Design, Synthesis, and Biochemical Evaluation of *N*-Substituted Maleimides as Inhibitors of Prostaglandin Endoperoxide Synthases†

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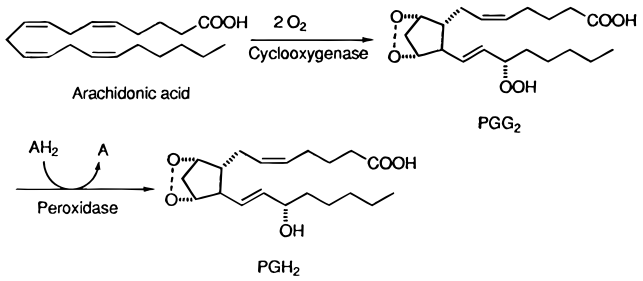
N-(Carboxyalkyl)maleimides are rapid as well as time-dependent inhibitors of prostaglandin endoperoxide synthase (PGHS). The corresponding *N*-alkylmaleimides were only time-dependent inactivators of PGHS, suggesting that the carboxylate is critical for rapid inhibition. Several *N*-substituted maleimide analogs containing structural features similar to those of the nonsteroidal anti-inflammatory drug aspirin were synthesized and evaluated as inhibitors of PGHS. Most of the aspirin-like maleimides inactivated the cyclooxygenase activity of purified ovine PGHS-1 in a time- and concentration-dependent manner similar to that of aspirin. The peroxidase activity of PGHS was also inactivated by the maleimide analogs. The cyclooxygenase activity of the inducible isozyme, i.e., PGHS-2, was also inhibited by these compounds. The corresponding succinimide analog of *N*-5-maleimido-2-acetoxy-1-benzoic acid did not inhibit either enzyme activity, suggesting that inactivation was due to covalent modification of the protein. The mechanism of inhibition of PGHS-1 by *N*-(carboxyheptyl)maleimide was investigated. Incubation of apoPGHS-1 with 2 equiv of *N*-(carboxyheptyl)[3,4-^{14}C]maleimide led to the incorporation of radioactivity in the protein, but no adduct was detected by reversed-phase HPLC, suggesting that it was unstable to the chromatographic conditions. Furthermore, hematin-reconstituted PGHS-1, which was rapidly inhibited by *N*-(carboxyheptyl)maleimide, displayed spontaneous regeneration of about 50% of the cyclooxygenase and peroxidase activities, suggesting that the adduct responsible for the inhibition breaks down to regenerate active enzyme. ApoPGHS-1, inhibited by *N*-(carboxyheptyl)maleimide, did not display regeneration of enzyme activity, but addition of hematin to the inhibited apoenzyme led to spontaneous recovery of about 50% of cyclooxygenase activity. These results suggest that addition of heme leads to a conformational change in the protein which increases the susceptibility of the adduct toward hydrolytic cleavage. ApoPGHS-1, pretreated with *N*-(carboxyheptyl)maleimide, was resistant to trypsin cleavage, suggesting that the carboxylate functionality of the maleimide binds in the cyclooxygenase channel. A model for the interaction of *N*-(carboxyheptyl)maleimide in the cyclooxygenase active site is proposed.

Introduction

Prostaglandin endoperoxide synthase (PGHS, EC 1.14.99.1) catalyzes the first two steps of prostaglandin biosynthesis.[1] Its cyclooxygenase activity is responsible for the incorporation of two molecules of dioxygen into the carbon framework of arachidonic acid, leading to the formation of the prostaglandin endoperoxide, PGG_2. The peroxidase activity of the enzyme then reduces PGG_2 in the presence of a reducing substrate to the corresponding alcohol PGH_2 (Scheme 1).[2,3] The protein is a homodimer of 70 kDa subunits and binds one heme per subunit.[4] Although heme is essential for both the cyclooxygenase and peroxidase activities, the substrate binding sites of the two activities are spatially distinct.[4,5]

The cyclooxygenase activity of PGHS is inhibited by a variety of compounds known as nonsteroidal anti-inflammatory drugs (NSAID's) which include aspirin, indomethacin, and ibuprofen.[6,7] It is generally believed that the anti-inflammatory effect of NSAID's is mediated by inhibition of the cyclooxygenase activity of the PGHS protein. Therapeutic intervention along the arachidonic acid cascade continues to be an area of intense activity toward the development of anti-inflammatory agents and has gained fresh impetus due to the recent discovery of a new form of the enzyme, i.e., PGHS-2.[8,9] PGHS-1 is constitutively expressed in most tissues,[10] whereas the PGHS-2 protein is inducible, and the biosynthesis of this isozyme is enhanced by inflammatory stimuli.[11]

Scheme 1



Previous studies in our laboratory have documented that *N*-substituted maleimides such as *N*-ethylmaleimide (NEM) and *N*-[7-(dimethylamino)-4-methylcoumarinyl]maleimide (DACM) (Figure 1) are time- and concentration-dependent inhibitors of PGHS.[12] However, a large excess of inhibitor (~300-fold) and prolonged incubation times (~120 min) are required to achieve

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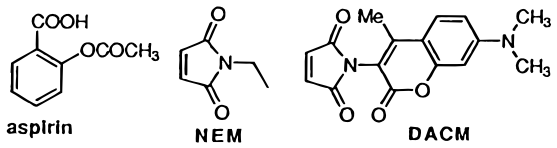


Figure 1. Chemical structures of aspirin, NEM, and DACM.

significant inhibition. These conditions result in modification of all the free cysteines in the protein, i.e., Cys[313], Cys[512], and Cys[540].

We have recently shown that maleimide derivatives tethered to a series of medium-length fatty acids exhibit much more potent cyclooxygenase inactivation. The most potent of these inhibitors, *N*-(carboxyheptyl)-maleimide, inhibits enyzme activity within seconds after mixing with a stoichiometric amount of PGHS protein.[13] The lack of PGHS inhibition by the *N*-(carboxyheptyl)-succinimide suggests that this rapid inhibition results from covalent modification of the protein. Varying the length of the alkyl chain in *N*-(carboxyalkyl)maleimides alters their potency as rapid inhibitors but does not significantly affect their ability to inhibit the enzyme on prolonged incubation (~30 min). Removal of the carboxylate eliminates rapid enzyme inhibition although *N*-alkylmaleimides exhibit slow, time-dependent inactivation.

The importance of the carboxylate group in rapid inactivation of PGHS by maleimides and the selective loss of cyclooxygenase activity at stoichiometric amounts of *N*-(carboxyheptyl)maleimide suggested that *N*-(carboxyalkyl)maleimides bind in the cyclooxygenase substrate access channel of the protein. Therefore, we prepared a series of maleimides tethered to salicylic or benzoate moieties that are known to bind in this channel.[14,15]

In addition to expanding the structure−activity for PGHS inhibition by *N*-substituted maleimides, the interaction of *N*-(carboxyheptyl)maleimide with purified ovine PGHS-1 was studied in greater detail with radiolabeled *N*-(carboxyheptyl)maleimide. Although incubation of apoPGHS-1 with radiolabeled inhibitor led to the incorporation of radioactivity in the protein, subsequent attempts to identify the amino acid residue(s) by peptide mapping were unsuccessful, presumably due to the instability of the enzyme/inhibitor adduct. Thus, studies were initiated with several *N*-(carboxyheptyl)maleimide analogs and carboxyheptyl analogs tethered to other electrophilic moieties such as the *N*-(carboxyheptyl)haloalkyl acetamides. This paper summarizes the results of our studies and places them in the perspective of current concepts of PGHS structure and function.

Results

Chemistry. The synthesis of the *N*-substituted maleimide derivatives was achieved by modifications of previously reported methodology.[16,17] Reaction of maleic anhydride (**1**) with the appropriate amine in the presence of glacial acetic acid at room temperature generated the corresponding *N*-substituted maleamic acids **2**−**18** in near quantitative yields (Scheme 2 and Table 1). The maleamic acid intermediates were subsequently cyclized to the corresponding *N*-arylmaleimides **19**−**25** (Table 2), *N*-alkylmaleimides **26**−**28** (Table 3), and *N*-(carboxyalkyl)maleimides **29**−**35** (see Table 3) by heating in the presence of acetic anhydride containing catalytic amounts of sodium acetate. In the case of the *N*-arylmaleimide analogs **21** and **22** (see Table 2), cyclization of the corresponding *N*-arylmaleamic acid derivatives **4** and **5** in the presence of acetic anhydride also resulted in the desired acetylation of the salicylate hydroxyl group.

The 2-[2-(*N*-maleimido)ethoxy]benzoic acid analog **19** required the synthesis of the intermediate (aminoethoxy)benzoic acid (**38**). The synthesis of **38** was achieved by the reaction of commercially available 4-chromanone (**36**) with sodium azide in the presence of acid to generate the seven-membered ring lactam **37**.[18] Acid-catalyzed hydrolysis of **37** afforded the desired amine (**39·HCl**) as its hydrochloride salt (Scheme 3).

