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Substituted Hippurates and Hippurate Analogs as Substrates and Inhibitors of Peptidylglycine α -Hydroxylating Monooxygenase (PHM)

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Abstract

Peptidyl α -hydroxylating monooxygenase (PHM) functions *in vivo* towards the biosynthesis of α -amidated peptide hormones in mammals and insects. PHM is a potential target for the development of inhibitors as drugs for the treatment of human disease and as insecticides for the management of insect pests. We show here that relatively simple ground state analogs of the PHM substrate hippuric acid ($\text{C}_6\text{H}_5\text{-CO-NH-CH}_2\text{-COOH}$) inhibit the enzyme with K_i values as low as 0.5 μM . Substitution of sulfur atom(s) into the hippuric acid analog increases the affinity of PHM for the inhibitor. Replacement of the acetylglycine moiety, $\text{-CO-NH-CH}_2\text{-COOH}$ with an S-(thioacetyl)thioglycolic acid moiety, $\text{-CS-S-CH}_2\text{-COOH}$, yields compounds with the highest PHM affinity. Both S-(2-phenylthioacetyl)thioglycolate and S-(4-ethylthiobenzoyl)thioglycolic acid inhibit the proliferation of cultured human prostate cancer cells at concentrations >100-fold excess of their respective K_i values. Comparison of K_i values between mammalian PHM and insect PHM shows differences in potency suggesting that a PHM-based insecticide with limited human toxicity can be developed.

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1. Introduction

Dozens of α -amidated peptides are known in mammals,¹ insects,² and cnidarians.³ The C-terminal α -amide moiety, required for the activity of most α -amidated peptide hormones,⁴ results from the oxidative cleavage of C-terminal glycine-extended precursor by the PAM/PHM/PAL system.⁵ The first step in the amidation reaction is the ascorbate-, copper-, and O₂-dependent hydroxylation of the glycyl α -carbon⁶ followed by the zinc-, calcium-, and iron-dependent dealkylation of the carbinolamide intermediate to the α -amidated peptide and glyoxylate (Fig. 1).⁷ The first step is catalyzed by peptidyl- α -hydroxylating monooxygenase (PHM) and the second step is catalyzed by peptidylamidoglycolate lyase (PAL).⁸ In mammals, PHM and PAL exist both as individual catalytic units and fused together as a bifunctional protein, peptidylglycine α -amidating monooxygenase (PAM). Mammalian PAM is coded for by a single gene; alternative splicing of the PAM mRNA and proteolytic processing of bifunctional PAM can produce the monofunctional enzymes, PHM and PAL.⁹ In insects and cnidarians, bifunctional PAM is not found; only monofunctional PHM and PAL are known, each coded by a distinct gene.¹⁰ Intriguingly, studies of PAM in the mollusk, *Lymnaea stagnalis*, show the expression of a complex protein with four PHM domains per PAL domain in a pentafunctional enzyme.¹¹ The functions underlying the complexities in the various forms of the PAM/PHM/PAL enzymes are not well understood.

PHM has potential as a novel therapeutic target because of the role played by specific α -amidated peptides in human disease, examples being luteinizing hormone-releasing hormone (LH-RH) and vasoactive intestinal peptide (VIP) in cancer,¹² substance P in rheumatoid arthritis,¹³ and corticotropin-releasing factor (CRF) in anxiety and depression.¹⁴ Inhibition of PAM would generate the glycine-extended precursors which are generally >1000-fold less potent than the mature α -amidated peptides.⁴ However, a drug targeted against PAM/PHM could prove to be unacceptably toxic by blocking the final maturation of *all* mammalian α -amidated peptide hormones. In fact, homozygous knock-out of the PAM gene leads to embryonic lethality between e14.5 and e15.5 in mice.¹⁵ The possibility that PHM may also be involved in the biosynthesis of oleamide and other bioactive lipid amides only heightens any concern about toxicity of an anti-PHM drug.¹⁶ While toxicity is likely an issue for an anti-PAM/PHM drug, most anticancer drugs are toxic and must be used carefully to minimize damage to healthy cells. The future development of “molecular zip codes” for the targeted delivery of toxic compounds to the site of disease¹⁷ should ameliorate such concerns and could render an anti-PHM compound a valuable weapon for the treatment of human diseases.

Of course, toxicity of an anti-PHM compound could be an asset in the development of a new class of insecticides. In fact, PHM has been suggested as an attractive target for the development of a novel insecticide,¹⁸ an idea supported by work demonstrating that the elimination of PHM expression is lethal in *Drosophila*.^{10b} Alignment of PHM sequences from *Drosophila* and mammalian PHM sequences shows 41% amino acid identity with 52% overall similarity^{10b} suggesting that there may be sufficient differences between the insect and mammalian enzymes to develop a PHM-directed insecticide with limited human toxicity.

Structure activity studies of PAM/PHM have been devoted to defining the requirement of a C-terminal glycine or D-alanine for catalysis,^{19,20} establishing the amino acid preferences at the penultimate and antepenultimate positions for the peptide substrates,²⁰ defining structural parameters for turnover-dependent inactivation by olefinic compounds,²¹ and developing N-substituted dipeptides with a C-terminal homocysteine as low nanomolar inhibitors.²² Since these earlier studies, we²³ and others²⁴ have shown that relatively simple *N*-acyl- and *N*-arylglycines are PHM substrates with (V/K)_{app} values comparable to the peptide substrates and three dimensional structures of oxidized and reduced PHM have been elucidated to provide a framework for structure-activity data.²⁵ In this report, we further define how simple,

substrate-like compounds bind to rat PAM with surprisingly strong affinity. The compound with the highest affinity reported here, *S*-(thiolauroyl)thioglycolate **55**, exhibits an $K_{i,s}$ of 540 ± 50 nM. We further show differences in $K_{i,s}$ values between rat PAM and insect PAM and that *S*-(2-phenylthioacetyl)thioglycolate **60** ($K_{i,s} = 7.9$ μ M to rat PAM) halts the proliferation of cultured human prostate DU 145 cells in a dose dependent manner. These new data, combined with ongoing work to define the transition-state for glycine hydroxylation²⁶ and to develop PAL-specific inhibitors,²⁷ provide a strong basis for the rational design of anti-PAM/PHM/PAL compounds to treat human disease or to control insect pests.

2. Results

2.1. Substituted hippurates and hippurate analogs as substrates

Katopodis and May²⁴ first demonstrated that hippurate **1**, 4-methoxyhippurate **20**, and 4-nitrohippurate **23** were oxidized by bifunctional PAM to produce the corresponding amide and glyoxylate in a 1:1 stoichiometry. Subsequent work in our laboratory has verified these results^{23a,23b} and we have further employed hippurate in mechanistic studies of PHM/PAM catalysis because significant kinetic isotope effects are found when using α -dideuterohippurate as a substrate.²⁸ Further investigation into the structure-activity relationships for hippurate oxidation have not been performed to date, despite the ease of synthesis or the commercial availability of many substituted hippurates and hippurate analogs. The information potential of a hippurate SAR study has likely been overlooked because it was thought that relatively small hippurate-based substrates would have limited contacts within the PHM active site, which lies between two $\sim$ 150-amino acid domains in an open, solvent accessible cleft.^{25a,25b} The peptide substrates for PHM *in vivo* can be large, the largest known being C-terminal glycine-extended sorbin (154 amino acids).²⁹

The data presented in Table 1 indicates that hippurate and a number of hippurate analogs stimulate the PAM-dependent consumption of O_2 in a dose-dependent manner. The dependence of the initial rate of O_2 consumption on the initial concentration of substrate, at one fixed concentration of ascorbate and O_2 , is well-described by the standard Michealis-Menten equation yielding the steady-state kinetic values for $K_{M,app}$, $V_{M,app}$, and $(V/K)_{app}$ included in Table 1. While we have not assayed for glyoxylate or amide formation in each case, previous work on PHM strongly suggests that the binding of the compounds in Table 1 do not simply stimulate the ascorbate-dependent consumption of O_2 , but instead the compounds are oxidized to the amide and glyoxylate.^{23,24}

We found a $\sim$ 100-fold variation in $(V/K)_{app}$ values for the compounds listed in Table 1 that results predominately from a $\sim$ 60-fold variation in the $K_{M,app}$ values of 18 mM for 2,6-difluorohippurate **3** to 0.3 mM for 3-indolylacetyl glycine **24** and L-pyroglutamyl-Gly **25**. The variation in the $V_{M,app}$ values is smaller for the compounds shown in Table 1, only being 2–3-fold. This pattern of results, the variation of $(V/K)_{app}$ resulting predominantly from the variation in the $K_{M,app}$, was also observed for a set of *N*-acylglycine PAM substrates.^{23a} Mechanistically, these results suggest there is little variation in the rate of product release for hippurate analogs and instead there is likely an effect of substrate structure on the microscopic rate constants leading to the first irreversible step in PHM catalysis.

2.2. Extended hippurate analogs as substrates

The presence of a penultimate hydrophobic amino acid is preferred by PHM. Using a set of peptides of the form *N*-dansyl-(Gly)₄-X-Gly in which the amino acid at position “X” was substituted with the 20 amino acids typically found in proteins, Tamburini *et al.* reported that X = Phe, Tyr, or Ile were ranked first, second, and fourth in terms of the their respective $(V/K)_{app}$ values for amidation.^{20b} Erion *et al.* further found that C-terminal homocysteine

dipeptide inhibitors, which were N-blocked with a hydrophobic group yielded the tightest binding compounds to PHM.²² The X-ray structure of PHM shows that several hydrophobic amino acids in the active site have extensive contact with the peptide substrate.^{25b} Based on all of these results, we hypothesized that moving the benzene ring (or other hydrophobic group) away from the amidoacetate moiety would yield a substrate that bound with higher affinity to PAM.

