In Vivo Characterization of Hydroxamic Acid Inhibitors of 5-Lipoxygenase

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The hydroxamic acid functionality can be incorporated into simple molecules to produce potent inhibitors of 5-lipoxygenase. The ability of many of these hydroxamates to inhibit leukotriene synthesis in vivo has been measured directly with a rat peritoneal anaphylaxis model. Despite their potent enzyme inhibitory activity in vitro, many orally dosed hydroxamic acids only weakly inhibited leukotriene synthesis in vivo. This discrepancy is attributable at least in part to the rapid metabolism of hydroxamates to the corresponding carboxylic acids, which are inactive against the enzyme. A study of the structural features that affect this metabolism revealed that 2-arylpropionohydroxamic acids are relatively resistant to metabolic hydrolysis. Several members of this class of hydroxamates are described that are orally active inhibitors of leukotriene synthesis.

The enzyme 5-lipoxygenase catalyzes the first step of the biochemical cascade in which arachidonic acid is converted to the leukotrienes. Numerous pathophysiological effects have been associated with the leukotrienes, and they have been implicated as important mediators in a variety of disease states including asthma, arthritis, psoriasis, and allergy.[1] As the first dedicated enzyme in the biosynthetic cascade leading to these important mediators, 5-lipoxygenase clearly represents an exciting target for therapeutic intervention.

We have previously reported that the hydroxamic acid moiety can be incorporated into a variety of simple stable molecules to produce extremely potent in vitro inhibitors of 5-lipoxygenase.[2] We described several structural features that influence inhibitory activity and demonstrated that the hydroxamic acid unit is required for the high potency observed. We now report the ability of simple hydroxamic acids to inhibit leukotriene synthesis in vivo. We also describe the absorption, metabolism, and duration characteristics of these compounds.

Results and Discussion

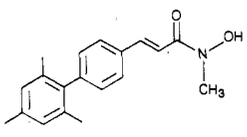
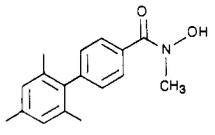
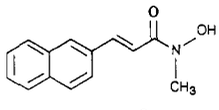
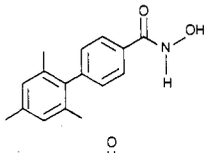
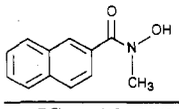
Chemistry. The hydroxamic acids described here were prepared by established procedures from the corresponding carboxylic acid. In general, the carboxylate was treated with oxalyl chloride and DMF in methylene chloride to generate an acid chloride. Without further purification, this was reacted with an appropriately substituted hydroxylamine in the presence of triethylamine to yield the desired hydroxamic acid.

Rat Peritoneal Anaphylaxis Model. Several laboratories have investigated the in vivo activity of lipoxygenase inhibitors by evaluating their effects against various pathophysiological responses (e.g., bronchoconstriction[3] and ear edema[4,5]) purported to be caused by leukotrienes. However, such studies can only provide indirect support for in vivo 5-lipoxygenase inhibitory activity since interpretation of results are complicated by the fact that compounds could be producing their effects through mechanisms other than 5-lipoxygenase inhibition. Therefore, we have chosen to evaluate in vivo 5-lipoxygenase inhibitory activity of compounds directly by measuring their ability to block leukotriene biosynthesis. This is done by using a rat peritoneal anaphylaxis model.[6-8]

Groups of rats were injected intraperitoneally (ip) with rabbit antibody to bovine serum albumin (BSA) followed 3 h later by an ip injection of BSA, which triggered the synthesis of leukotrienes in the peritoneal cavity. The rats were sacrificed 15 min after this challenge, and the peritoneal fluids were collected and processed. The amount of leukotrienes was determined routinely by radioimmunoassay and expressed as the amount of immunoreactive LTC_4 equivalents. To evaluate oral effectiveness, selected inhibitors were administered by gavage 1 h prior to antigen challenge.