Scheme 2

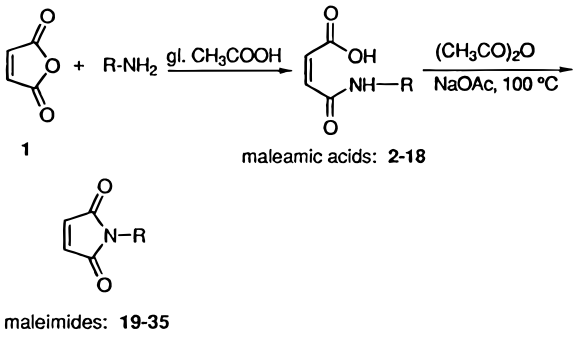
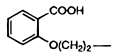
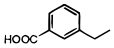
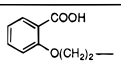
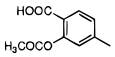
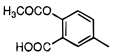
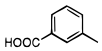
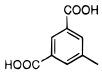
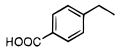


Table 1. Chemical Properties and Purification Methods for the Intermediate *N*-Substituted Maleamic Acids

Entry	R	% yield[a]	recryst solvent	mp, °C	formula
2		64	EtOH	161-162	$C_{13}H_{13}NO_6$
3		78	EtOH	163-165	$C_{12}H_{13}NO_3$
4		50	EtOH	219-220	$C_{11}H_9NO_6$
5		46	EtOH	224-226	$C_{11}H_9NO_6$
6		81	EtOH	172-174	$C_{11}H_9NO_5$
7		73	EtOH	> 300	$C_{12}H_9NO_7$
8		70	EtOH / H_2O	219-220	$C_{12}H_{11}NO_5$
9	—$(CH_2)_7$-CH_3	81	2-PrOH / H_2O	84-86	$C_{12}H_{21}NO_3$
10	—$(CH_2)_8$-CH_3	79	2-PrOH / H_2O	74-76	$C_{13}H_{23}NO_3$
11	—$(CH_2)_9$-CH_3	88	2-PrOH / H_2O	81-82	$C_{14}H_{25}NO_3$
12	—$(CH_2)_5$-COOH	71	2-PrOH / H_2O	171-173	$C_{10}H_{15}NO_5$
13	—$(CH_2)_6$-COOH	84	2-PrOH / H_2O	170-172	$C_{11}H_{17}NO_5$
14	—$(CH_2)_7$-COOH	80	2-PrOH / H_2O	179-181	$C_{12}H_{19}NO_5$
15	—$(CH_2)_8$-COOH	85	2-PrOH / H_2O	165-167	$C_{13}H_{21}NO_5$
16	—$(CH_2)_9$-COOH	76	2-PrOH / H_2O	148-151	$C_{14}H_{23}NO_5$
17	—$(CH_2)_{10}$-COOH	85	2-PrOH / H_2O	159-161	$C_{15}H_{25}NO_5$
18	—$(CH_2)_{11}$-COOH	85	2-PrOH / H_2O	160-162	$C_{16}H_{27}NO_5$

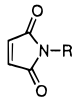
[a] All compounds gave satisfactory H NMR, GC-MS or FAB-MS, and elemental analyses.

Table 2. Chemical Properties, Purification Methods, and Inhibition Constants for Time-Dependent Cyclooxygenase Inhibition by Phenyl-Substituted Maleimides

Entry	R	% yield[a]	recryst solvent	mp, °C	formula	Cyclooxygenase Inhibition IC_{50}, (μM)[b,c]
19		32	EtOH	145-147	$C_{13}H_{11}NO_5$	250
20		51	EtOH	80-81	$C_{12}H_{11}NO_2$	50
21		61	EtOH	135-137	$C_{13}H_9NO_6$	> 300
22		59	EtOH	190-192	$C_{13}H_9NO_6$	29[d]
23		68	EtOH	145-146	$C_{11}H_7NO_4$	...[e]
24		75	EtOH	> 350	$C_{12}H_7NO_6$	...[e]
25		41	EtOH	189-190	$C_{12}H_9NO_4$	27

[a] All compounds gave satisfactory ^{1}H NMR, GC-MS or FAB-MS, and elemental analyses. [b] The IC_{50} values were obtained after a 30 min preincubation between the enzyme and inhibitor. [c] The corresponding IC_{50} values for inhibition of peroxidase activity were similar. [d] The corresponding succinimide analog **68** failed to inhibit either activity. [e] No significant inhibition at 300 μM.

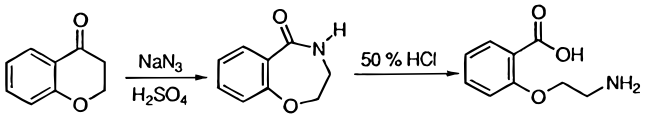
Table 3. Chemical Properties, Purification Methods, and Cyclooxygenase Inhibition Constants for *N*-Alkyl- and *N*-(Carboxyalkyl)maleimides



entry	R	% yield[a]	recryst solvent	mp, °C	formula	IC_{50}, μM (cyclooxygenase)
26	$(CH_2)_7CH_3$	68	2-PrOH/H_2O	36−38	$C_{12}H_{19}NO_2$	9[b]
27	$(CH_2)_8CH_3$	49	2-PrOH/H_2O	45−47	$C_{13}H_{21}NO_2$	12[b]
28	$(CH_2)_9CH_3$	51	2-PrOH/H_2O	48−49	$C_{14}H_{23}NO_2$	14[b]
29	$(CH_2)_5COOH$	49	2-PrOH/H_2O	88−91	$C_{10}H_{13}NO_4$	>800[c]
30	$(CH_2)_6COOH$	50	2-PrOH/H_2O	91−92	$C_{11}H_{15}NO_4$	570[c]
31	$(CH_2)_7COOH$	44	2-PrOH/H_2O	79−80	$C_{12}H_{17}NO_4$	0.1[c]
32	$(CH_2)_8COOH$	52	2-PrOH/H_2O	94−97	$C_{13}H_{19}NO_4$	26[c]
33	$(CH_2)_9COOH$	45	2-PrOH/H_2O	91−93	$C_{14}H_{21}NO_4$	37[c]
34	$(CH_2)_{10}COOH$	58	2-PrOH/H_2O	92−94	$C_{15}H_{23}NO_4$	40[c]
35	$(CH_2)_{11}COOH$	60	2-PrOH/H_2O	93−95	$C_{16}H_{25}NO_4$	140[c]

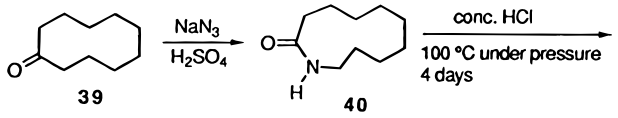
[a] All compounds gave satisfactory ^{1}H NMR, GC−MS, or FAB-MS, and elemental analyses. [b] IC_{50} values were obtained after a 30 min preincubation of the enzyme with inhibitor. [c] IC_{50} values for rapid cyclooxygenase inhibition.

Scheme 3



36 **37** **38**

Scheme 4



39 **40**

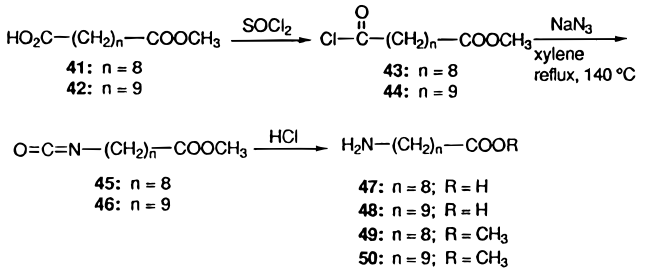
$H_2N-(CH_2)_9-COOH$

48

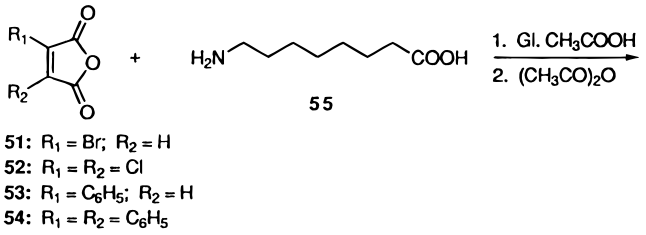
The synthesis of the intermediate ω-amino acids **47** and **48** was required for the synthesis of the *N*-(carboxyalkyl)maleimide derivatives **32** and **33**. The acid-catalyzed reaction of commercially available cyclodecanone **39** with sodium azide generated the 11-membered ring lactam, azacycloundecan-2-one (**40**). However acid-catalyzed hydrolysis of **40** under pressure afforded low yields of the desired ω-amino acid **48** (Scheme 4).

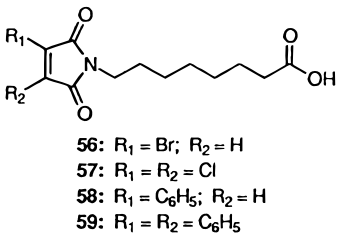
Subsequently a Curtius rearrangement strategy[19,20] was employed in the synthesis of **47** and **48**. Reaction of the monoesterified dicarboxylic acids **41** and **42** in the presence of excess thionyl chloride afforded the corresponding acid chlorides **43** and **44** in nearly quantitative yields. Isocyanates **45** and **46** were obtained by heating the acid chlorides **43** and **44** in the presence of sodium azide. Acid-catalyzed reaction of the isocyanates **45** and **46** led to the hydrolysis of the isocyano as well as the ester functionality to generate the desired ω-amino acids **47** and **48** (Scheme 5). Large-scale acid-catalyzed hydrolysis of the isocyanate derivatives **45** and **46** also generated the ω-amino acid methyl esters **49** and **50**, respectively, as contaminants (see Scheme 5). Repeated recrystallizations of the impure ω-amino acid hydrochloride salts from methanol/ethyl ether led to the isolation of the pure compounds.

Scheme 5

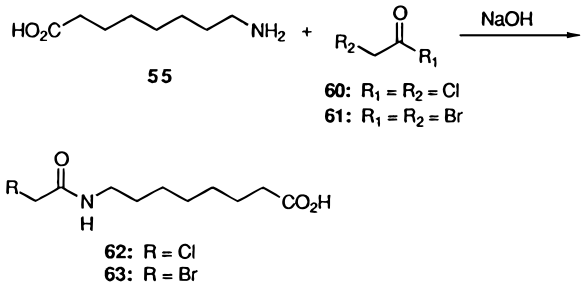


Scheme 6





Scheme 7

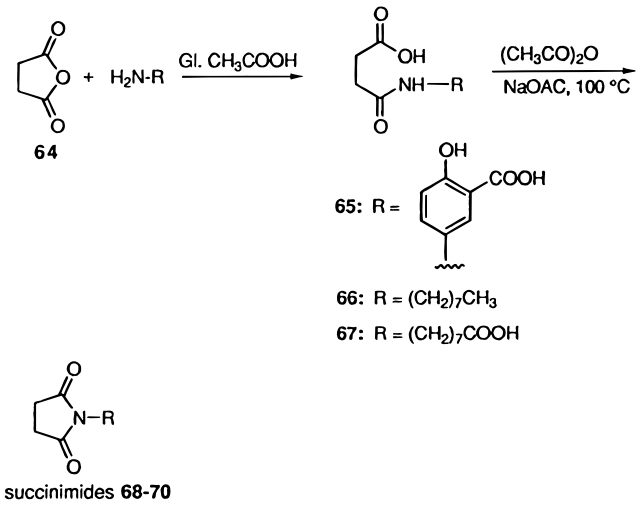


The synthesis of the *N*-(carboxyheptyl)-3,4-disubstituted-maleimide analogs **56**−**59** was achieved in a slightly different fashion. The substituted maleic anhydride analogs **51**−**54** were heated under reflux with 8-aminocaprylic acid (**55**) in glacial acetic acid for 24 h, as no reaction was observed at room temperature. The intermediate maleamic acids were not isolated; instead they were cyclized to the corresponding maleimides **56**−**59** in the presence of acetic anhydride (Scheme 6).

