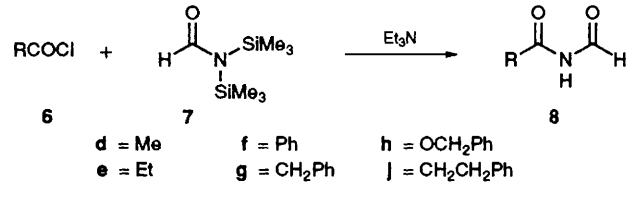


**Table 1** Synthetic yields of *N*-formyl amides **8** and inhibition of PHM from honeybees

| Compound | Yield (%)[a] | $IC_{50}$ (μmol $dm^{-3}$)[b] |
|---|---|---|
| **8d** | 78 | >2500 |
| **8e** | 84 | >2500 |
| **8f** | 77 | 890 |
| **8g** | 90 | 820 |
| **8h** | 59 | 800 |
| **8j** | 84 | 570 |

[a] Isolated yields of pure material characterized by satisfactory IR, $^1$H NMR, MS, and elemental analysis. [b] Concentration required for 50% inhibition in initial rate studies; see ref. 9 for assay procedure.

formation [*i.e.* conversion of *N*-dansyl-L-Phe-L-Phe-Gly (**1c**) to *N*-dansyl-L-Phe-L-Phe-$NH_2$ (**2c**) and glyoxylate] in submillimolar concentrations. However, the presence of a phenyl group in **8** enhances binding, and compound **8j**, which may mimic phenylalanine, is the most active inhibitor among the non-peptidic analogues tested. As with **5a**, *N*-formyl amide **8j** inactivates insect PHM in a time dependent process, which requires copper ion and ascorbate and shows protection by substrate **1c**. Repeated washing using ultrafiltration to remove **8j** does not restore activity. Hence it is a mechanism-based irreversible inactivator of PHM.

The mechanism of PHM inactivation by *N*-formyl amides is presently unknown and could involve a cofactor-dependent direct formylation of an active site nucleophile. Alternatively, it may proceed by hydrogen abstraction from the formyl group to generate an acyl radical which transfers an electron to a second active site copper[14] to produce a very reactive isocyanate. This may be the electrophile for active site acylation. Although presently there is no direct evidence to distinguish between these two pathways, the moderate reactivity of the *N*-formyl amides and the absolute requirement for copper and ascorbate for inactivation suggest that a process involving enzymatic oxidation of these inhibitors occurs. Studies on the mechanism of inactivation and on design of irreversible inactivators which are selective for insect PHM rather than the mammalian enzyme are in progress.

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## Footnotes

† Compound **4a** (40.0 mg, 0.094 mmol) was treated at 0 °C with 90% $H_2O_2$ (1.0 ml) followed by concentrated $H_2SO_4$ (0.2 ml) and glacial acetic acid (2.0 ml). This was stirred for 2 d at 20 °C, ice (4 g) was added, and the mixture was extracted with EtOAc (2 × 5 ml). The extracts were dried ($MgSO_4$), solvent was evaporated, and the resulting product was purified by reverse phase HPLC (Water $C_{18}$ μ-Bondapak Radial Pak, 25 mm × 10 cm 10 μm cartridge, solvent A; 0.1% TFA, solvent B; MeCN–$H_2O$–TFA, 80:20:0, $t_r$ = 32.0 min at 2.0 ml $min^{-1}$ flow rate, 50% B isocratic elution) to give **5a** (16.0 mg, 45%). IR (KBr) 3420, 1748, 1684, 1546 $cm^{-1}$; $^1H$ NMR (400 MHz, [$^2H_6$]DMSO), δ 1.70 (s, 3 H, $CH_3CO$), 2.40 (m, 1 H, aryl-CH), 2.56 (m, 1H, aryl-CH), 2.74 (m, 1 H, aryl-CH), 3.05 (m, 1 H, aryl-CH), 4.53 (m, 2 H, 2 × NCHCO), 6.95–7.40 (m, 10 H, aromatic), 7.96 (d, *J* = 7.3 Hz, 1 H, NH), 8.58 (d, *J* = 7.2 Hz, 1 H, NH), 9.04 (d, *J* = 8.6 Hz, 1 H, NH), 11.42 (d, *J* = 7.3 Hz, 1 H, CHO); $^{13}C$ NMR (100 MHz, [$^2H_6$]DMSO), δ 22.30, 36.36, 37.68, 53.58, 54.33, 126.09, 126.54, 127.86, 128.07, 128.99, 129.26, 136.97, 163.15, 168.95, 171.48, 173.31; FAB-MS 382.17 ($MH^+$).

‡ Kinetic analysis of the extent of time dependent inhibition at varying concentrations of **5a** (*e.g.* 0.125–0.75 mmol $dm^{-3}$) according to literature procedures (see refs. 15 and 16) gives the inactivation rate constant, $k_{inact}$ = 0.030 $min^{-1}$;

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