Initial Studies. Table I lists five of the more potent in vitro inhibitors of 5-lipoxygenase described previously.[2]

Table I. Antihibitory Activity of Selected Hydroxamates against RBL-1 5-Lipoxygenase in Vitro and Leukotriene Biosynthesis in Vivo with the Rat Peritoneal Anaphylaxis Model

structure	no.	in vitro IC_{50}, μM[a]	in vivo % inhibn at 100 mg/kg po[b]
	1	0.022 (0.018–0.026)	NS[c]
	2	0.064 (0.054–0.074)	41 ± 8%
	3	0.10 (0.091–0.11)	66 ± 6%
	4	0.29 (0.27–0.30)	NS[c]
	5	1.3 (1.1–1.5)	NS[c]

[a] IC_{50} with 95% confidence limits in parentheses calculated by the method of Finney.[10] [b] Percent inhibition values ± SEM in the rat peritoneal anaphylaxis model. [c] NS = not significantly different from controls ($p > 0.05$). Typically, 30–40% inhibition was required for statistical significance.

(1) For a review of the biochemistry and pharmacology of the leukotrienes see: Sirois, P. *Adv. Lipid Res.* **1985**, *21*, 79.
(2) Summers, J. B.; Mazdiyasni, H.; Holms, J. H.; Ratajczyk, J. D.; Dyer, R. D.; Carter, G. W. *J. Med. Chem.* **1987**, *30*, 574.
(3) Richie, D. M.; Sierchio, J. N.; Capetola, R. J.; Rosenthale, M. E. *Agents Actions* **1981**, *11*, 4.
(4) Young, J. M.; Wagner, B. M.; Spires, D. A. *J. Invest. Dermatol.* **1983**, *80*, 48.
(5) Opas, E. E.; Bonney, R. J.; Humes, J. L. *J. Invest. Dermatol.* **1985**, *84*, 253.
(6) Young, P. R.; Dyer, R. D.; Carter, G. W. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* *44*, 1185.
(7) Rapp, H. J. *J. Physiol. (London)* **1961**, *158*, 35.
(8) Orange, R. P.; Valentine, M. D.; Austen, K. F. *Science (Washington, D. C.)* **1967**, *157*, 318.

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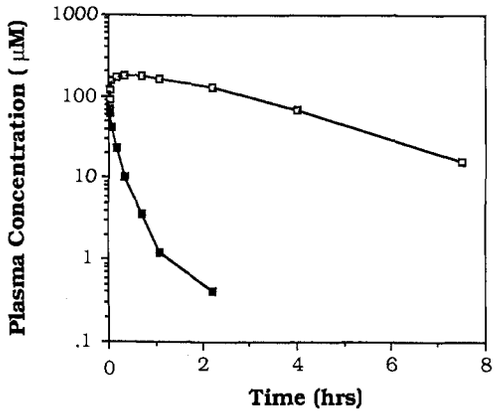


Figure 1. Rat plasma concentrations of **3** (■) and its carboxylic acid metabolite (□) following an iv dose of **3** at 20 mg/kg.

Table I also describes the activity of these compounds in the rat peritoneal anaphylaxis model. Despite the fact that these compounds are among the most potent in vitro inhibitors of the enzyme ever reported, they have little or no activity in this in vivo model. Compounds **1**, **4**, and **5** cause no significant inhibition after oral doses of 100 mg/kg, and **2** and **3** are only moderately active at this dose. These in vivo results are typical of the compounds described in our previous paper.

Although the compounds are not effective after oral administration, they produce potent inhibition when injected ip. By this route of administration, the hydroxamates are deposited directly at the site of leukotriene biosynthesis. For example, **3** has an ED_{50} of 0.2 mg/kg when given ip. Thus hydroxamates can inhibit leukotriene synthesis in vivo if they reach the location of the enzyme in sufficient concentrations.

Pharmacokinetic Evaluation of Hydroxamic Acid 3. To understand the discrepancy between in vitro and oral in vivo potencies, hydroxamate **3** was selected for pharmacokinetic investigation. Rats were dosed orally and intravenously with the compound in order to study the rates and extent of absorption, metabolism, and excretion. When plasma samples from animals dosed with **3** were examined by HPLC, it was found that the hydroxamic acid group is readily metabolized to the corresponding carboxylic acid. This carboxylic acid does not inhibit 5-lipoxygenase in vitro at doses up to 300 μM.

The metabolic conversion of hydroxamates to carboxylates was extremely rapid. Figure 1 shows the concentrations of **3** and the corresponding carboxylic acid at various times following a 20 mg/kg intravenous (iv) dose of **3**. As early as 1 min after dosing, there is more carboxylic acid than hydroxamic acid present in the plasma. Two hours after dosing, **3** is no longer detectable. From these data, the estimated plasma half-life of **3** is about 5 min in the rat. The carboxylic acid circulates much longer and has a half life of about 2.2 h.