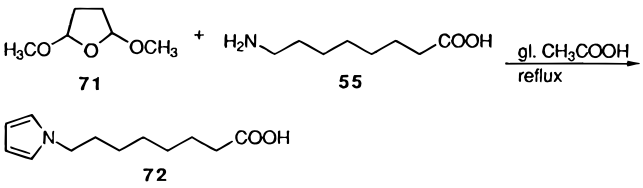
The synthesis of the (haloacetamido)octanoic acid analogs **62** and **63** was achieved by a base-catalyzed reaction of 8-aminocaprylic acid (**55**) with the corresponding acid halides **60** and **61**, respectively (Scheme 7).

As part of our enzymological studies, the synthesis of the succinimide analogs, namely, 2-acetoxy-5-*N*-(succinimido)benzoic acid (**68**), *N*-octylsuccinimide (**69**), and *N*-(carboxyheptyl)succinimide (**70**), was required. The succinimides were synthesized by the condensation of the appropriate amine with succinic anhydride (**64**) to yield the corresponding *N*-succinamic acids analogs **65**−**67** which were cyclized to the corresponding succinimides **68**−**70** in a similar manner as described in the maleimide synthesis (Scheme 8).

Scheme 8



Scheme 9



The *N*-(carboxyheptyl)pyrrole (**72**) derivative was synthesized by heating a reaction mixture containing 2,5-dimethoxytetrahydrofuran (**71**) and 8-aminocaprylic acid (**55**) in the presence of glacial acetic acid (Scheme 9).

Enzymology. A. *N*-Salicylylmaleimides. Treatment of hematin-reconstituted apoPGHS-1 with a 100-fold excess of the *N*-substituted maleimide derivatives **19**−**25** led to a time-dependent loss of cyclooxygenase activity at a rate comparable to the time-dependent inactivation of the cyclooxygenase activity by a similar concentration of aspirin. The aryl-substituted maleimide analogs **19**−**25** also displayed time-dependent inactivation of the peroxidase activity of holoPGHS-1 at rates similar to the loss of cyclooxygenase activity. None of the intermediate *N*-substituted maleamic acids **2**−**18** displayed inhibition of enzyme activity. Representative inhibition data are shown for the 5-*N*-maleimido-2-acetoxy-1-benzoic acid analog **22** in Figure 2.

The potency of time-dependent inhibition by maleimide analogs **19**−**25** was estimated by incubating the holoprotein with several concentrations of the maleimide analogs for 30 min followed by measurement of the remaining cyclooxygenase activity. The IC_{50} values were determined from plots of percent remaining enzyme activity versus inhibitor concentration (see Table 2). Of particular interest was the inactivation of PGHS by isomeric maleimide analogs **21** and **22**. The maleimide analog **22** in which the carboxylic acid moiety was *meta* with respect to the maleimide ring displayed a greater time-dependent inhibitory potency than the corresponding maleimide analog **21** in which the carboxylic acid moiety was *para* to the maleimide ring (compound **21**, IC_{50} (cyclooxygenase) = >200 μM; compound **22**, IC_{50} (cyclooxygenase) = 29 μM). The succinimide analog of **22**, i.e., **68**, failed to inactivate either

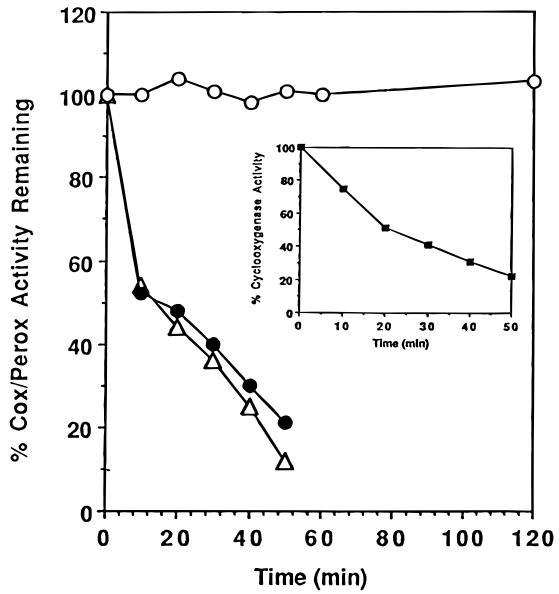


Figure 2. Time course of loss of cyclooxygenase and peroxidase activity of purified ovine PGHS-1 in the presence of *N*-5-maleimido-2-acetoxy-1-benzoic acid (**22**) and *N*-5-succinimido-2-acetoxy-1-benzoic acid (**68**): open circles, compound **68**; closed circles, cyclooxygenase inhibition by compound **22** (16 μM); open triangles, peroxidase inhibition by **22** (16 μM). Inset: Time-dependent cyclooxygenase inhibition of PGHS-1 by 16 μM aspirin.

cyclooxygenase or peroxidase activity, suggesting that inhibition of the protein by **22** was due to covalent modification. The lack of PGHS inhibitory properties by **68** also suggests that covalent modification of the protein by **22** was due to addition of an amino acid residue across the α,β-unsaturated carbonyl moiety in the maleimide ring system rather than acetylation of an amino acid residue. Incorporation of a methylene group between the maleimide ring and the phenyl group generated the *N*-(2-methylbenzyl)maleimide (**20**) and the *N*-(4-carboxybenzyl)maleimide (**25**) which displayed better PGHS inhibitory properties, in general, than those of the *N*-phenyl analogs.

The maleimide analogs also inhibited the cyclooxygenase activity of human PGHS-2 in a time-dependent fashion, although higher concentrations were required to achieve significant cyclooxygenase inhibition. Representative data comparing time-dependent cyclooxygenase inhibition of ovine PGHS-1 and human PGHS-2 are shown for *N*-(2-methylbenzyl)maleimide (**20**) in Figure 3. IC_{50}'s were not determined for most of the compounds against PGHS-2 because of inadequate supplies of the enzyme.

B. *N*-Alkylmaleimides. The maleimide analogs substituted with medium-length alkyl chains **26**−**28** also led to time- and concentration-dependent inhibition of the cyclooxygenase and the peroxidase activities of the PGHS-1 protein. The IC_{50} values for the time-dependent inhibition were determined in a manner similar to that described for the *N*-arylmaleimide derivatives (see Table 3). The *N*-alkylmaleimides were better time-dependent inactivators of both activities than the corresponding maleimides tethered to the salicylyl or aryl moiety. Among the three *N*-alkylmaleimides evaluated as PGHS inhibitors, *N*-octylmaleimide (**26**) was the most potent time-dependent inhibitor of the cyclooxygenase and peroxidase activities of PGHS-1

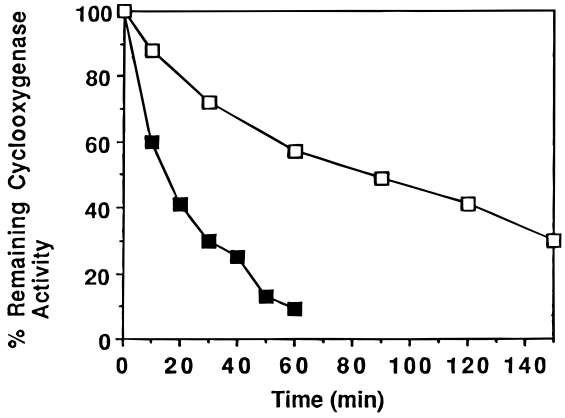


Figure 3. Time course of loss of cyclooxygenase activity of purified ovine PGHS-1 and human PGHS-2 following addition of **20**: open squares, human PGHS-2; closed squares, ovine PGHS-1.

(IC_{50} = 9 μM). The lack of PGHS inhibition by *N*-octylsuccinimide (**69**) suggests that inhibition by **26** is mediated by covalent modification of the protein.

C. *N*-(Carboxyheptyl)halomaleimide and Haloacetamidocaprylic Acid Analogs. Incubation of hematin-reconstituted PGHS-1 or PGHS-2 with the *N*-(carboxyheptyl)maleimide (**31**) analogs which included *N*-(carboxyheptyl)-3-substituted-maleimides **56** and **58**, *N*-(carboxyheptyl)-3,4-disubstituted-maleimides **57** and **59**, the open chain, haloacetamidocaprylic acid derivatives **62** and **63**, and *N*-(carboxyheptyl)pyrrole (**72**) did not result in significant time-dependent cyclooxygenase inhibition even at a high inhibitor/enzyme ratio.