The data presented in Table 2 validate our hypothesis; virtually all of the extended hippurate analogs exhibit a $(V/K)_{app}$ value that is higher than that for hippurate. Most informative is a set of related compounds that position the benzene ring away from the amidoacetate moiety with an increasingly longer methylene spacer, $-(CH_2)_x-$, with $X = 0-7$. The $(V/K)_{app}$ for O_2 consumption increases ~30-fold from $6.2 \pm 0.1 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ for hippurate 1 to $2.0 \pm 0.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for 6-phenylhexanoylglycine **50**. This set of compounds indicates that there is an optimum position for the benzyl ring as the $(V/K)_{app}$ for phenaceturic acid **42** is approximately equal to that for 4-phenylbutyrylglycine **43** and the $(V/K)_{app}$ for 8-phenyloctanoylglycine **46** is ~2.5-fold lower than the value we measured for 6-phenylhexanoylglycine **50**.

Not surprisingly, replacement of a simple saturated methylene linker between the phenyl group and the amidoacetate moiety has an effect on the $(V/K)_{app}$ for O_2 consumption. The presence of a more peptide-like linker increases the $(V/K)_{app}$, most apparent in comparing hippuryl-Gly **49** to 4-phenylbutyrylglycine **43**. Replacement of a $-CH_2-$ moiety with a $-NH-$, $-O-$, or $-S-$ has a mixed effect on the $(V/K)_{app}$, with both increases and decreases being observed. The $(V/K)_{app}$ values for CBZ-glycine **33**, *N*-[(benzylmercapto)carbonyl]glycine **32**, and phenylmercaptoacetylglutamate **37** are higher than that for hydrocinnamoylglycine **31**, while the value for 2-propylmercaptoacetylglutamate **29** is lower than that for isocaproylglycine **35** and the value for phenylhydantoic acid **38** is also lower than the $(V/K)_{app}$ for phenaceturic acid **42**. Previously, we have reported lower $(V/K)_{app}$ values for methoxycarbonylglycine ($CH_3-O-CO-NH_2-CH_2-COOH$) relative to propionylglycine ($CH_3-CH_2-CO-NH_2-CH_2-COOH$) and 5-butyldihydroxyacid ($CH_3-(CH_2)_3-NH-CO-NH_2-CH_2-COOH$) relative to hexanoylglycine ($CH_3-(CH_2)_5-CH_2-CO-NH_2-CH_2-COOH$).^{23a}

Finally, replacement of the phenyl group with other hydrophobic groups (a furan, an acetyl group, or a branched aliphatic group) also has a mixed effect on the $(V/K)_{app}$. The $(V/K)_{app}$ for isocaproylglycine **35** is higher than hydrocinnamoylglycine **31** while the $(V/K)_{app}$ is lower when comparing acetyl-Gly-Gly **28** and *t*BOC-Gly-Gly **36** to hippuryl-Gly **49**, 2-propylmercaptoacetylglutamate **29** to phenylthioacetylglutamate **48**, and 3-(2-furyl)acryloylglycine **26** to cinnamoylglycine **40**.

The ratio of the $(V/K)_{app}$ values for the best substrate included in Table 2, phenylhexanoylglycine **50**, to the substrate with the lowest $(V/K)_{app}$ value, 3-(2-furyl)acryloylglycine **26**, is about 70. As was observed in Table 1 and with the *N*-acylglycines, the variation in the $(V/K)_{app}$ is largely due to differences in the $K_{M,app}$ values, ranging from 24 μM for phenylthioacetylglutamate **48** to 4.1 mM for *N*-(α -methylhydrocinnamoyl)glycine **27** (a ratio of 170). The $V_{M,app}$ values only vary about ~20-fold from 0.69 s^{-1} for *N*-[(benzylmercapto)carbonyl]glycine **32** to 14 s^{-1} for *N*-(α -methylhydrocinnamoyl)glycine **27**.

2.3. N-substituted D-alanines as substrates

It has long been known that C-terminal D-alanine extended peptides were also PHM substrates,¹⁹ but this aspect of PHM chemistry has not been explored in detail. The fact that simple N-blocked glycines are substrates (Tables 1 and 2) provided us with an opportunity to examine the structure-function relationships for PHM-mediated oxidation of the N-substituted D-alanines. As shown in Table 3, the simple N-substituted D-alanines, acetyl-D-alanine **51**, benzoyl-D-alanine **52**, and CBZ-D-alanine **53** are very poor substrates with $(V/K)_{app}$ values

that are 0.05–0.3% of the corresponding N-substituted glycines. In contrast to the simple N-substituted D-alanines, the one C-terminal D-alanine extended peptide included in our study, *N*-dansyl-Tyr-Val-D-Ala, is a respectable substrate with a $(V/K)_{app}$ value that is ~6% of the value for *N*-dansyl-Tyr-Val-Gly. As we have observed before, the relatively low $(V/K)_{app}$ values for the D-alanine-extended peptides is largely due to a $K_{M,app}$ effect. The replacement of the pro-R proton of the glycine with a CH₃ group seems to greatly decrease the affinity of PHM for its substrate.

2.4. Inhibition of PHM by analogs of the hippurate-based substrates

Previous work on PHM^{22,23d,30} and another copper-dependent, mechanistically-related enzyme, dopamine β -monooxygenase (D β M),³¹ has indicated that incorporation of a sulfur atom into a hippurate analog is likely to increase the extent of inhibition for any compound developed as a PHM inhibitor. The sulfur atom is thought to either directly interact with enzyme-bound copper leading to the formation of an E-Cu-inhibitor complex or to chelate the enzyme-bound copper to generate inactive copper-free PHM and the Cu-inhibitor complex free in solution.

The three inhibitors reported here which bind with the greatest affinity, 0.5–3.5 μ M, all share the replacement of the amidoacetate moiety, $-\text{CO}-\text{NH}-\text{CH}_2-\text{COOH}$, with the (thiocarbonyl) thioglycolate moiety, $-\text{CS}-\text{S}-\text{CH}_2-\text{COOH}$ (Table 4). In fact, in all of the inhibitors included in Table 4 with a $K_{i,s}$ of $\leq 10 \mu\text{M}$, have the carbonyl of the amidoacetate moiety replaced with a thiocarbonyl, C=S. The analog of the benzoyl-D-alanine **52** ($K_{M,app} = 84 \text{ mM}$) that incorporated the $-\text{CS}-\text{S}-$ in place of $-\text{CO}-\text{NH}-$, *S*-(thiobenzoyl)-(*R,S*)-thiolactate **66**, is a surprisingly effective inhibitor with a $K_{i,s}$ value of 58 μM (Table 4).

The relative contribution made by the different structural components of the inhibitors and substrates can be estimated from the inhibitors listed in Table 4. For example, the PHM X-ray structure shows that the glycine carboxylate of the substrate interacts with the positively charged guanidinium of Arg-240.^{25b} Previous data had shown that esterification of the substrate carboxylate decreased the affinity of PHM for the compound approximately 15-fold, as the $K_{i,s}$ for the methyl ester of *N*-dansyl-Tyr-Val-Gly was 54 μM compared to a $K_{M,app}$ of 3.5 μM for *N*-dansyl-Tyr-Val-Gly.^{20a} The data included in Table 4 indicate that the contribution to binding made by the substrate carboxylate is not straightforward. Glutathione ethyl ester **67**, $K_{i,s}$ of 66 μM , is lower than the $K_{M,app}$ for glutathione, 130 μM ,³⁰ and the $K_{i,s}$ for salicylhydroxamate **71**, 300 μM , is also lower than the $K_{M,app}$ for salicylurate of 860 μM .^{23b} Clearly, there must be interactions other than the substrate carboxylate and Arg-240 that are important for binding in the PHM active site. Contributions made by other structural components of the substrates and inhibitors are included in the discussion.

2.5. Inhibition of PAM-catalyzed carbinolamide dealkylation

The recombinant enzyme used in this research is bifunctional rat PAM containing both the monofunctional PHM and PAL domains.^{9a} This affords us the opportunity to determine whether the inhibitors shown in Tables 1–4 were targeted specifically against PHM or PAL, or inhibited both activities. PHM-specific inhibition is determined by following the ascorbate-dependent consumption of O₂, PAL-specific inhibition is determined by following the ascorbate-independent dealkylation of α -hydroxyhippurate, while the inhibition of *N*-dansyl-Tyr-Val-Gly amidation represents the inhibition of both PHM and PAL activities (see Fig. 1).

All of the compounds included in Tables 1–4 were screened for inhibition of PAL activity by measuring the amount of glyoxylate produced in a single time interval from 2.0 mM α -hydroxyhippurate at 0 or 750 μM inhibitor. Under these conditions, <0.4 mM glyoxylate was produced in the absence of inhibitor and <5% inhibition of PAL activity was found for all the

compounds in Tables 1–4. Thus, the inhibition observed for the compounds included in this study is specific for PHM. There is one exception, *S*-(phenylthiocarbamoyl)thioglycolate **61**. This compound weakly inhibits PAL activity with an IC_{50} of 3.5 ± 0.2 mM, which is 400-fold higher than the $K_{i,app}$ for the inhibition of rat PHM. So even for **61**, there is a strong preference for PHM.