These results are not unique to the rat. Similar studies were conducted with both dogs and monkeys. In both species, **3** was rapidly metabolized to the carboxylic acid and had a half-life of less than 5 min in the plasma after intravenous dosing.

When **3** was administered orally to rats, it was quickly and efficiently absorbed. By comparison of the areas under the plasma concentration curves of **3** and its metabolite after iv and oral administration, 60% of **3** was estimated to be absorbed from the gastrointestinal tract of the rat. However, because of the rapid metabolism, the parent compound **3** could not be detected in plasma samples after oral doses of 20 mg/kg. As early as 5 min after oral dosing with **3**, only the carboxylic acid was present.

Plasma concentrations do not necessarily reflect the concentration of compounds present in the peritoneal cavity, the site where in vivo reaction takes place. However, on the basis of the results described above, it would appear that the lack of oral activity of these hydroxamic acids is likely to be attributable to their rapid metabolism. Compounds such as **3** show only marginal activity in the rat peritoneal anaphylaxis model because they are quickly converted to inactive metabolites.

Influence of Structural Features on Rate of Metabolism. A wide variety of hydroxamic acids of diverse structures was surveyed in order to identify those hydroxamates that were less vigorously metabolized to the corresponding carboxylic acid. Rats were dosed orally with selected compounds (typically at 100 mg/kg), and blood samples were removed from the tail vein at various times. These samples were examined by HPLC to determine the concentrations of parent drug and corresponding carboxylic acid.

After several hydroxamates were surveyed the nonsteroidal antiinflammatory drug ibuproxam (**6**)[9] was found to be relatively resistant to this metabolism. Twenty min after an oral dose of 100 mg/kg, plasma concentrations of 49 μM were observed, and at 1 h after dosing, the plasma concentrations of **6** were still 27 μM. These concentrations are considerably higher than those observed at similar times for hydroxamates such as **3**. Hydroxamate **6** is also metabolized to the corresponding carboxylic acid. Plasma concentrations of the corresponding carboxylate 20 min and 1 h after dosing were 114 and 146 μM, respectively.

Table II summarizes the results of a study designed to evaluate the structural features of **6**, which contribute to its relative resistance to metabolism and elevated plasma concentrations. The most important structural feature in this regard appears to be the spacer unit that connects the carbonyl group of the hydroxamate to the phenyl ring. All of the compounds shown in Table II producing significant plasma levels after oral dosing possessed either a CH_2 or a $CHCH_3$ spacer unit, viz. **6**, **7**, and **10**.

When the spacer unit was eliminated as in **13** or lengthened as in **14** or **15**, the hydroxamates were more readily metabolized to the corresponding carboxylic acid. With these compounds, only the carboxylate was detected in the plasma 20 or 60 min after dosing. Compounds such as these that have a two-carbon spacer or no spacer at all are structurally similar to our early compounds (e.g., **1–5**), which were also rapidly metabolized.

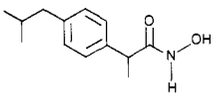
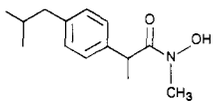
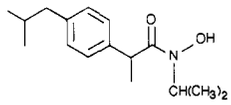
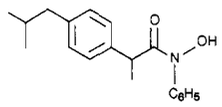
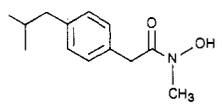
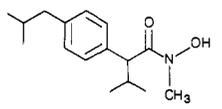
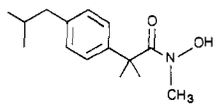
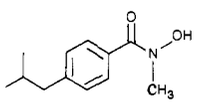
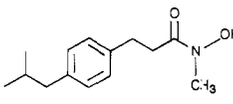
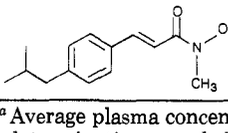
When a branching group larger than methyl was incorporated into this spacer unit, the hydroxamate appeared to be poorly absorbed by the rat. For example, **11** exhibited only very low plasma concentrations of the parent hydroxamate or its carboxylate metabolite after oral dosing. No other metabolites were detected. Similary **12**, which has two methyl groups branching from the spacer, gave low plasma levels.

Compounds that have a group larger than methyl attached to the hydroxamate nitrogen also appeared to be poorly absorbed. Hydroxamates **8** and **9** exhibit no measurable amount of parent compound, carboxylic acid, or any metabolite at 20 or 60 min following oral dosing.