D. Interaction of *N*-(Carboxyheptyl)maleimide and Related Analogs with PGHS-1. 1. Protection against Trypsin Cleavage. Trypsin cleaves the 70 kDa apoPGHS-1 protein at Arg^{277} into two fragments of 33 and 38 kDa.[21] A variety of PGHS inhibitors which include compounds categorized as slow, tight-binding inhibitors (e.g., SQ 29,535[22]), compounds which covalently modify the PGHS protein (e.g., acylindomethacin analogs[23]), and reversible inhibitors (e.g., ibuprofen and mefenamic acid[24]) are capable of protecting against PGHS cleavage by trypsin. Protection of the apoprotein from trypsin cleavage by these inhibitors is due to their ability to bind to the apoprotein in the cyclooxygenase active site channel and induce a long-range conformational change that renders a flexible loop near the entrance to the peroxidase channel resistant to tryptic cleavage. Reconstitution of apoPGHS with the heme prosthetic group also induces resistance of the protein to trypsin cleavage.[25] In order to assess whether *N*-(carboxyheptyl)maleimide (**31**) and related analogs **56**−**59** and **72** prevent trypsin cleavage at Arg^{277}, apoenzyme rapidly inactivated with 2 equiv of the inhibitor of interest was compared to untreated apoenzyme for its sensitivity to trypsin. The trypsin protection assay was modified to increase the rate of tryptic cleavage in the absence of inhibitor.[24] The rapid cleavage of apoPGHS that occurred under these conditions enabled us to detect protection by PGHS inhibitors at times as short as 5 s after their addition. In a typical experiment, apoPGHS (2 μM) and inhibitor (4 μM) were incubated for 5 s. Trypsin (3.1 μM) was added, and aliquots were removed at 5, 100, and 300 s. The reaction mixtures were analyzed by polyacrylamide gel electrophoresis, and the percentage of each band was

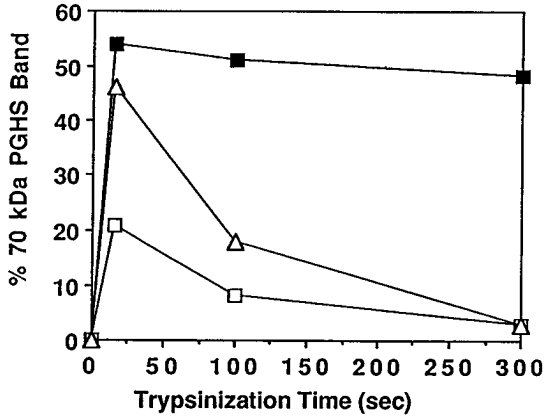


Figure 4. Reversibility of trypsin protection by *N*-(carboxyheptyl)maleimide (**31**), *N*-(carboxyheptyl)succinimide (**70**), and *N*-(carboxyheptyl)pyrrole (**72**): closed squares, **31**; open triangles, **72**; open squares, **70**.

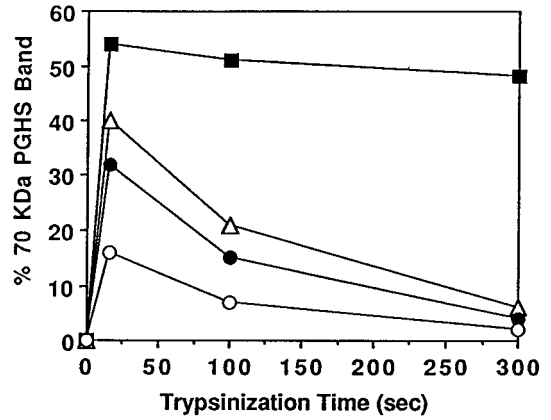


Figure 5. Reversibility of trypsin protection by *N*-(carboxyheptyl)maleimide and related analogs **56**−**59**: closed squares, **31**; open triangles, **57**; closed circles, **56**; open circles, **58**.

measured by densitometry. Approximately 70−80% cleavage occurred in the absence of an inhibitor which was sufficient for assessing protective effects of inhibitors. Figure 4 depicts the graphical representation of a gel on which 2 equiv of *N*-(carboxyheptyl)maleimide (**31**), *N*-(carboxyheptyl)succinimide (**70**), and *N*-(carboxyheptyl)pyrrole (**72**) incubated with the apoPGHS-1 protein for 5 s followed by addition of trypsin. Aliquots were removed at 5, 100, and 300 s and analyzed for cleavage at Arg^{277}. Approximately 55% of the 70 kDa protein band was protected from trypsin cleavage following a 5 s incubation of the apoenzyme with **31**, whereas incubation of the apoenzyme for 5 s with **70** and **72** led to 20% and 45% trypsin protection, respectively. Furthermore, the extent of trypsin protection of PGHS provided by **31** remained constant during 300 s of trypsin treatment, whereas that by **70** and **72** decreased by 97% (see Figure 4).

A similar result was obtained when apoenzyme was treated with 2 equiv of the mono- and disubstituted carboxyheptylmaleimide analogs **56**−**59**. After a 5 s preincubation with the protein, the *N*-(carboxyheptyl)-3,4-dichloromaleimide derivative **57** displayed 40% protection, whereas the *N*-(carboxyheptyl)-3-bromomaleimide (**56**) and the *N*-(carboxyheptyl)-3-phenylmaleimide (**58**) analogs demonstrated 33% and 15% trypsin protection, respectively (Figure 5). Incubation of the apoenzyme with *N*-(carboxyheptyl)-3,4-diphenylmaleimide analog **59** did not lead to any trypsin protection. Furthermore the extent of trypsin protection offered by these compounds decreased upon increments in the length of trypsinization time (see Figure 5).

2. Reaction of Holo- and ApoPGHS-1 with *N*-(Carboxyheptyl)[3,4-^{14}C]maleimide ([3,4-^{14}C]-31). Synthesis of [3,4-^{14}C]-31. In order to assess whether the inhibition of PGHS-1 by **31** was a result of covalent modification of the protein and to identify the residue(s) modified, *N*-(carboxyheptyl)[3,4-^{14}C]maleimide ([3,4-^{14}C]-**31**) was prepared. The synthesis of [3,4-^{14}C]-**31** was achieved in a fashion identical to that described for **31**. Briefly, [3,4-^{14}C]-**1** (50 μCi, 1.2 mCi mmol^{-1}) was reacted with **55** in glacial acetic acid to generate the intermediate maleamic acid [3,4-^{14}C]-**14**. The ^{1}H NMR (DMSO-d_6) of this intermediate was identical with the NMR spectrum of **14**. The maleamic acid was then cyclized to the corresponding maleimide [3,4-^{14}C]-**31** by heating at 90 °C in the presence of acetic anhydride and sodium acetate. The crude product was purified by column chromatography in 30% yield and its purity was estimated by ^{1}H NMR (see the Experimental Section).

Detection of Covalent Modification of PGHS-1 by SDS Page. Hematin-reconstituted PGHS-1 was incubated at room temperature for 5 min with 2, 5, and 50 equiv of [3,4-^{14}C]-**31**, and the samples were run on a nondenaturing 5% polyacrylamide gel with or without 1% SDS treatment. The gel was dried, exposed for 6 days, and scanned on a phosphorimager for detection of radioactive protein bands. Incubation of holoenzyme with concentrations of [3,4-^{14}C]-**31** which led to rapid cyclooxygenase inhibition and protection of the apoenzyme from trypsin cleavage did not lead to incorporation of radioactivity in the holoPGHS-1 protein. Although rapid inhibition of holoPGHS-1 with a 50-fold excess of [3,4-^{14}C]-**31** did result in the formation of a radioactive 70 kDa protein band, this incorporation was likely due to modification of an amino acid residue(s), different from the one that leads to rapid cyclooxygenase inhibition at stoichiometric concentrations of **31**.

Since *N*-(carboxyheptyl)maleimide (**31**) was shown to bind directly to apoPGHS-1 and prevent subsequent trypsin cleavage of the apoenzyme, the interaction of apoPGHS-1 with [3,4-^{14}C]-**31** was also examined. ApoPGHS-1 was incubated with 2, 4, and 10 equivalents of [3,4-^{14}C]-**31**, and the samples were run on a native gel with or without 1% SDS treatment. Upon analysis of the exposed plates on the phosphorimager, all of the inhibitor concentrations utilized led to the incorporation of radioactivity in the apoprotein (Figure 6). The amount of radioactivity that was incorporated in the SDS-treated samples was greater than that incorporated in the non-SDS- treated samples.

Attempts To Monitor the Formation of Modified ApoPGHS-1 by HPLC. Since incubation of apoPGHS-1 with [3,4-^{14}C]-**31** led to the detection of radiolabeled apoprotein by SDS PAGE, attempts were made to detect the radiolabeled apoenzyme by reverse phase HPLC for the purposes of identifying the radiolabeled amino acid residue by peptide mapping. ApoPGHS-1 was incubated at room temperature for 1 min with 2 or 4 equiv of [3,4-^{14}C]-**31** and then injected on a Vydac C4 column eluted with a water−acetonitrile gradient containing 0.1% TFA. Under these conditions, untreated apoenzyme eluted at 21 min. No radioactivity coeluted

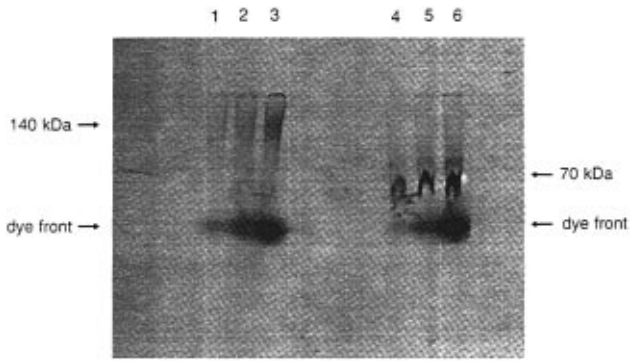


Figure 6. SDS PAGE of [3,4-^{14}C]-**31**-treated apoPGHS-1. Top panel, SDS-treated: lane 1, apoPGHS-1 + 2 equiv of **31**, lane 2, apoPGHS-1 + 4 equiv of **31**; lane 3, apoPGHS-1 + 10 equiv of **31**. Bottom panel, no SDS treatment: lane 1, apoPGHS-1 + 2 equiv of **31**; lane 2, apoPGHS-1 + 4 equiv of **31**; lane 3, apoPGHS-1 + 10 equiv of **31**.

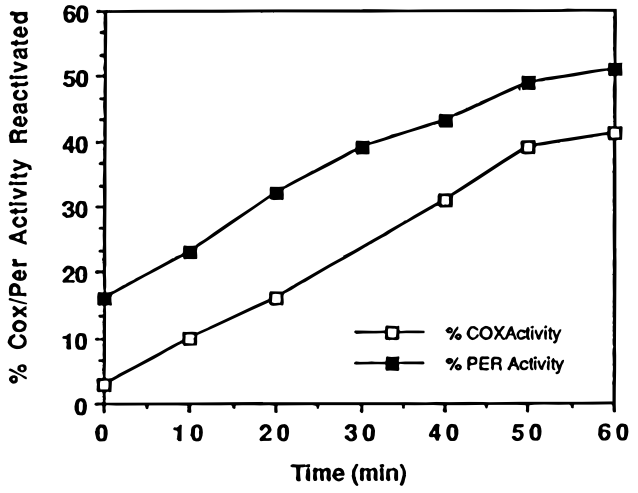


Figure 7. Spontaneous recovery of the cyclooxygenase and peroxidase activity of **31**-treated holoPGHS-1.

with the protein peak when [3,4-^{14}C]-**31**-treated apoPGHS-1 was injected on the column, suggesting that if the apoenzyme was indeed covalently modified by **31**, then the enyme/inhibitor adduct was unstable to the conditions of chromatography.