2.6. *In silico* modeling of the PHM • substrate and the PHM • inhibitor complexes

Using Glide and Q-site, we docked 79 of the 80 compounds included in Tables 1–4 into the active site of reduced rat PHM. One compound, (nicotinamidomethyl)phosphonic acid **80**, would not dock into the active site. This compound showed no inhibition of PHM at 28 mM. For the remaining 79 compounds that would dock into PHM, the Glide emodel values varied 4-fold, from –30 to –120, and the Glide gscore values varied 2.4-fold, from –3.1 to –7.4 (Table S1, supplementary material). The docking values for 72 of the 79 of the compounds were within a more narrow range of Glide emodel values between –55 to –80. Of the seven compounds with Glide emodel values not in the –55 to –80 range, 5 did not terminate in a glycine: *N*-dansyl-Tyr-Val-D-Ala **54**, *N*-(thiobenzoyl)-(D,L)-alanine **59**, glutathione ethyl ester **67**, *S*-(4-methylthiobenzoyl)thioglycolic acid ethyl ester **70**, and salicylhydroamic acid **71**.

2.7. Inhibition of insect PHM

There has always been a concern that anti-PHM inhibitors would be too toxic for clinical use because such inhibitors would block the biosynthesis of all the α -amidated peptide hormones and the primary fatty acid amides. However, the toxicity of anti-PHM inhibitors could be a tremendous benefit in another venue - the development of novel insecticides because a large majority of insect peptide hormones are α -amidated.² For this approach to work, there needs to be sufficient differences between mammalian PHM and insect PHM to enable the development of insect-specific anti-PHM inhibitors.

We determined the $K_{i,s}$ values for seven of the PHM inhibitors from Table 4 for the *B. germanica* enzyme and have compared these values to those determined for the rat enzyme (Table 5). There are differences in the $K_{i,s}$ values between *B. germanica* and rat; the largest difference being a 4–7-fold difference for *S*-(4-methylthiobenzoyl)thioglycolic acid **57** and for *S*-(3-phenylthiopropionyl)thioglycolic acid **62**. There are also differences between the $K_{i,s}$ values for the other five compounds included in Table 5, but the magnitude of the differences is smaller.

2.8. Antiproliferative effects of selected PHM inhibitors on human prostate cancer cells (DU 145)

The probability that an American male will die of prostate cancer is ~10%.³² There are two basic types of prostate cancer: androgen-dependent and androgen-independent. Androgen-dependent prostate cancer cells die when deprived of androgen and, consequently, this form of prostate cancer can be successfully treated with luteinizing hormone-releasing hormone (LH-RH) agonists that bind to the androgen receptors.³³ Unfortunately, most patients relapse into androgen-independent prostate cancer with few good options for treatment.³⁴

LH-RH is not the only α -amidated peptide that stimulates the proliferation of prostate cancer. Analogs of both gastrin-releasing peptide (GRP) and growth hormone-releasing hormone (GH-RH) also show promise for the treatment of prostate cancer.³⁵ Instead of an approach that targets individual α -amidated peptide hormones, another strategy to treat prostate cancer that would block the production of all α -amidated peptide hormones would be to inhibit PHM. To this end, we have evaluated the effectiveness of two of the PHM inhibitors of Table 4, *S*-(2-phenylthioacetyl)thioglycolate **60** ($K_{i,s} = 7.9$ μ M) and *S*-(4-methylthiobenzoyl)thioglycolic

acid **57** ($K_{i,s} = 3.5 \mu\text{M}$), as growth inhibitors for human androgen-independent prostate cancer cells (DU 145 cells).

S-(2-Phenylthioacetyl)thioglycolate **60** shows significant anti-proliferative effects on the growth of DU 145 cells at concentrations $>500 \mu\text{M}$ (Fig. 2). The dose required to inhibit the proliferation of the DU 145 cells is ~ 60 -fold higher than the $K_{i,s}$ for the inhibition of mammalian PHM. There are many reasons that **60** would be considerably less effective as an anti-proliferative than for the inhibition of PHM *in vitro*; one possibility being that the inhibitor does not easily cross the cell membrane. To address this possibility, we examined the anti-proliferative activity of a more hydrophobic inhibitor, *S*-(4-methylthiobenzoyl)thioglycolate **57** and its ethyl ester **70**. Despite exhibiting a lower $K_{i,s}$ *in vitro*, **57** is less effective than **60** in inhibiting the growth of DU 145 cells, requiring $\sim 1.0 \text{ mM}$ to reduce the number of viable cells to 50% (Fig. 3). The ethyl ester **70** shows only $\leq 10\%$ growth inhibition at $100 \mu\text{M}$ (Fig. 3).

3. Discussion

3.1. Structure-function relationships towards the design of PHM inhibitors

The *in silico* docking studies show that 72 of the 79 compounds (91%) dock in the PHM active site and exhibit a relatively narrow range of Glide emodel and gscore values (Table S1, supplementary material). For the remaining 7 compounds, the Glide emodel and gscore values are still within a factor of 2 of the range for other compounds. The docking results indicate that the orientation of the substrates and inhibitors within the active site of reduced PHM is similar for 79 docked compounds included in this study. This means that a comparison of their respective binding affinities will provide valuable insights about structural determinants important for the design of tight-binding PHM inhibitors.

Note that 5 of the 7 “out of range” compounds do not terminate in a glycine moiety suggesting that there are likely (perhaps subtle) differences in active site positioning for the compounds that do not terminate in a glycine.

Previous work had shown that peptide substrates with a penultimate hydrophobic amino acid exhibited the highest V/K for oxidation.²⁰ The PHM X-ray structure containing bound *N*-acetyl-3,5-diiodotyrosylglycine showed that the diiodotyrosyl moiety was located in a hydrophobic pocket consisting of Asn-316 and Tyr-318.^{25b} Modeling studies carried out in our laboratory using Glide point toward a more extensive hydrophobic pocket within the PHM active site consisting also of Met-208, Leu-206, Ile-306, and Met-314.[¶] We purposely included a hydrophobic moiety in virtually all of the substrates and inhibitors included in this study to take advantage of the known preference exhibited by PHM for hydrophobic substrates and to take advantage of the hydrophobic pocket within the PHM active site.

Incorporation of a sulfur atom at any position in the compound increases binding affinity. The magnitude of the increase varies considerably, from a $\Delta(\Delta G)$ of -0.15 kcal/mol for the **35/29** pair to $\sim -6 \text{ kcal/mol}$ for the **52/59** pair (Table 6). Thiols and the -CS-S- functionality are best for increasing affinity to PHM, with $\Delta(\Delta G)$ of $\sim -2.5 \text{ kcal/mol}$ for thiols and -2 to -5 kcal/mol for -CS-S- (Table 7). The increase in potency due to the incorporation of sulfur atom(s) in the inhibitor is likely due to: (a) abstraction of the copper from PHM yielding inactive copper-free enzyme, (b) coordination of PHM-bound copper to form the PHM·Cu-inhibitor complex, or (c) a combination of the chelation and coordination effects. Research to develop inhibitors for the related enzyme, D β M, reached similar conclusions. Sulfur-containing compounds lead to relatively tight-binding D β M inhibitors, those exhibiting the lowest K_i values generally possessed a thiol moiety.^{31c,31d} Furthermore, inhibition of D β M by the sulfur-containing

[¶]McIntyre, N. R., Lowe, E. W., Jr., and Merkler, D. J., manuscript in preparation

inhibitors could not be attributed only to chelation of enzyme-bound copper to yield inactive apo-D β M.^{31b,36}

Replacement of α -methylene with a -NH- spacer, R-CH₂-CO- vs. R-NH-CO-, decreases binding affinity. The magnitude of this effect is relatively small, with an average ratio of K_{CH₂}/K_{NH} of ~0.5 yielding an average $\Delta(\Delta G)$ of approximately -0.6 kcal/mol (Table 8). The decrease in affinity most likely results from an unfavorable interaction between the unpaired electrons of the nitrogen and the hydrophobic amino acids of the PHM active site.^{25b}

3.2. Effect of the PHM inhibitors on cultured prostate cancer cells

Two of our inhibitors, *S*-(2-phenylthioacetyl)thioglycolate **60** and *S*-(4-methylthiobenzoyl)thioglycolate **57**, were antiproliferative against cultured human prostate carcinoma cells; however, these compounds were 60–300-fold less potent than their respective K_{i,s} values in decreasing cell viability by 50%. Similarly, Jeng *et al.*³⁷ reported that their PHM inhibitors were ~200-fold less potent relative to their *in vitro* IC₅₀ values in inhibiting the PHM-dependent formation of an α -amidated peptide, substance P, in cultured rat dorsal root ganglion cells. While some of the discrepancy between the *in vitro* K_{i,s} (or IC₅₀) values and the potency in cellular assays is likely a result of limited cellular availability, other factors such as the potential partial activity of the amidated peptide precursors,⁴ increased expression of PHM, or other non-PHM targets for these molecules could decrease *in vivo* potency. These data combined with similar results from Jeng *et al.*³⁷ and concerns about toxicity have led to questions about PHM being a viable anticancer target. However, the development of molecular “zip-codes” to specifically target a drug to its site of action could render an anti-PHM drug useful against such difficult-to-treat cancers as small-cell lung cancer,³⁸ prostate cancer,³⁹ or pancreatic cancer.⁴⁰ The structure-activity data reported here will prove useful towards the design of a tight-binding anti-PHM drug.