3. Reversibility of Inhibition of 31-Treated ApoPGHS-1 in the Presence of Hematin. The possibility that the lack of incorporation of radioactivity in the holoPGHS-1 upon reaction with [3,4-^{14}C]-**31** was the result of an unstable enzyme/inhibitor adduct was also investigated. The cyclooxygenase and the peroxidase activities of holoPGHS-1 were rapidly inhibited by 2 and 8 equiv, respectively, of **31** in 100 mM Tris buffer (pH 8), and the inhibited holoPGHS-1 was allowed to stand at room temperature. Aliquots of this reaction mixture were periodically analyzed for cyclooxygenase and peroxidase activity. Under these conditions, a spontaneous regeneration of 45% cyclooxygenase and 55% peroxidase activity of the inhibited holoenzyme was observed (Figure 7).

The recovery of cyclooxygenase and peroxidase activity was also examined using **31**-inactivated apoPGHS-1 protein. As shown in Figure 8, incubation of **31**-inactivated apoprotein in 100 mM Tris buffer (pH 8) did not lead to the spontaneous recovery of either enzyme activity. In fact, a slight time-dependent decrease in the cyclooxygenase and peroxidase activity was observed. Overall, these results suggested that the presence of the heme prosthetic group in the holoPGHS-1 protein renders the enzyme/inhibitor adduct unstable. In order to test the possibility that the presence of the heme prosthetic group is responsible for the dissociation of the enzyme/inhibitor adduct, an incubation mixture of **31**-inactivated apoenzyme in 100 mM Tris buffer (pH 8) was analyzed for spontaneous regeneration of cyclooxygenase activity following the addition of hematin. The results of the experiment are summarized in Figure 8. In the absence of hematin, apoenzyme inactivated by **31** did not display any recovery of its cyclooxygenase activity. However, addition of 2 equiv of hematin to this reaction mixture led to the spontaneous recovery of the cyclooxygenase activity. Approximately 50% recovery of cyclooxygenase activity was observed after 60 min. Furthermore, the extent of regenerated cyclooxygenase activity in the apoenzyme was comparable to the cyclooxygenase recovery in the holoprotein which had been inhibited by **31**. These results indicate that addition of the heme prosthetic group to the **31**-inhibited apoenzyme destabilizes the enzyme/*N*-(carboxyheptyl)maleimide adduct.

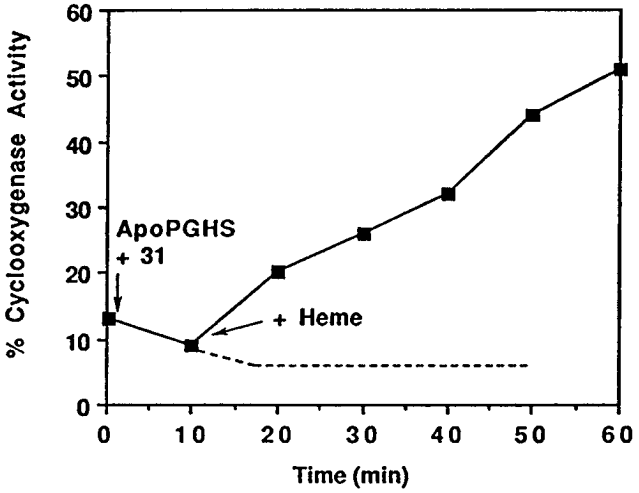


Figure 8. Spontaneous recovery of the cyclooxygenase activity of **31**-treated apoPGHS-1 following addition of hematin.

Discussion

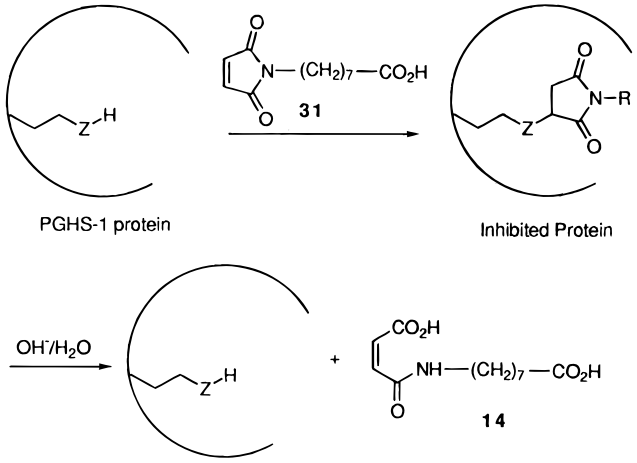
Reaction of holoPGHS-1 with *N*-substituted maleimide analogs results in a complex series of events which lead to the loss of cyclooxygenase and peroxidase activities. Coadministration of arachidonic acid and *N*-(carboxyalkyl)maleimides **29**−**35** results in a rapid dose-dependent loss of cyclooxygenase activity. The rapid loss of cyclooxygenase activity is a feature displayed only by the *N*-(carboxyalkyl)maleimides and not by the *N*-alkylmaleimides or the *N*-arylmaleimides. This nearly instantaneous inactivation occurs concomitant with a reaction(s) leading to slower time-dependent inactivation. The slower time-dependent inactivation has been observed previously[12] with NEM and DACM and was observed in the present study with the aspirin-like *N*-arylmaleimide analogs **19**−**25** and the long-chain *N*-alkylmaleimide derivatives **26**−**28** which lack the carboxylate substituent. Saturation of the maleimide double bond affords the corresponding succinimide derivative with no inhibitory potency as exemplified by *N*-5-succinimido-2-acetyl-1-benzoic acid (**68**), *N*-octylsuccinimide (**69**), and *N*-(carboxyheptyl)succinimide (**70**).

These results suggest that rapid as well as slow inactivation results from covalent modification of the holoprotein.

Reaction of holoPGHS-1 with a stoichiometric concentration (0.1 μM) of *N*-(carboxyheptyl)maleimide (**31**) led to rapid inactivation of the cyclooxygenase activity but not peroxidase activity. A similar behavior is displayed by aspirin, which covalently modifies PGHS, and by several other NSAIDs, such as *S*-flurbiprofen, which do not covalently modify the protein.[7] All of these agents are thought to inhibit the cyclooxygenase activity by binding in the fatty acid substrate channel. Raising the concentration of **31** also led to rapid inactivation of the peroxidase activity ($IC_{50} \sim 3$ μM). Rapid cyclooxygenase inactivation was a feature displayed only by *N*-(carboxyalkyl)maleimides. *N*-Alkylmaleimides such as *N*-octylmaleimide (**26**) which lack the carboxylate moiety displayed only the slow time-dependent inactivation event, suggesting an important role for the carboxylate moiety in the rapid inhibition of the cyclooxygenase activity. The lack of cyclooxygenase or peroxidase inhibition by *N*-(carboxyheptyl)succinimide (**70**) suggested that the rapid inactivation of holoPGHS-1 by **31** resulted from the covalent modification of the holoprotein.

Stoichiometric concentrations of **31** also led to the protection of apoPGHS-1 from trypsin cleavage. Furthermore, trypsin resistance in the apoenzyme following treatment with **31** was irreversible, suggesting that the apoprotein/inhibitor adduct was stable. The ability of **31** to protect apoPGHS from cleavage by trypsin supports the hypothesis that it binds in the fatty acid substrate access channel. Subsequent studies on the cyclooxygenase inhibition of apoPGHS-1 with stoichiometric concentrations of **31** also led to rapid inactivation of the cyclooxygenase activity. Furthermore, treatment of apoprotein with a stochiometric concentration of [3,4-^{14}C]-**31** revealed the incorporation of radioactivity in the 70 kDa apoprotein band when analyzed by SDS PAGE. However, when the hematin-reconstituted apoprotein was treated with [3,4-^{14}C]-**31**, no radioactive protein bands were detected. These results suggested that the protein/inhibitor adduct was unstable in the presence of the heme prosthetic group. The instability of the apoenzyme/inhibitor adduct in the presence of heme was further examined in the **31**-inactivated apoenzyme, which was treated with hematin. Following addition of 1 equi of hematin, a spontaneous regeneration of the cyclooxygenase activity was discernible. Instability of the covalent enzyme/inhibitor linkage was also detected in **31**-inhibited holoPGHS-1 as periodic analysis of the cyclooxygenase and the peroxidase activities of the holoprotein resulted in the regeneration of both the cyclooxygenase and the peroxidase activities. Compounds **56**−**58** were synthesized in an attempt to produce inhibitors that would yield a stable linkage to the protein. Unfortunately, none of the compounds inhibited cyclooxygenase activity. Two explanations can be put forth to account for the instability of the covalent adduct in the holoenzyme relative to the apoprotein: (1) Reconstitution of the apoenzyme following addition of heme results in a conformational change in the protein. This change in conformation juxtaposes electron rich amino acid residues with the covalent enzyme/**31** adduct, thus resulting in a hydrolytic cleavage to generate

Scheme 10



active enzyme and the inactive form of **31**, i.e., the maleamic acid **14**. This proposal is supported by previously reported results that addition of nucleophiles (e.g., amino acids) across the maleimide ring destabilizes the 5-membered ring resulting in a hydrolytic cleavage above neutral pH which leads to the generation of the corresponding maleamic acid (Scheme 10).[26] (2) A second possibility could be a direct involvement of the heme prosthetic group in destabilizing the covalent linkage between **31** and PGHS, if **31** modifies a amino acid residue in proximity of the heme binding site.

Overall, these results suggest that *N*-(carboxyheptyl)-maleimide (**31**) binds in the fatty acid substrate access channel and modifies an amino acid residue other than cysteine(s). The latter conclusion is supported by previous studies in our laboratory that established that the maleimides NEM and DACM form adducts with the cysteine residues of PGHS that are stable to the proteolytic cleavage and chromatography steps of peptide mapping. Furthermore, incubation of **31** with each of the three possible cysteine-to-serine site-directed mutants of PGHS leads to inhibition of cyclooxygenase activity with dose-responses similar to those of native enzyme.[27] Finally, solution of an *S*-flurbiprofen/PGHS cocrystal revealed that none of the three free cysteines (Cys^{313}, Cys^{512}, and Cys^{540}) resides in the fatty acid binding site.[4]

The solution of a *S*-flurbiprofen/PGHS-1 cocrystal by Garavito and co-workers[4] provides insight to the location of the substrate access channel and the active site. The cyclooxygenase active site is found in the interior of the protein, at the apex of a hydrophobic channel that extends inward from the lipid bilayer. At the apex of the proposed cyclooxygenase channel is Tyr^{385}, the proposed cyclooxygenase oxidant.[1] Tyr^{385} is positioned directly in front of the heme prosthetic group and separates the channel from the heme edge. Furthermore, a second tyrosine residue, Tyr^{348}, appears to be hydrogen bonded to Tyr^{385}, and both residues have some extent of solvent exposure. The carboxylate of *S*-flurbiprofen is ion-paired to Arg^{120} which is the only positively-charged residue in the cyclooxygenase substrate binding channel. The importance of the carboxylate in the rapid inactivation of PGHS-1 by **31** suggests that it is associating with Arg^{120}. The inhibitory potency of *N*-(carboxyalkyl)maleimides is exquisitely sensitive to the length of the alkyl chain, implying a highly specific orientation in the active site channel. Figure 9

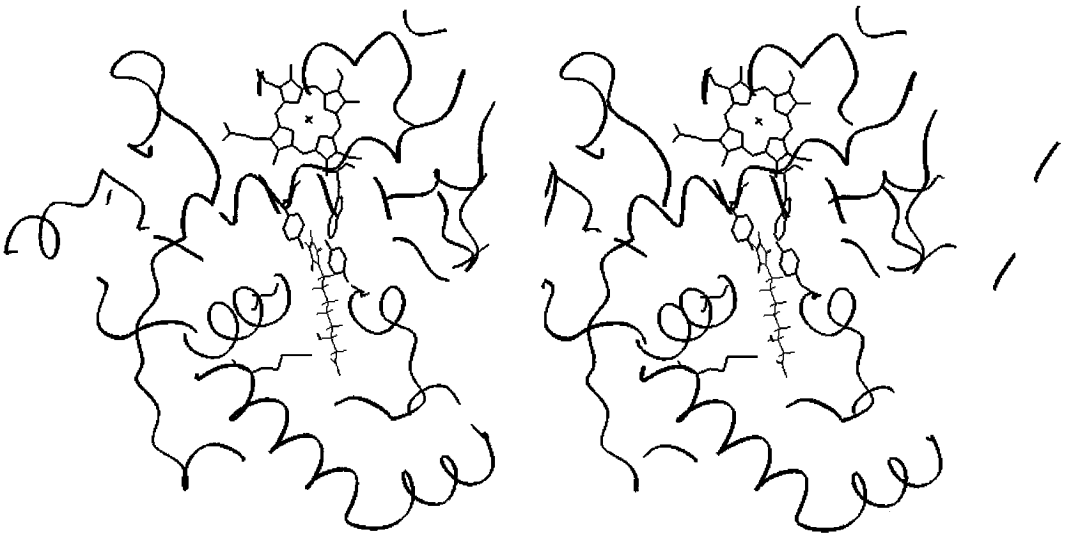


Figure 9. Cyclooxygenase active site of PGHS with *N*-(carboxyheptyl)maleimide (**31**). *N*-(Carboxyheptyl)maleimide (**31**) is shown modeled after *S*-flurbiprofen in the active site. The carboxylate of **31** was coordinated to the positively charged Arg^{120} side chain, and the maleimide ring was extended toward the tyrosines and the tryptophan residue. The least energy conformation indicates that the maleimide was stacked alongside the tyrosine and the tryptophan rings in the cyclooxygenase active site.

displays a model for the interaction of **31** with PGHS. The carboxylate of **31** was positioned next to Arg^{120}, and the maleimide ring was extended toward the tyrosines at the apex of the channel. The complex was subjected to energy minimization. The lowest energy conformation of **31** bound to Arg^{120} indicated that the maleimide ring was stacked alongside the aromatic side chains (Tyr^{348}, Tyr^{385}, and Trp^{387}) at the apex of the cyclooxygenase channel (Figure 9). The inhibitor-docking study predicts that initial noncovalent binding of **31** in the cyclooxygenase active site could occur between the carboxylate of **31** and the positively charged Arg^{120} such that the maleimide ring is extended upward toward the apex of the channel, in a manner analogous to that proposed for the fatty acid substrate, arachidonic acid. Following binding, **31** must react with an amino acid residue at the apex to form a covalent adduct. It is tempting to speculate that the target for covalent modification is a tyrosine residue.

Experimental Section

Chemistry. Melting points were determined using a Gallenkamp melting point apparatus and are uncorrected. Unless stated otherwise, starting materials were obtained from Aldrich Chemical Co., Milwaukee, WI, and Lancaster Synthesis Inc., Windham, NH, and were used without further purification. Solvents were obtained from Fisher Scientific, Pittsburgh, PA, and were HPLC grade. All other chemicals were reagent grade or better.

^{1}H NMR spectra were recorded on a Bruker WP-360 or WP-200 instrument; chemical shifts are expressed in parts per million relative to internal tetramethylsilane (TMS), and spin multiplicities are given as s (singlet), bs (broad singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Electron impact mass spectra (EI-MS) were recorded on a Delsi Nermag R10−10 C instrument. Fast atom bombardment mass spectra (FAB-MS) were recorded on a VG70HF mass spectrometer. Column chromatography was performed using silica gel (60−100 mesh) from Fisher. Thin layer chromatography (TLC) was performed on silica gel GF 254 (Analtech). Microanalyses, performed by Atlantic Microlab, Inc., Norcross, GA, were within 0.4% of the theoretical values calculated for C, H, and N.

General Procedure for the Synthesis of *N*-Substituted Maleamic Acids and Succinamic Acids. A reaction mixture containing either maleic anhydride (**1**, 1 g, 10.19 mmol) or succinic anhydride (**64**, 1g, 10 mmol) and the appropriate amine (10.19 mmol) in 20 mL of glacial acetic acid was stirred overnight. The solid which precipitated out of the reaction mixture was filtered, washed thoroughly with water and recrystallized from 2-propanol/water. Under these conditions the corresponding succinamic acid derivatives did not precipitate out of the reaction, and hence the solvent was evaporated and the residue was crystallized from 2-propanol/water to afford the desired product.

General Procedure for the Synthesis of *N*-Substituted Maleimides and Succinimides. A reaction mixture containing the appropriate maleamic acid (5.23 mmol) or succinamic acid in 5 mL of acetic anhydride and sodium acetate (3 mmol) was heated at 90 °C for 2 h. The reaction was cooled and quenched with water. The aqueous solution was extracted with diethyl ether (3 × 40 mL). The combined organic extracts were dried (Na_2SO_4) and filtered, and the solvent was evaporated. The residue was chromatographed on silica gel with EtOAc/hexane. The fractions containing the pure product were combined and concentrated to afford essentially pure maleimide. The product was further recrystallized from 2-propanol/water. In the case of the maleimide analogs **21**, **22**, and **24**, purification on silica gel was not required as the compounds crystallized out from EtOH.

2,3-Dihydro-1,4-benzoxazepin-5(4*H*)-one (37). To a reaction mixture containing 4-chromanone (**36**, 2 g, 13.4 mmol) in 15 mL of concentrated H_2SO_4 at 0 °C was added sodium azide (1.131 g, 17.4 mmol), and the reaction mixture was stirred initially at 0 °C for 1 h and then stirred at room temperature for 2 h. The reaction was quenched by the slow addition of water and then subsequently basified with 10% NaOH. The aqueous solution was extracted with Et_2O (2 × 150 mL). The combined ether layers were dried (Na_2SO_4) and filtered. The solvent was removed in vacuo, and the crude product was recrystallized from EtOAc/hexane to afford 1.3 g (61%) of **37** as white needles: mp 118−120 °C (lit.[18] mp 118−120 °C); ^{1}H NMR ($CDCl_3$) δ 8.0 (d, 1H ArH), 7.6−7.7 (bs, 1H, NH), 7.4 (t, 1H, ArH), 7.2 (t, 1H, ArH), 7.0 (d, 1H, ArH), 4.4 (t, 2H, CH_2), 3.5 (t, 2H, CH_2); EIMS M^+ 163 (98), 134 (99), 105 (100).

(Aminoethoxy)benzoic Acid Hydrochloride (38·HCl). A solution of 2,3-dihydro-1,4-benzoxazepin-5(4*H*)-one (**37**, 2.5 g, 15.3 mmol) in 75 mL of 6 N HCl was heated in an oil bath at 110 °C for 24 h. The reaction mixture was diluted with water, and the aqueous solution was washed with diethyl ether (2 × 50 mL). The aqueous solution was evaporated in vacuo, and the residue was recrystallized from EtOH/Et_2O to afford 2.89 g (87%) of **38·HCl** as a white crystalline solid: mp 182−183 °C; ^{1}H NMR (DMSO-d_6) δ 11.9 (bs, 1H, COOH), 8.2−8.3

(bs, 2H NH_2), 7.6 (d, 1H, ArH), 7.5 (t, 1H, ArH), 7.2 (t, 1H, ArH), 7.0 (d, 1H, ArH), 4.3 (t, 2H, CH_2), 3.1 (t, 2H, CH_2); EIMS M^+ 182 (40), 164 (32), 133 (100). Anal. ($C_9H_{12}ClNO_3$) C, H, N.

Maleamic Acid (2). An aqueous solution of the (aminoethoxy)benzoic acid hydrochloride salt (**38**·HCl, 0.72 g, 3.3 mmol) in 10 mL of water was neutralized with aqueous potassium carbonate. The aqueous solution was evaporated under pressure, and the resultant solid in 20 mL of glacial acetic acid was treated with maleic anhydride (**1**, 0.32 g, 3.3 mmol). The reaction mixture was stirred overnight, and the precipitated solid was filtered, washed with water, and dried. The crude product was recrystallized from EtOH/H_2O to afford **2** in 64% yield: mp 161−162 °C; ^{1}H NMR (DMSO-d_6) δ 9.3 (bs, 1H, NH), 7.4−7.7 (m, 2H, ArH), 7.0−7.3 (m, 2H, ArH), 6.4 (d, 1H, olefinic H), 6.2 (d, 1H, olefinic H), 4.2 (t, 2H, CH_2), 3.6 (t, 2H, CH_2); FABMS MH^+ 280 (70), 262 (60), 164 (98), 133 (100). Anal. ($C_{13}H_{12}NO_6$) C, H, N.