3.3. PHM inhibitors as novel insecticides

Most bioactive insect peptides possess a C-terminal amide² and are dependent on PHM for their biosynthesis. While there are reports of α -amidated peptides from ginseng root⁴¹ and alfalfa,⁴² we could not detect PHM activity in cell homogenates from freshly harvested alfalfa, identify a protein by Western analysis from alfalfa extracts using mammalian anti-PHM antibodies, nor identify a gene in the *Arabidopsis* genome that showed significant homology to human or insect PAM (Carpenter & Merkler, unpublished). These data strongly suggest that PHM does not exist in plants and that plant α -amidated peptides, if such molecules do exist, are likely produced via a non-PHM-dependent pathway. Thus, a PHM-targeted insecticide could prove relatively non-toxic to plants.

The catalytic cores of rat and *Drosophila* PHM show 41% sequence identity and 52% sequence similarity.^{10b} The sequence differences between mammalian and insect PHM coupled with the differences in K_{i,s} values between rat and *B. germanica* PHM (Table 5) strongly suggest insect specific-PHM inhibitors can be developed and could prove useful as alternative insecticides. While toxicity of an anti-PHM compound is a concern in the design of human drug, insect toxicity *would be the goal* of a PHM-directed insecticide.

4. Conclusion

We report here that relatively simple, substrate-like compounds can bind to mammalian PHM with K_{i,s} values <1 μ M. A more complete understanding of the PHM mechanism should yield transition-state information leading to transition-state mimics that would exhibit better affinity for PHM. The structure-activity data reported here and elsewhere^{20–24} will prove invaluable to the design of the tight-binding transition state analogs. Specific PHM-directed compounds

could prove useful for the treatment of human disease, particularly certain forms of difficult-to-treat cancers, and for the development of novel insecticides.

5. Experimental Section

5.1. Materials

Bovine catalase, sodium ascorbate, MES, hippuric acid **1**, aceturic acid **2**, *t*BOC-Gly **5**, 2-methylhippuric acid **6**, 4-aminohippuric acid **7**, *N*-(2-furoyl)glycine **8**, 4-methylhippuric acid **11**, 3-chlorohippuric acid **14**, 3-methylhippuric acid **17**, 4-bromohippuric acid **18**, *N*-(2-thienylcarbonyl)glycine **19**, 4-methoxyhippuric acid **20**, 2-hydroxyhippuric acid **22**, 4-nitrohippuric acid **23**, 3-(2-furyl)acryloylglycine **26**, 2-propylmercaptoacetyl glycine **29**, *N*-[(2-phenylcyclopropyl)carbonyl]glycine **30**, cinnamoylglycine **40**, acetyl-D-alanine **51**, 6-*O*-palmitoyl-L-ascorbic acid **63**, *S*-(thiobenzoyl)thioglycolic acid **64**, glutathione ethyl ester **67**, salicylhydroxamic acid **71**, *O*-benzamidoglycolic acid **73**, Pro-Leu-Gly hydroxamic acid **74**, (*S*)-*N*-CBZ-4-amino-2-hydroxybutyric acid **75**, 3-benzoylpropionic acid **77**, (CBZ-hydrazido) glycine **78**, (nicotinamidomethyl)phosphonic acid **80**, and (*R,S*)- α -hydroxyhippuric acid were from Sigma-Aldrich Co.; 2-aminohippuric acid **9**, 4-hydroxyhippuric acid **12**, L-pyroglutamyl-Gly **25**, acetyl-Gly-Gly **28**, *t*BOC-Gly-Gly **36**, hippuryl-Gly-Gly **39**, hippuryl-Gly **49**, and benzoyl-D-alanine **51**, were from Bachem Bioscience Inc.; 2,6-difluorohippuric acid **3**, 4-trifluoromethylhippuric acid **13**, 4-chlorohippuric acid **21**, phenylhydantoic acid **38**, 2-pyridylmercaptoacetyl glycine **47**, and phenylthioacetyl glycine **48** were from Maybridge Chemicals; *N*-dansyl-Tyr-Gly and 2-iodohippuric acid **4** were from Fluka Laboratory Chemicals; 3-indolylacetyl glycine **24**, CBZ-glycine **33**, chloroacetyl-Gly-Gly **34**, and CBZ-D-alanine **53** were from Research Organics Inc.; nicotinuric acid **16**, phenaceturic acid **42**, and 4-nitrobenzoyl-Gly-Gly **44** were from TCI America; *S*-phenylmercaptoacetic acid **72** was from Pfaltz and Bauer Rare and Fine Chemicals; *N*-phenylthiohydantoic acid **69** was from Eastern Chemical Corp.; *N*-benzylglycine **79** was from Indofine Chemical Co.; and *N*-[(benzylmercapto)carbonyl]glycine **32** was from SPECS. *N*-Dansyl-Tyr-Val-D-Ala^{6e} and *S*-(thiobenzoyl)-4-mercapto-4-cyanopentanoic acid **58**⁴³ were synthesized as described. Human prostate DU 145 cells were purchased from the American Type Culture Collection. Adult German cockroaches (*Blattella germanica*) were purchased in lots of 100 from Carolina Biological Supply Co.

5.2. Enzymes

Recombinant type A rat medullary thyroid carcinoma PAM was produced and purified as described⁴⁴ and was a gift from Unigene Laboratories, Inc. (Fairfield, NJ, see www.unigene.com). The rat PAM used in these studies had a specific activity ≥ 3.0 μ moles of O₂ consumed/min/mg at 37 °C under the following conditions: 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X 100, 10 μ g/mL bovine catalase, 1.0 μ M Cu(NO₃)₂, 5.0 mM sodium ascorbate, and 11.0 mM aceturic acid.

Partially purified PAM from *B. germanica* was obtained by first homogenizing one hundred cockroaches (typical combined mass of 5–7 g) at 4 °C in 150 mL of 20 mM TES/NaOH pH 7.4, 10 mM D-mannitol, 0.3 mg/mL PMSF, 2 μ g/mL leupeptin, and 16 μ g/mL benzamidine (Buffer C). The resulting homogenate was filtered through cheese cloth, frozen and thawed three times using a dry ice-methanol bath, and then centrifuged for 20 min at 4,000 $\times$ g to remove cellular debris. The supernatant was brought to 30% of saturation by the slow addition of solid ammonium sulfate, precipitated proteins collected by centrifugation, and discarded. Solid ammonium sulfate was added to the supernatant to bring the solution to 50% saturation and the precipitated proteins again collected by centrifugation. The pellet was resuspended in Buffer C and then stored frozen at –80 °C. *B. germanica* PAM used in these studies had a specific activity ≥ 10 nmoles of *N*-dansyl-Tyr-Val-NH₂ produced/min/mg at 37 °C under the

following conditions: 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X 100, 58 µg/ml bovine catalase, 1.6 µM Cu(NO₃)₂, 10 mM sodium ascorbate, and 10.0 µM *N*-dansyl-Tyr-Val-Gly. The PAL specific activity of the *B. germanica* preparation was 2–3 nmoles of glyoxylate produced/min/mg at 37 °C under the following conditions: 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, and 2.0 mM (*R,S*)- α -hydroxyhippuric acid.

5.3. Growth and treatment of the human prostate DU 145 cells

The DU 145 prostate cancer cells were grown in Eagle's minimum essential medium (EMEM) with Earle's Salts (CaCl₂, KCl, MgSO₄, and Na₂HPO₄) and 2 mM glutamine. The growth media also contained 1.0 mM sodium pyruvate, 0.1 mM amino acids (contains all 20 amino acids), 1.5 g/L sodium bicarbonate, 10% FBS, 10,000 U/mL penicillin, and 10,000 µg/mL of streptomycin.⁴⁵ For control experiments, 10 µM 4-HPR, a compound known to be antiproliferative for DU 145 cells,⁴⁶ was also added to the growth media.

DU 145 cells were grown to 80% confluency, trypsinized, counted, and diluted to give 50,000 cells per well. The cells were allowed to adhere to the bottom of the wells for 24 hours. The media was then replaced with and without the desired inhibitor added. Cells were then counted at 8, 24, 48, 72, and 96 hours. At each time point, the media was removed and the cells washed twice with PBS. Trypsin/EDTA was added to detach cells. Media (2 mL) was added to each well, mixed, and removed. A sample from each well was taken out and Trypan blue and HBS were added. The viable and non-viable cells were counted on a hemacytometer. Viable cells appeared clear and non-viable cells appeared blue.⁴⁷

5.4. Determination of $K_{M,app}$, $V_{M,app}$, and $(V/K)_{app}$ for the rat bifunctional PAM substrates

Reactions at 37.0 ± 0.1 °C were initiated by the addition of 15–50 µg of rat PAM into 2.4 mL of 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, 10 µg/mL bovine catalase, 1.0 µM Cu(NO₃)₂, 5.0 mM sodium ascorbate, and the oxidizable substrate (generally 0.3 $K_{M,app}$ to 5.0 $K_{M,app}$). Initial rates were measured by following the PAM-dependent consumption of O₂ using a Yellow Springs Instrument Model 53 oxygen monitor. $V_{M,app}$ values were normalized to controls performed at 11.0 mM aceturic acid. Ethanol was added to protect the catalase against ascorbate-mediated inactivation and Triton X-100 was included to prevent nonspecific absorption of PAM to the sides of the oxygen monitor chambers.