2-[2-(*N*-Maleimido)ethoxy]benzoic Acid (19). A reaction mixture containing **2** (730 mg, 2.6 mmol) in 8 mL of acetic anhydride and sodium acetate (132 mg, 1.61 mmol) was heated at 90 °C for 2 h. The reaction mixture was cooled and diluted with water. The aqueous solution was extracted with Et_2O (3 × 25 mL). The combined organic solution was washed with brine and then with water. The organic solution was dried ($MgSO_4$) and filtered, and the solvent was evaporated to afford a yellow oil. The yellow oil was chromatographed on silica gel and eluted with EtOAc/hexanes to afford essentially pure **19** as a white solid which was further recrystallized from ethanol (0.21 g, 31%): mp 145−147 °C; ^{1}H NMR ($CDCl_3$) δ 8.2 (d, 1H, C_6-H, ArH), 7.5−7.6 (t, 1H, C_5-H, ArH), 7.2−7.3 (t, 1H, C_4-H, ArH), 7.0 (d, 1H, C_2-H, ArH), 6.7 (s, 2H, olefinic H), 4.4 (t, 2H, CH_2), 4.2 (t, 2H, CH_2); FABMS MH^+ 262 (26), 244 (84), 124 (99), 79 (100). Anal. ($C_{13}H_{11}NO_5$) C, H, N.

Sebacic Acid Chloride Monomethyl Ester (43). A reaction mixture containing sebacic acid monomethyl ester (**41**, 0.5 g, 2.31 mmol) and thionyl chloride (0.55 g, 4.62 mmol) was warmed to 50 °C and allowed to stir at that temperature for 3 h. The excess thionyl chloride was removed under reduced pressure to afford essentially pure **43** as a colorless oil (0.54 g, 98%): ^{1}H NMR ($CDCl_3$) δ 3.64 (s, 3H, OCH_3), 2.84 (t, 2H, CH_2 adjacent to COCl), 2.28 (t, 2H, methylene adjacent to $COOCH_3$), 1.28−1.7 (complex multiplet, 12 H, methylenes).

Undecanedioic acid chloride monomethyl ester (44) was obtained in 96% yield: ^{1}H NMR ($CDCl_3$) δ 3.7 (s, 3H, OCH_3), 2.81 (t, 2H, methylene adjacent to COCl), 2.3 (t, 2H, methylene adjacent to $COOCH_3$), 1.24−1.8 (complex multiplet, 14 H, methylenes).

9-Isocyano-1-methylnonanoate (45). To a solution of the above acid chloride (**43**, 0.54 g, 2.31 mmol) in 10 mL of dry xylene was added sodium azide (0.15 g, 2.34 mmol) and this reaction mixture was heated under reflux for 2 h. The insoluble residue was filtered off and the solvent was removed under reduced pressure to afford **45** as a semisolid material (0.47g, 97%): ^{1}H NMR ($CDCl_3$) δ 3.54 (s, 3H, OCH_3), 3.26 (t, 2H, methylene adjacent to NCO), 2.3 (t, 2H, methylene adjacent to $COOCH_3$), 1.28−1.7 (complex multiplet, 12H, methylenes).

Methyl 10-isocyanodecanoate (46) was obtained in 97% yield: ^{1}H NMR ($CDCl_3$) δ 3.6 (s, 3H, OCH_3), 3.36 (t, 2H, methylene adjacent to NCO), 2.3 (t, 2H, methylene adjacent to $COOCH_3$), 1.28−1.7 (complex multiplet, 14H, methylenes).

9-Amino-1-nonanoic Acid Hydrochloride (47·HCl). A reaction mixture containing crude **45** (0.47 g, 2.2 mmol) in 4 mL of concentrated HCl was heated under reflux for 2.5 h. The reaction mixture was cooled, and the solvent was removed under reduced pressure to afford the crude hydrochloride salt of **47** which was purified by recrystallization from CH_3OH/Et_2O to afford the desired product as a white crystalline solid in 64% yield: mp 133−134 °C; ^{1}H NMR (DMSO-d_6) δ 2.74 (t, 2H, methylene adjacent to NH_2), 2.15 (t, 2H, methylene adjacent to COOH), 1.2−1.52 (complex multiplet, 12H, methylenes). Anal. ($C_9H_{20}ClNO_2$) C, H, N.

Azacycloundecan-2-one (40). To a reaction mixture containing cyclodecanone (**39**, 1 g, 6.4 mmol) in 10 mL of concentrated H_2SO_4 at 0 °C was added sodium azide (0.54 g, 8.41 mmol), and the reaction mixture was stirred at 0 °C for 1 h and subsequently at room temperature for 2 h. The reaction mixture was diluted with cold water and treated dropwise with cold 10% NaOH until a pH of 9 was obtained. The aqueous solution was extracted with Et_2O (3 × 60 mL). The combined ether extracts were dried (Na_2SO_4) and filtered, and the solvent was removed in vacuo to afford a white solid which was recrystallized from CH_3OH/Et_2O to yield pure **40** as a crystalline white solid in 69% yield: mp 161−162 °C; ^{1}H NMR ($CDCl_3$) δ 5.6 (bs, 1H, NH), 3.3 (q, 2H, CH_2N), 2.2 (t, 2 H, methylene adjacent to CO), 1.7 (m, 2H, methylene), 1.3−1.6 (m, 12H, methylenes); EI-MS M^+ 169 (100), 125 (65), 112 (67).

10-Amino-1-decanoic Acid Hydrochloride Salt (48·HCl). A reaction mixture containing **40** (0.1 g, 0.6 mmol) in 5 mL of concentrated HCl was heated in a sealed tube at 100 °C for 4 days. The reaction mixture was cooled and diluted with water to precipitate unreacted starting material. The mixture was filtered, and the aqueous solution was evaporated in vacuo. The residue was recrystallized from CH_3OH/Et_2O to afford **48**·HCl as a white crystalline solid (30 mg, 20%): mp 157−159 °C; ^{1}H NMR (DMSO-d_6) δ 7.7 (bs, 2H, NH_2), 2.77 (q, 2H, CH_2 adjacent to NH_2), 2.2 (t, 2H, CH_2 adjacent to COOH), 1.49 (m, 4H, methylenes), 1.24 (complex multiplet, 10H, methylenes); FAB-MS 188 (MH^+ − HCl). Anal. ($C_{10}H_{22}ClNO_2$) C, H, N.

General Procedure for the Synthesis of *N*-(Carboxyheptyl)maleimide Derivatives. A reaction mixture containing the appropriate maleic anhydride (1.25 mmol) and 8-aminocaprylic acid (**55**, 1.25 mmol) in 25 mL of glacial acetic acid was refluxed for 10 h. The reaction mixture was cooled, and acetic anhydride (2 mL) was added to the reaction mixture which was refluxed for an additional 2 h. The solvent was removed under reduced pressure, and the residue was chromatographed on silica gel and eluted with 40% EtOAc/hexane to afford the desired maleimide which was recrystallized from 80% EtOH.

***N*-(Carboxyheptyl)-3-bromomaleimide (56)** was obtained in 61% yield: mp 92−94 °C; ^{1}H NMR ($CDCl_3$) δ 6.6 (s, 1H, olefinic H), 3.5 (t, 2H, NCH_2), 2.3 (t, 2H, CH_2 adjacent to COOH), 1.2−1.6 (m, 10H, methylenes); GC−EIMS (temperature program: 100−320 °C with a temperature ramp of 15 °C/min for 15 min) showed a single peak with t_R = 9.1 min; *m/z* 317 (^{79}Br, $M^{\bullet+}$) (10), 319 (^{81}Br, $M^{\bullet+}$ + 2) (10), 190 (90), 123 (89), 55 (100). Anal. ($C_{12}H_{16}BrNO_4$) C, H, N.

***N*-(Carboxyheptyl)-3,4-dichloromaleimide (57)** was obtained in 75% yield: mp 95−97 °C; ^{1}H NMR ($CDCl_3$) δ 3.6 (t, 2H, NCH_2), 2.3 (t, 2H, CH_2 adjacent to COOH), 1.24−1.63 (m, 10H, methylenes); GC−EIMS (temperature program: 100−320 °C with a temperature ramp of 15 °C/min for 15 min) showed a single peak with t_R = 9.1 min; *m/z* 307 (^{35}Cl, $M^{\bullet+}$) (20), 309 (^{37}Cl, $M^{\bullet+}$ + 2) (13), 311 ($M^{\bullet+}$ + 4), 178 (90), 123 (89), 55 (100). Anal. ($C_{12}H_{15}Cl_2NO_4$) C, H, N.

***N*-(Carboxyheptyl)-3-phenylmaleimide (58)** was obtained in 50% yield: mp 85−87 °C; ^{1}H NMR ($CDCl_3$) δ 7.9 (m, 2H, ArH), 7.4 (m, 3H, ArH), 6.7 (s, 1H, olefinic H), 3.55 (t, 2H, NCH_2), 2.3 (t, 2H, CH_2 adjacent to COOH), 1.24−1.66 (m, 10H, methylenes); GC−EIMS (temperature program: 100−320 °C with a temperature ramp of 15 °C/min for 15 min) showed a single peak with t_R = 7.6 min; *m/z* 297 (M − 18) (25), 186 (100), 103 (85), 91 (60), 55 (60). Anal. ($C_{18}H_{21}NO_4$) C, H, N.

***N*-(Carboxyheptyl)-3,4-diphenylmaleimide (59)** was obtained in 32% yield: mp 110−111 °C; ^{1}H NMR ($CDCl_3$) δ 7.46 (m, 4H, ArH), 7.32 (m, 6 H, ArH), 3.62 (t, 2H, NCH_2), 2.33 (t, 2H, CH_2 adjacent to COOH), 1.24−1.66 (m, 10H, methylenes); FAB-MS 392 (MH^+). Anal. ($C_{24}H_{25}NO_4$) C, H, N.

General Procedure for the Synthesis of Haloacetamidocaprylic Acid Analogs. A reaction mixture containing 8-aminocaprylic acid (**55**, 1.9 mmol) in 2 mL of 1 M NaOH at 0 °C was treated with the appropriate haloacetyl halide (2 mmol) dropwise, and the reaction mixture was stirred under nitrogen overnight. The reaction mixture was diluted with water and acidified with 1 N HCl. The aqueous solution was extracted with Et_2O (3 × 20 mL), the combined organic solution was washed with water, dried (Na_2SO_4), and filtered, and the solvent was removed in vacuo. The resultant oil was

chromatographed on silica gel and eluted with 40% EtOAc/hexane to afford the desired product.

8-(Chloroacetamido)caprylic acid (62) was obtained in 71% yield: mp 73−75 °C; ^{1}H NMR ($CDCl_3$) δ 6.6 (bs, 1H, NH), 4.0 (s, 2H, $ClCH_2$), 3.3 (q, 2 H, NCH_2), 2.3 (t, 2H, methylene adjacent to COOH), 1.33−1.65 (m, 10H, methylenes). Anal. ($C_{10}H_{18}ClNO_3$) C, H, N.

8-(Bromoacetamido)caprylic acid (63) was obtained in 40% yield: mp 72−74 °C; ^{1}H NMR ($CDCl_3$) δ 6.25 (bs, 1H, NH), 3.4 (s, 2H, $BrCH_2$), 3.3 (q, 2H, NCH_2), 2.3 (t, 2H, methylene adjacent to COOH), 1.33−1.66 (m, 10H, methylenes); Anal. ($C_{10}H_{18}BrNO_3$) C, H, N.

***N*-(Carboxyheptyl)pyrrole (72).** A reaction mixture containing 2,5-dimethoxytetrahydrofuran (**71**, 0.5 g, 4 mmol) and 8-aminocaprylic acid (**55**, 0.6 g, 4 mmol) in 25 mL of glacial acetic acid was heated under reflux for 3 h during which time the reaction mixture turned black. The solvent was removed under reduced pressure, and the residue was dissolved in 100 mL of brine. The aqueous solution was extracted with CH_2Cl_2 (3 × 50 mL). The combined organic solution was washed with water, dried (Na_2SO_4), and filtered, and the solvent was removed under reduced pressure to afford a oil. The oil was chromatographed on silica gel and eluted with EtOAc/hexane (40:60) to afford pure **72** as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 6.6 (bs, 2H, pyrrole-CHN), 6.1 (bs, 2H, pyrrole CH β to N), 3.8 (t, 2H, NCH_2), 2.3 (t, 2H, CH_2 adjacent to COOH), 1.73 (m, 2H, methylene), 1.6 (m, 2H, methylene), 1.2−1.3 (m, 6H, methylenes); FAB-MS MH^+ 210 (100), 192 (20), 80 (25).

***N*-(Carboxyheptyl)[3,4-^{14}C]maleamic Acid ([3,4-^{14}C]-14).** A reaction vial containing [3,4-^{14}C]-**1** (3 mg, 40 μmol, 1.2 mCi mmol^{-1}) in 100 μL of glacial acetic acid was treated with **55** (5.9 mg, 40 μmol), and this reaction mixture was stirred at ambidient temperature overnight, during which time the maleamic acid derivative precipitated out of the reaction mixture. The solvent was removed under reduced pressure to afford [3,4-^{14}C]-**14** as a white solid. ^{1}H NMR (DMSO-d_6) analysis of the crude white solid revealed quantitative conversion to the intermediate maleamic acid.

***N*-(Carboxyheptyl)[3,4-^{14}C]maleimide ([3,4-^{14}C]-31).** To the crude maleamic acid [3,4-^{14}C]-**14** were added NaOAc (2 mg, 25 μmol) and acetic anhydride (400 μL), and this reaction mixture was heated at 90 °C for 2 h. Pure [3,4-^{14}C]-**31** (specific activity = 1.2 mCi/mmol; 30% by liquid scintillation measurement) was obtained following a workup identical to the one described in the general procedure for the synthesis of *N*-substituted maleimides.

Enzymology Studies. Sheep seminal vesicles were purchased from Oxford Biomedical Research, Inc. (Oxford, MI). Arachidonic acid was purchased from Nu Chek Prep (Elysian, MN). Hematin, hydrogen peroxide, and guaiacol were purchased from Sigma Chemical Co. (St. Louis, MO). PGHS-1 was purified from sheep seminal vesicles as described earlier.[29] The specific activity of the protein was 20.9 (μMO_2/min)/mg, and the percentage of holoenzyme was 13.5%. ApoPGHS-1 was prepared as described earlier.[30] Apoenzyme was reconstituted by the addition of hematin to the assay mixtures. Human PGHS-2 was a generous gift from J. Gierse, Monsanto (St. Louis, MO). The cyclooxygenase activity of the human PGHS-2 was ∼18−20 (μmol AA/min)/mg.

Cyclooxygenase Activity. Oxygen consumption was measured at 37 °C with a Gilson model 5/6 oxygraph (Gilson Medical Electronics, Inc., Middleton, WI) equipped with a clark electrode and a thermostated cuvette. Enzyme aliquots (20 μg) were added to 100 mM Tris-HCl at pH 8 containing 500 μM phenol and 1 μM hematin in a final volume of 1.3 mL. Oxygen uptake was initiated by the addition of 100 μM sodium arachidonate, and the initial reaction velocity was determined from the linear portion of the oxygen uptake curve.

Peroxidase Activity. Assays were performed at 25 °C on a Shimadzu UV 160U by measuring the initial rates of oxidation of guaiacol at 436 nm. Enzyme aliquots were added to 100 mM Tris-HCl (pH 8) containing 1 μM heme and 500 μM guaiacol in 1 mL disposible cuvettes. Reaction was initiated by the addition of 400 μM hydrogen peroxide.

Time-Dependent Inactivation of PGHS-1 and PGHS-2 by *N*-(Substituted) Maleimides. ApoPGHS-1 (5 μM) or apoPGHS-2 (5 μM) in 100 mM sodium phosphate buffer pH 7.8 containing 0.1% Tween 20 was treated with 2 equiv of hematin from a 500 μM stock solution in DMSO, and this mixture was allowed to stand at 25 °C for 5 min. The inhibitor of interest in DMSO was added to this mixture, and the time course for inactivation was determined by testing 0.16 μM enzyme aliquots for cyclooxygenase and peroxidase activity.

Rapid Inactivation of PGHS-1 by *N*-Substituted Maleimides. ApoPGHS (0.25 μM) in 100 mM Tris HCl containing 500 μM phenol at pH 8 was treated with 1 μM hematin from a 500 μM stock solution in DMSO and allowed to reconstitute for 1 min followed by simultaneous addition of the inhibitor and 100 μM sodium arachidonate.

Incorporation of Radioactivity in HoloPGHS-1 and ApoPGHS-1 Following Inactivation by *N*-(Carboxyheptyl)-[3,4-^{14}C]maleimide ([3,4-^{14}C]-31). A nondenaturing 5% polyacrylamide gel was run according to the method of Davis et al.[31] Hematin-reconstituted PGHS-1 or apoPGHS-1 (4 μM) was incubated at room temperature with 2, 5, and 50 equiv of [3,4-^{14}C]-**31** for 5 min, and the samples were run on the native gel with or without 1% SDS pretreatment. The gel was dried, exposed for 6 days, and scanned on the phosphorimager for detection of radioactive 70 kDa protein bands.

Chromatography Conditions for the Modified Enzyme. Apo- or holoprotein (80 μg) treated with 2, 10, or 50 equiv of [3,4-^{14}C]-**31** was injected on a Vydac C4 column (0.46 × 25 cm) and eluted with a solvent system of A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile and a linear gradient from 40% to 60% acetonitrile in 30 min (flow rate, 1 mL/min). The HPLC system was connected to a Varian 2050 UV detector (λ = 230 nm) and to a Radiomatic Flo-one β radioactive flow detector.

Proteolytic Digestion of ApoPGHS-1 with Trypsin. ApoPGHS (2 μM) in 100 mM Tris-HCl pH 8 was treated with 2 equiv of inhibitor in DMSO for 5, 30, 60, or 300 s at 25 °C. Proteolytic digestion was initiated by the addition of 3.1 μM trypsin at 25 °C for 15 s. Trypsinization was quenched by the addition of 1.8 mM PMSF followed by incubation at 4 °C for 5 min.

Reversibility of PGHS Resistance to Trypsin Protection. ApoPGHS-1 (2 μM) in 100 mM Tris-HCl pH 8 was incubated with 2 equiv of inhibitor at 25 °C for 5 s. Trypsin (3.1 μM) was added, and aliquots were removed at 5, 30, 60, or 300 s. Trypsinization was quenched by the addition of 1.8 mM PMSF followed by incubation at 4 °C for 5 min.

Control experiments were performed to confirm that the proteolytic activity of trypsin was unaffected by incubation with inhibitors. A reaction mixture containing trypsin with inhibitor (2 μM) was added to a cuvette containing 0.5 mM *N*-α-*p*-tosyl-L-arginine methyl ester in 100 mM Tris-HCl at pH 8. The initial rate of proteolysis was monitored at 247 nm. The rates of proteolysis in the presence or absence of inhibitors were essentially the same.

Gel Electrophoresis. PGHS-1 aliquots (2 μg) were heated at 95 °C for 3 min in SDS sample buffer containing dithiothreitol and were loaded on a 10% polyacrylamide minigel. Electrophoresis was performed according to the procedure of Laemmli.[32] The 70 kDa uncleaved protein band and the 38 and 33 kDa protease-cleaved protein bands were scanned on an E-C Apparatus Model EC910 scanning densitometer and quantitated with Hoeffer GS370 Data System software.

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Supporting Information Available: ^{1}H NMR spectral data, mass spectral data, and yields of compounds **3**−**18**, **20**−**35**, and **66**−**70** (7 pages). Ordering information is given on any current masthead page.

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