5.5. Bifunctional rat PAM inhibition assays

The inhibition of rat PAM was determined by either measuring the inhibition of O₂ consumption using aceturic acid as an oxidizable substrate or the inhibition of *N*-dansyl-Tyr-Val-Gly amidation. Measuring O₂ consumption is more convenient, but is less sensitive and requires relatively high amounts of enzyme and inhibitor. Measuring *N*-dansyl-Tyr-Val-Gly amidation is a more sensitive assay for PAM activity and, thus, is most useful when enzyme and/or inhibitor are limiting.

Assays to define the inhibition of O₂ consumption were initiated by the addition of 15–50 µg of rat PAM to a solution composed of 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1.0% (v/v) ethanol, 0.001 % (v/v) Triton X-100, 10 µg/mL bovine catalase, 1.0 µM Cu(NO₃)₂, 6.0 mM sodium ascorbate, 8.0 mM aceturic acid, and a range of inhibitor concentrations (generally 0.5 $K_{i,s}$ to 10 $K_{i,s}$). For compounds of lower aqueous solubility, the reaction solution contained 2.5 % (v/v) DMSO. A few experiments employed a lower substrate concentration, 5.0 mM aceturic acid, to better define the inhibition for the compounds that exhibited relatively low affinity to PAM. The final volume of the reaction solution after the addition of enzyme was 2.4 mL and the reactions were maintained at 37.0 ± 0.1 °C.

Assays for the inhibition of *N*-dansyl-Tyr-Val-Gly amidation were initiated by the addition of 50–100 ng of rat PAM to a solution composed of 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1.0 % (v/v) ethanol, 0.001 % (v/v) Triton X-100, 10 µg/mL bovine catalase, 1.0 µM Cu (NO₃)₂, 6.0 mM sodium ascorbate, 8 µM *N*-dansyl-Tyr-Val-Gly, and a range of inhibitor concentrations (generally 0.5 *K_{i,s}* to 10 *K_{i,s}*). For compounds of lower aqueous solubility, the reaction solutions contained 2.5% (v/v) DMSO. A few experiments employed a lower substrate concentration, 5.0 µM *N*-dansyl-Tyr-Val-Gly, to better define the inhibition for the compounds that exhibited relatively low affinity to PAM. The final volume of the reaction solutions after the addition of enzyme was 0.5 mL and the reactions were maintained at 37.0 ± 0.1 °C throughout the course of the experiment. At the desired time, an aliquot was removed from the reaction solution and added to a vial containing one-fifth volume of 6% (v/v) trifluoroacetic acid to terminate the reaction. The acidified aliquots were assayed for the percent conversion to *N*-dansyl-Tyr-Val-NH₂ by reverse phase HPLC as described by Jones *et al.*⁴⁸

5.6. *B. germanica* PHM Inhibition Assays

Assays to define the inhibition of *N*-dansyl-Tyr-Val-Gly amidation were initiated by the addition of 70–200 µg of partially purified *B. germanica* PAM to a solution composed of 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1.0 % (v/v) ethanol, 0.001 % (v/v) Triton X-100, 58 µg/mL bovine catalase, 1.6 µM Cu(NO₃)₂, 8.0 mM sodium ascorbate, 10 µM *N*-dansyl-Tyr-Val-Gly, and a range of inhibitor concentrations (generally 0.5 *K_{i,s}* to 10 *K_{i,s}*). The final volume of the reaction solution after the addition of enzyme was 0.5 mL and the reactions were maintained at 37.0 ± 0.1 °C throughout the course of the experiment. At the desired time, an aliquot was removed from the reaction solution and added to a vial containing one-fifth volume of 6% (v/v) TFA acid to terminate the reaction. The acidified aliquots were assayed for the percent conversion to *N*-dansyl-Tyr-Val-NH₂ by reverse phase HPLC as described by Jones *et al.*⁴⁸

5.7. Inhibition of PAL activity (carbinolamide dealkylation)

Dealkylation reactions at 37.0 ± 0.1 °C were initiated by the addition of 1.5–3.0 µg rat PAM into 0.3 mL of 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, 2.0 mM (*R,S*)- α -hydroxyhippuric acid, and 0 or 750 µM inhibitor. A control lacking enzyme was included to measure both the contaminating glyoxylate in the (*R,S*)- α -hydroxyhippuric acid and any non-enzymatic carbinolamide dealkylation. The reactions were terminated after 20 min. at 37.0 ± 0.1 °C by the addition of 60 µL of 6% (v/v) TFA acid. Dealkylation activity of the partially purified *B. germanica* preparation was measured similarly except that the assays were initiated by the addition of 54 µg protein into a 0.6 mL reaction volume and the reactions were incubated for 6 hrs at 37.0 ± 0.1 °C before an aliquot was removed for glyoxylate quantification.

The concentration of glyoxylate in the acid quenched samples was determined spectrophotometrically via the method of Christman *et al.*⁴⁹ as modified by Katopodis and May.²⁴ Standard curves of [glyoxylate] vs. A₅₂₀ were constructed in the appropriate buffers using a glyoxylate solution that had been calibrated by measuring the glyoxylate-dependent oxidation of NADH ($\Delta\epsilon_{340} = 6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) as catalyzed by lactate dehydrogenase.

The single time point assays for glyoxylate served only to screen for PAL inhibition from the set of compounds under study. Only one compound, *S*-(phenylthiocarbamoyl)thioglycolic acid **61**, showed appreciable inhibition (~40%) of the rat PAM dealkylation activity from the initial screen. To more fully characterize this inhibition, 8.3 µg of rat PAM was added to 1.0 mL of 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, 2.0 mM (*R,S*)- α -hydroxyhippuric acid, and 0–6.0 mM *S*-(phenylthiocarbamoyl)thioglycolic acid **61**. At 5 min intervals, a 0.15 mL aliquot was removed, and added to a vial containing 30 µL

of 6 % (v/v) TFA to terminate the reaction. Glyoxylate was quantified in the acidified samples as described above.

5.8. Analysis of initial rate kinetic and inhibition data

Initial velocities, v , obtained as a function of the initial substrate concentrations, $[S]$, were fit to eq. 1 using KaleidaGraph^{TM}.

$$v = (V_{M,app} [S]) / (K_{M,app} + [S]) \quad (1)$$

The $K_{M,app}$ is the apparent Michaelis constant for S at constant fixed initial concentrations of the co-substrates (5.0 mM sodium ascorbate and 200 μ M O_2 for PAM). The $V_{M,app}$ is the apparent catalytic rate at saturating $[S]$ under the conditions of the experiment.

Values for $K_{i,s}$ were obtained by a KaleidaGraph^{TM} fit of the data for the dependence of the initial velocity, v , on the concentration of inhibitor, $[I]$, at one fixed, initial substrate concentration, $[S]$ to eq. 2. We have assumed, in using eq. 2, that the inhibitors are competitive with the oxidizable substrate, aceturic acid or *N*-dansyl-Tyr-Val-Gly.

$$v = (V_{M,app} [S]) / \{K_{M,app} + K_{M,app} ([I]/K_{i,s}) + [S]\} \quad (2)$$

The IC_{50} values for the inhibition of *N*-dansyl-Tyr-Val-Gly amidation by 6-*O*-palmitoyl-L-ascorbate and the inhibition of (*R,S*)- α -hydroxyhippuric acid dealkylation by *S*-(phenylthiocarbamoyl)thioglycolic acid **61** were calculated as described by Cortés *et al.*⁵⁰

5.9. In silico docking

The crystal structure for reduced peptidylglycine α -hydroxylating monooxygenase (PHM) was obtained from the Protein Data Bank (<http://www.rcsb.org/pdb/>, 1SDW).^{25c} All co-crystallized species determined to be redundant for ligand binding were removed (nickel, water, glycerol, and substrate). Formal charges for enzyme-bound copper ions and bond orders were corrected, and hydrogens were added using *Maestro* (www.schrodinger.com). Further receptor refinements were carried out utilizing ProteinPrep from within *Maestro*. *Glide* and *Q-site* from the FirstDiscovery 3.0 suite (www.schrodinger.com) were used for quantum polarized ligand docking (QPLD) to generate highly accurate ligand binding modes.⁵¹

5.10. Synthesis of isocaproylglycine 35

To a stirred, chilled solution of isocaproyl chloride (1.35 g, 10 mmole) in 10 mL of dichloromethane was added glycine methyl ester hydrochloride (1.25 g, 10 mmole), followed by the dropwise addition of triethylamine (2.2 g, 22 mmole). The mixture was stirred at room temperature overnight and then treated with 20 mL of 1.0 M HCl. The dichloromethane layer was washed with a sodium bicarbonate solution, dried with sodium sulfate, and stripped of solvent. The yield of methyl isocaproylglycinate was 1.32 g (70%) and the structure confirmed by ¹H NMR. A portion of the crude ester (0.93 g, 5 mmole) was stirred with 1.0 M NaOH (5.5 mL) until all dissolved (25 min), chilled, and acidified with 6.0 M HCl (2 mL). The oil, which separated slowly, crystallized. Recrystallized twice from ether:pentane (2:1), isocaproylglycine (0.4 g, 50%) had mp 91–92, ¹H NMR (CDCl₃): δ 0.86–0.88 (m, 6H), 1.47–1.57 (m, 6H), 2.27 (t, $J=8.0$ Hz, 2H), 4.01–4.03 (m, 2H), 6.51–6.54 (m, 1H), and 8.03–8.24 (br. s., 1H).

5.11. General synthesis of substrates and inhibitors

Glycine conjugate **27** (*N*-(α -methylhydrocinnamoyl)glycine) was prepared as described by McIntyre *et al.*^{23d} Glycine conjugates **10**, **15**, **31**, **43**, **45**, **46**, and **50** were prepared similarly. Acylation of cysteine and serine requires careful pH control,⁵² otherwise considerable racemization will occur.⁵³ At pH 9.5, unracemized **68** and **76** were obtained satisfactorily. Thioacylglycine **48** (phenylthioacetyl)glycine) was prepared using a literature procedure,⁵⁴ and **41** and **59**⁵⁵ were prepared in a similar fashion. *S*-(Thioacyl)thioglycolic acids, **55**,⁵⁶ **57**,⁵⁷ **60**,^{54,57} **62**,⁵⁷ and **66**⁵⁸ were made using the literature procedures cited here. The ethyl ester of **57**, *S*-(4-methylthiobenzoyl)thioglycolic acid ethyl ester **70**,⁵⁹ was an adventitious by-product when the acid was prepared in ethanol using thioacetic acid in place of H₂S using the procedure of Jensen and Pedersen.⁵⁷ Thiourea **69**⁶⁰ (*N*-phenylthiohydantoic acid) and dithiocarbamates **56** and **61** were prepared as described,⁶¹ as was carbamate **65**⁶² (*O*-(phenylcarbamoyl)glycolic acid). Several of the compounds detailed here are mentioned in the prior literature as synthons or as isolates from biological systems, but physical and spectroscopic data were not reported. We detail this information in the next section: *Physical and spectroscopic properties*.

5.12. Physical and spectroscopic properties

The solvent used in obtaining the NMR spectra was CDCl₃, unless otherwise noted. Satisfactory elemental analysis, C, H, N, and S, was obtained for all compounds.

10: ¹H NMR δ 1.58 (t, *J*=7.6 Hz, 3H), 2.60 (q, *J*=7.6 Hz, 2H), 4.10 (d, *J*=4.7 Hz, 2H), 7.05 (br. s, 1H), 7.16 (d, *J*=7.7 Hz, 2H), and 7.67 (d, *J*=7.7 Hz, 2H).

31: mp 118 (Davies *et al.*,⁶³ mp 116–118).

41: mp 125–126. ¹H NMR δ 2.86 (t, *J*=7.2 Hz, 2H), 2.97 (t, *J*=7.0 Hz, 2H), 4.19 (d, *J*=4.8 Hz, 2H), 7.06–7.15 (m, 5H), and 9.37 (s, 1H).

48: mp 142 (Fry,⁵³ mp 141–142).

50: mp 88–90.

55: mp 66–68 (Leon,⁵⁶ mp 64.5–66).

56: mp 157–158 (Cherbuliez *et al.*,^{61b} mp 159–160). ¹H NMR (CDCl₃-[D₆]-DMSO) δ 2.64 (t, *J*=6.9 Hz, 2H), 3.41 (t, *J*=6.9 Hz, 2H), 7.09–7.49 (m, 5H), and 11.00 (s, 1H). ¹³C NMR δ 30.16, 34.00, 124.28, 126.56, 128.71, 139.35, and 173.11.

57: mp 116–117 (Jensen and Pedersen,⁵⁷ mp 118–119). ¹H NMR δ 2.40 (s, 3H), 4.24 (s, 2H), 7.20 (d, *J*=8.1 Hz, 2H), 7.97 (d, *J*=8.2 Hz, 2H), and 10.21 (br. s, 1H). ¹³C NMR δ 21.57, 38.58, 127.09, 129.13, 141.51, 144.10, 173.79, 243.

59: mp 124 (Barrett,⁵⁵ mp 124).

60: mp 55^{||}. ¹H NMR δ 4.04 (s, 2H), 4.33 (s, 2H), 7.21–7.32 (m, 5H), and 10.3 (br. s, 1H). ¹³C NMR δ 38.50, 57.27, 127.46, 128.64, 129.11, 136.30, 173.24, 233.10.

61: mp 145 (fast), 192–193 (slow) (Cherbuliez *et al.*,^{61a} compound cyclizes to a thioxothiazolidine, mp 193). ¹H NMR (CDCl₃-[D₆]-DMSO) δ 3.96 (s, 2H), 7.09–7.45 (m, 5H), 9.28 (br. s., 1H), and 10.79 (s, 1H). ¹³C NMR δ 39.54, 124.03, 128.31, 128.73, 129.40, and 170.68.

62: mp 88–90 (Jensen and Pedersen,⁵⁷ mp 92–93). ¹H NMR δ 2.95–3.15 (m, 4H), 3.87 (s, 2H), 6.7–7.21 (m, 5H), and 10.1 (s, 1H).

65: mp 141–143 (Fischer and Fischer,⁶² mp 142–143). ¹H NMR (CDCl₃-[D₆]-DMSO) δ 4.51 (s, 2H), 6.91 (t, J=7.3 Hz, 1H), 7.17 (t, J=7.7 Hz, 2H), 7.42 (d, J=8.0 Hz, 2H), and 9.62 (s, 1H). ¹³C NMR δ 60.73, 118.62, 122.79, 128.79, 139.05, 153.18, and 170.13.

68: changes form at 173, melts at 182 (according to Fischer and Fischer,⁶² **68** shrinks at 173 and melts at 179–182).

69: mp 142–144 (fast), >200 (slow, compound cyclizes) (Dash *et al.*,⁶⁰ mp 141). ¹H NMR (CDCl₃-[D₆]-DMSO) δ 4.21 (d, J=5.0 Hz, 2H), 7.05 (t, J=7.3 Hz, 2H), 7.21–7.42 (m, 5H), 7.62 (t, J=4.7 Hz, 1H), and 9.67 (s, 1H). ¹³C NMR δ 46.11, 123.46, 124.72, 128.85, 139.15, 171.40, **70:** mp 33–35, bp 185–190/1mm. ¹H NMR δ 1.29 (t, J=7.1 Hz, 3H), 2.37 (s, 3H), 4.18–4.26 (m, 4H), 7.18 (d, J=8.0 Hz, 2H), and 7.96 (d, J=7.9 Hz, 2H).

76: mp 150 (Kameda *et al.*,⁶⁴ mp 149–150).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

tBOC	<i>tert</i> -butoxycarbonyl
CBZ	carbobenzyloxy

^{||}Thioglycolate **60** was first prepared by Kjær⁵⁴ and, subsequently, in a more reliably reproducible procedure by Jensen and Pedersen.⁵⁷ Both authors report melting points of 77–80 °C. We have prepared **60** several times using both published procedures and our product always melted at 53–55 °C (a sample purified for X-ray structure determination melted at 55.1 °C). The structure of our compound is beyond question, confirmed by elemental analysis, ¹H and ¹³C NMR, and proven absolutely by a single-crystal X-ray structure kindly determined by Dr. Mike Zawarotko (Department of Chemistry, University of South Florida). Our compound reacted with ammonia, morpholine, and glycine to yield derivatives corresponding perfectly to those described by Kjær.⁵⁴ It is possible that **60** is dimorphic and that we have isolated a lower melting form. We were unable, however, to persuade the **60** made in our laboratories to transform into a higher melting form.

Dansyl	[5-(dimethylamino)-1-naphthalenyl]sulfonyl
DβH	dopamine β-monooxygenase
4-HPR	<i>N</i> -(4-hydroxyphenol)retinamide
MES	4-morpholineethanesulfonic acid
PAL	monofunctional peptidylamidoglycolate lyase
PAM	bifunctional peptidylglycine α-amidating monooxygenase
PHM	monofunctional peptidylglycine α-hydroxylating monooxygenase
SAR	structure-activity relationship

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Figure 1. The reactions catalyzed by PHM and PAL

Bifunctional PAM is comprised of the two monofunctional enzymes. The possible role of Fe(III) in PAL catalysis is unclear.

Figure 2. The effect of *S*-(2-phenylthioacetyl)thioglycolate 60 on the growth of human prostate DU 145 cells

Cells were grown to 50,000 cells per well and then treated with the indicated concentration of *S*-(2-phenylthioacetyl)thioglycolate or *N*-(4-hydroxyphenyl) retinamide (4-HPR). Cell growth was measured at 8, 24, 48, 72, and 96 hr. 4-HPR was included as a positive control and 10 μ M completely eliminates cell growth at 48 hr, consistent with published results.⁴⁶

Figure 3. The effect of *S*-(4-methylthiobenzoyl)thioglycolate 57 and its ethyl ester 70 on the growth of human prostate DU 145 cells

Cells were grown to 50,000 cells per well and then treated with the indicated concentration of *S*-(4-methylthiobenzoyl)thioglycolate or *S*-(4-methylthiobenzoyl)thioglycolate ethyl ester. Cell growth was measured at 8, 24, 48, 72, and 96 hr.

Table 1

Hippurate and Hippurate Analogs as Substrates^a

Structure	$K_{M,app}$ (mM)	$V_{M,app}$ (s ⁻¹)	$(V/K)_{app}$ (M ⁻¹ s ⁻¹)	Rel. (V/K) _{app}
	R = 2,6-F ₂ (C ₆ H ₃)	4.3 ± 0.6	(2.3 ± 0.2) × 10 ²	0.04
	R = CH ₃	8.0 ± 0.2	(8.6 ± 0.3) × 10 ²	0.1
	R = 2-(C ₆ H ₄)	3.1 ± 0.1	(9.1 ± 0.7) × 10 ²	0.1
	R = (CH ₃) ₃ CO	6.3 ± 0.3	(1.7 ± 0.05) × 10 ³	0.3
	R = 2-CH ₃ (C ₆ H ₄)	7.9 ± 0.3	(1.8 ± 0.04) × 10 ³	0.3
	R = 4-HN(C ₆ H ₄)	5.6 ± 0.2	(2.2 ± 0.1) × 10 ³	0.4

Structure	K_{Mapp} (mM)	V_{Mapp} (s^{-1})	$(V/K)_{app}$ ($M^{-1} s^{-1}$)	Rel. (V/K) _{app}
	2.7 ± 0.1	7.5 ± 0.07	$(2.8 \pm 0.1) \times 10^3$	0.5
R = 2-H ₂ N(C ₆ H ₄)	1.4 ± 0.1	4.2 ± 0.1	$(2.9 \pm 0.1) \times 10^3$	0.5

 Structure	$K_{M,app}$ (mM)	$V_{M,app}$ (s^{-1})	$(V/K)_{app}$ ($M^{-1} s^{-1}$)	Rel. (V/K) _{app}
	R = 4-CH ₃ CH ₂ (C ₆ H ₄)	3.6 ± 0.3	(3.1 ± 0.5) × 10 ³	0.5
	R = 4-CH ₃ (C ₆ H ₄)	5.7 ± 0.3	(3.2 ± 0.2) × 10 ³	0.5
	R = 4-HO(C ₆ H ₄)	5.8 ± 0.1	(3.5 ± 0.2) × 10 ³	0.6
	R = 4-CF ₃ (C ₆ H ₄)	2.8 ± 0.1	(3.6 ± 0.5) × 10 ³	0.6
	R = 3-Cl(C ₆ H ₄)	7.8 ± 0.3	(3.6 ± 0.2) × 10 ³	0.6
	R = 4-CH ₃ (CH ₂) ₂ (C ₆ H ₄)	5.1 ± 0.4	(4.2 ± 0.6) × 10 ³	0.7

Structure	K_{Mapp} (mM)	V_{Mapp} (s^{-1})	$(V/K)_{app}$ ($M^{-1} s^{-1}$)	Rel. (V/K) _{app}
	1.9 ± 0.1	8.0 ± 0.2	$(4.2 \pm 0.2) \times 10^3$	0.7

Structure	$K_{M,app}$ (mM)	$V_{M,app}$ (s^{-1})	$(V/K)_{app}$ ($M^{-1} s^{-1}$)	Rel. $(V/K)_{app}$
R = 3-CH₃(C₆H₄) R = 4-Br(C₆H₄)	1.4 ± 0.1	6.4 ± 0.2	(4.7 ± 0.2) × 10 ³	0.8
	0.52 ± 0.06	2.5 ± 0.09	(4.9 ± 0.4) × 10 ³	0.8
	1.6 ± 0.1	9.3 ± 0.2	(6.0 ± 0.3) × 10 ³	1.0
R =				

Structure	$K_{M,app}$ (mM)	$V_{M,app}$ (s^{-1})	$(V/K)_{app}$ ($M^{-1} s^{-1}$)	Rel. (V/K) _{app}
	$R = C_6H_5$	8.2 ± 0.08	$(6.2 \pm 0.1) \times 10^3$	1.0
	$R = 4-CH_3O(C_6H_4)$	5.3 ± 0.08	$(6.4 \pm 0.2) \times 10^3$	1.0
	$R = 4-Cl(C_6H_4)$	4.1 ± 0.1	$(6.6 \pm 0.2) \times 10^3$	1.1
	$R = 2-HO(C_6H_4)$	4.9 ± 0.05	$(7.1 \pm 0.1) \times 10^3$	1.1
	$R = 4-O_2N(C_6H_4)$	4.2 ± 0.04	$(8.9 \pm 0.2) \times 10^3$	1.4
	 $R =$	7.3 ± 0.2	$(2.5 \pm 0.1) \times 10^3$	4.0

Structure	$K_{M,app}$ (mM)	$V_{M,app}$ (s^{-1})	$(V/K)_{app}$ ($M^{-1} s^{-1}$)	Rel. $(V/K)_{app}$
	0.31 ± 0.03	9.3 ± 0.4	$(3.0 \pm 0.2) \times 10^3$	4.8

Biorg Med Chem. Author manuscript; available in PMC 2009 December 1.

ts of the initial rate data to eqn. 1.

Table 2

Extended Hippurate Analogs as Substrates

Structure	$K_{M,app}$ (mM)	$V_{M,app}$ (s^{-1})	$(V/K)_{app}$ ($M^{-1} s^{-1}$)	Rel. (V/K) _{app}
	1.2 ± 0.1	3.4 ± 0.1	(2.8 ± 0.1) × 10 ³	0.45
	R = (C ₆ H ₅)CH ₂ CH(CH ₃)	18 ± 0.9	(4.3 ± 0.1) × 10 ³	0.69
	R = CH ₃ CONHCH ₂	8.9 ± 0.2	(5.9 ± 0.2) × 10 ³	1.0
	R = C ₆ H ₅	8.2 ± 0.08	(6.2 ± 0.1) × 10 ³	1.0
	R = (CH ₃) ₂ CHSCH ₂	1.8 ± 0.02	(8.2 ± 0.3) × 10 ³	1.3
	R =	10 ± 0.3	(1.1 ± 0.07) × 10 ⁴	1.8
	R = (C ₆ H ₅)CH ₂ CH ₂	9.9 ± 0.4	(1.1 ± 0.06) × 10 ⁴	1.8
	R = (C ₆ H ₅)CH ₂ S	0.69 ± 0.022	(1.2 ± 0.14) × 10 ⁴	1.9
	R = (C ₆ H ₅)CH ₂ O	8.6 ± 0.1	(1.5 ± 0.03) × 10 ⁴	2.4
	R = ClCH ₂ CONHCH ₂	8.9 ± 0.2	(2.1 ± 0.1) × 10 ⁴	3.3
	R = (CH ₃) ₂ CHCH ₂ CH ₂	6.6 ± 0.3	(2.3 ± 0.2) × 10 ⁴	3.7
	R = (CH ₃) ₃ COCONHCH ₂	7.5 ± 0.2	(2.5 ± 0.1) × 10 ⁴	4.0

Structure	$K_{M,app}$ (mM)	$V_{M,app}$ (s^{-1})	$(V/K)_{app}$ ($M^{-1} s^{-1}$)	Rel. (V/K) _{app}
	$R = (C_6H_5)SCH_2$	4.6 ± 0.2	$(2.5 \pm 0.1) \times 10^4$	4.0
	$R = (C_6H_5)NH$	9.7 ± 0.9	$(2.7 \pm 0.4) \times 10^4$	4.4
	$R = (C_6H_5)CONHCH_2-CONHCH_2$	8.8 ± 1.3	$(2.9 \pm 0.2) \times 10^4$	4.7
	$R = (C_6H_5)CH=CH$	8.4 ± 0.3	$(3.6 \pm 0.3) \times 10^4$	5.8
	$R = (C_6H_5)CONHCH_2-CONHCH_2$	1.6 ± 0.2	$(4.2 \pm 1.4) \times 10^4$	6.8
	$R = (C_6H_5)CH_2$	7.4 ± 0.09	$(4.6 \pm 0.09) \times 10^4$	7.4
	$R = C_6H_5(CH_2)_3$	11 ± 0.4	$(7.0 \pm 0.7) \times 10^4$	11
	$R = O_2NC_6H_4CONHCH_2$	8.7 ± 0.2	$(7.6 \pm 0.4) \times 10^4$	12
	$R = C_6H_5(CH_2)_4$	12 ± 0.4	$(7.6 \pm 0.4) \times 10^4$	12
	$R = C_6H_5(CH_2)_7$	8.0 ± 0.3	$(7.6 \pm 0.4) \times 10^4$	12
	$R =$	3.0 ± 0.1	$(8.3 \pm 0.7) \times 10^4$	13
	$R = (C_6H_5)CONHCH_2$	2.4 ± 0.2	$(1.0 \pm 0.2) \times 10^5$	16
		12 ± 0.8	$(1.6 \pm 0.1) \times 10^5$	26

Structure	$K_{M,app}$ (mM)	$V_{M,app}$ (s^{-1})	$(V/K)_{app}$ ($M^{-1} s^{-1}$)	Rel. (V/K) _{app}
R = C₆H₅(CH₂)₅	0.051 ± 0.007 <i>0.043 ± 0.005</i>	11 ± 0.4	(2.0 ± 0.2) × 10 ⁵	32

s value shown in italics.

1.3 mM

Table 3

N-Substituted D-Alanines as Substrates^a

Structure	$K_{M,app}$ (mM)	$V_{M,app}$ (s^{-1})	$(V/K)_{app}$ ($M^{-1} s^{-1}$)	$\frac{(V/K)_{app,Gly}}{(V/K)_{app,D-Ala}}$
R = CH ₃	1400 ± 160	1.3 ± 0.1	0.95 ± 0.05	900
R = C ₆ H ₅	84 ± 23	0.27 ± 0.03	3.2 ± 0.6	1,900
R = C ₆ H ₅ CH ₂ O	35 ± 5.0	1.4 ± 0.1	41 ± 5	370
R = Dansyl-Tyr-Val	0.031 ± 0.004	0.91 ± 0.06	(3.0 ± 0.2) × 10 ⁴	17

^a (BOC-D-alanine, we obtained a rate of O₂ consumption of 0.63 s⁻¹, which is approximately equal to the rate of O₂ consumption obtained

compared directly to *N*-dansyl-Tyr-Val-Gly amidation, using the exact same reaction conditions and source of PAM. The steady-state kinetic

parameters: $K_{M,app} = 2.7 \pm 0.4 \mu M$, $V_{M,app} = 1.4 \pm 0.6 s^{-1}$, and $(V/K)_{app} = (5.0 \pm 0.7) \times 10^5 M^{-1} s^{-1}$.

^b 470 ± 90 μM.

PAM Inhibitors

Table 4

	Structure
caproic acid (55)	
captoproic acid (56)	
logglycolic acid (57)	

	Structure
cyanopentanoic acid (58)	
(S)-phenylalanine (59)	
phenylglycolic acid (60) (2.5%)	
phenylglycolic acid (61)	
phenylglycolic acid (62) (2.5%)	

	Structure
corbate ^b (63)	
id ^a (64) (2.5% DMSO)	
mycolic acid (65)	

	Structure
thiolactic acid (66) NIH-PA Author Manuscript	 <chem>OC(=O)CSCC(=S)c1ccccc1</chem>
Ester (67) NIH-PA Author Manuscript NIH-PA Author Manuscript	 <chem>CCOC(=O)CCNC(=O)[C@H](CS)C(=O)N[C@@H](C)C(=O)O</chem>

	Structure
cysteine (68)	 <chem>O=C(O)[C@H](NC(=O)c1ccccc1)CCSC(=O)c2ccccc2</chem>
(69) (2.5% DMSO)	 <chem>NC(=S)[C@H](N)CC(=O)O</chem>
glycolic acid ethyl ester	 <chem>CCOC(=O)CCSC(=O)c1ccc(C)cc1</chem>

	Structure
c acid (71)	 <chem>O=C(O)C(=O)Nc1ccccc1O</chem>
etic Acid (72)	 <chem>OC(=O)CSc1ccccc1</chem>

	Structure
butyric acid (75) (CBZ-	 <chem>O=C(O[C@H](O)CCNC(=O)OCC1=CC=CC=C1)C(=O)O</chem>
erine (76)	 <chem>O=C(O[C@H](O)CNC(=O)C1=CC=CC=C1)C(=O)O</chem>
ic acid (77)	 <chem>O=C(OCCC(=O)C1=CC=CC=C1)C(=O)O</chem>

	Structure
lycine (78)	
ne (79)	
phosphonic acid ^c (80)	

^a $K_{M,app}$ for aceturic acid in 2.5% DMSO = 15.0 mM and the $K_{M,app}$ for *N*-dansyl-Tyr-Val-Gly in 2.5% DMSO = 14.9 μ M. In the absence of DMSO, the $K_{M,app}$ for aceturic acid = 9.3 mM and the $K_{M,app}$ for *N*-dansyl-Tyr-Val-Gly = 3.5 μ M. Values obtained in DMSO are shown in italics. In some cases, we obtained $K_{i,s}$ values in the presence and absence of DMSO. For such compounds, two values are shown with the value in black representing that measured in the absence of DMSO.

^b Inhibition constant included in the table is the IC₅₀ because it is not clear that 6-*O*-palmitoyl-L-ascorbate should be competitive with the aceturic acid of *N*-dansyl-Tyr-Val-Gly. Preliminary experiments indicate that 6-*O*-palmitoyl-L-ascorbate is a non-competitive inhibitor vs. *N*-dansyl-Tyr-Val-Gly and ascorbate at ambient O₂ (Malka and Merkler, unpublished).

^c No O₂ consumption observed at 0.9 or 28 mM (nicotinamidomethyl)phosphonate. No inhibition of O₂ consumption of 200 μ M *N*-octanoylglycine ($K_{M,app}$ = 200 μ M) was observed at 28 mM (nicotinamidomethyl)phosphonate. A $K_{i,s}$ of 75 mM would have decreased the initial rate ~15%, which was not seen.

Table 5
Comparison of $K_{i,s}$ Values Between Insect and Mammalian PHM

Compound	$K_{i,s}$ (μ M) vs. <i>B. germanica</i> PHM	$\frac{\text{Rat } K_{i,s}}{\text{Insect } K_{i,s}}$
<i>S</i> -(3-Phenylthiopropionyl)thioglycolic acid (62)	0.87 ± 0.11	11
<i>S</i> -(2-Phenylthioacetyl)thioglycolic acid (60)	3.6 ± 0.6	2.1
<i>S</i> -(Thiobenzoyl)thioglycolic acid (64)	92 ± 10	1.9
<i>S</i> -(Phenylthiocarbamoyl)thioglycolic acid (61)	5.4 ± 0.3	1.6
<i>S</i> -(Phenylthiocarbamoyl)-3-mercaptopropionic acid (56)	5.5 ± 0.8	0.47
<i>S</i> -(4-Methylthiobenzoyl)thioglycolic acid (57)	14 ± 1	0.25
<i>S</i> -Phenylmercaptoacetic Acid (72)	56 ± 6	6.9

Table 6
Sulfur Atom Effect on PHM Affinity

Compound Pair ^{a,b}	$\frac{K_{\text{no,sulfur atom}}^c}{K_{\text{sulfur atom}}}$	$\Delta(\Delta G)^d$ (kcal/mol)
52/59 ^c	2.3×10^4	-6.2
<i>N</i> -(isobutyryl)glycine/ <i>tiopronin</i> ^f	48	-2.4
<i>N</i> -(2-ethylhydrocinnamoyl)glycine/ <i>thiorphan</i> ^f	37	-2.2
31/41	24	-2.0
31/32	16	-1.7
42/48	6.7	-1.2
31/37	4.8	-0.98
<i>N</i> -(isobutyryl)glycine/ <i>S</i> -2-methyltiopronin ^f	3.5	-0.77
38/69	3.3	-0.73
35/29	1.3	-0.15

^aFor the compound pairs, the first compound listed does not incorporate a sulfur atom while the second does.

^bThe compounds highlighted in blue contain a thiol and those in red contain a thiocarbamoyl.

^cThis represents the ratio of the K_M or $K_{i,s}$ values for the PHM substrate or inhibitor containing no sulfur to the corresponding analog containing a sulfur atom.

^d $\Delta(\Delta G) = -RT \ln(K_{\text{no,sulfur atom}}/K_{\text{sulfur atom}})$ with $T = 310$ K.

^e59 is a mixture of enantiomers, *N*-(thiobenzoyl)-(D,L)-alanine. In calculating the ratio of the equilibrium constants, we assumed that L-isomer had little affinity for PHM²⁰ and, thus, used a $K_{i,s}$ value of 3.6 μM for the D-isomer (half the value show in Table 4).

^f K_M values from McIntyre *et al.*^{23d}

Table 7

Effect of -CS-S- PHM Affinity		
Compound Pair	$\frac{K_{\text{CO-NH-}}}{K_{\text{CS-S-}}}$	$\Delta(\Delta G)^a$ (kcal/mol)
1/64	33	-2.1
38/61	42	-2.3
31/62	98	-2.8
<i>N</i> -(lauroyl)glycine ^b /55	110	-2.9
11/57	510	-3.8
52/66 ^c	2.9×103	-4.9

^a $\Delta(\Delta G) = -RT \ln(K_{\text{CO-NH-}}/K_{\text{CS-S-}})$ with $T = 310 \text{ K}$.

^b $K_{\text{M,app}}$ value for *N*-(lauroyl)glycine is from Wilcox *et al.*^{23a}

^cCompound **66** is a mixture of enantiomers, *S*-(thiobenzoyl)-(*R,S*)-thiolactic acid. In calculating the ratio of the equilibrium constants, we assumed that *R*-isomer had little affinity for PHM and, thus, used a $K_{\text{i,s}}$ value of $29 \mu\text{M}$ for the *S*-isomer (half the value show in Table 4).

Table 8

Effect of -NH- vs. -CH ₂ - on PHM Affinity		
Compound Pair	$\frac{K_{\text{-CH}_2-}}{K_{\text{-NH-}}}$	$\Delta(\Delta G)^a$ (kcal/mol)
60/61	0.91	-0.05
42/38	0.44	-0.50
<i>N</i> -(hexanoyl)glycine/5-butylhydantoic acid ^b	0.42	-0.53
<i>N</i> -(acetyl)glycine/hydantoic acid ^b	0.28	-0.78
48/69	0.22	-0.94

^a $\Delta(\Delta G) = -RT\ln(K_{\text{-CH}_2-}/K_{\text{-NH-}})$ with T = 310 K.

^b $K_{M,\text{app}}$ values are from Wilcox *et al.*^{23a}