Orally Active Hydroxamic Acid Inhibitors of Leukotriene Synthesis. On the basis of the results of the study described above, the $CHCH_3$ spacer unit was selected as the fragment that provided hydroxamic acids most re-

(9) Orzalesi, G.; Selleri, R.; Caldini, O.; Volpato, I.; Innocenti, F.; Colome, J.; Sacristan, A.; Varez, G. *Arzneim.*-Forsch. **1977**, *27*, 1006.

Table II. Plasma Concentrations of Analogues of Ibuproxam 6 and Their Carboxylic Acid Metabolites in μM following Oral Dosing at 100 mg/kg

structure	no.	hydroxamic acid[a] 20 min	hydroxamic acid[a] 60 min	carboxylic acid[a] 20 min	carboxylic acid[a] 60 min
	**6**	49 (48–50)	27 (4–62)	114 (113–115)	146 (89–244)
	**7**	32 (3–67)	14 (1–27)	41 (18–60)	36 (8–71)
	**8**	<4[b]	<4[b]	<4[b]	<4[b]
	**9**	<3[b]	<3[b]	<4[b]	<4[b]
	**10**	46 (13–81)	12 (6–22)	162 (42–223)	138 (88–223)
	**11**	5.0 (2.7–6.5)	7.9 (3.4–10)	3.7 (2.6–4.3)	7.5 (4.7–10)
	**12**	5.8 (0–11.6)	<1[b]	9.3 (0–18.6)	<1[b]
	**13**	<5[b]	<5[b]	432 (348–512)	404 (226–628)
	**14**	<10[b]	<10[b]	349 (305–386), 86 (81–89)[c]	80 (32–144), 36 (8–51)[c]
	**15**	<4[b]	<4[b]	1.9 (1.5–2.4)	4.5 (3.9–5.3)

[a] Average plasma concentration from four determinations. The range of experimental values is in parentheses. [b] Plasma concentrations for all determination were below the indicated level of detection. [c] Additional metabolite: carboxylic acid corresponding to **15**.

sistant to metabolic hydrolysis. In order to establish the generality of this observation and to evaluate the effect of reduced metabolism on in vivo potency, several hydroxamates were prepared that incorporated this unit. Table III shows six 2-arylpropionyl hydroxamic acids bearing the $CHCH_3$ group as a spacer between the aryl ring and the hydroxamate functionality. The table lists the plasma concentrations of parent drug and carboxylate metabolite, the in vitro 5-lipoxygenase inhibitor potency, and the ability to block in vivo leukotriene biosynthesis with the rat peritoneal anaphylaxis model.

All of these 2-arylpropionyl hydroxamic acids exhibited detectable levels of hydroxamate circulating in plasma 1 h after oral administration of a 100 mg/kg dose. This is consistent with the observation that the $CHCH_3$ spacer offers some protection from metabolic hydrolysis. Furthermore, when these compounds were evaluated in the rat peritoneal anaphylaxis model, all but one demonstrated oral activity. Only **6** failed to show at least 50% inhibition after oral administration of a 100 mg/kg dose. However, with **7**, the *N*-methyl analogue of **6**, 65% inhibition of leukotriene synthesis was observed. We have previously shown that *N*-methyl hydroxamates are between 4- and 30-fold more potent inhibitors of 5-lipoxygenase in vitro than their *N*-protio congeners.[2] In this case, **7** was about five times more potent at inhibiting the enzyme than **6**. Apparently this increase in in vitro potency has directly translated into an increase in in vivo potency.

Hydroxamate **19** was one of the most potent orally active inhibitors of leukotriene biosynthesis listed in Table III. It showed significant inhibition of leukotriene biosynthesis at doses as low as 28 mg/kg and exhibited an oral ED_{50} of 40 mg/kg.

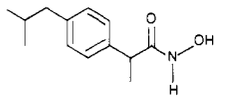
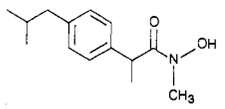
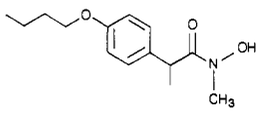
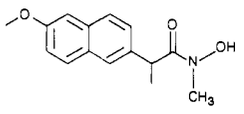
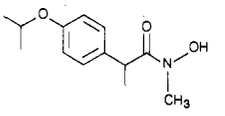
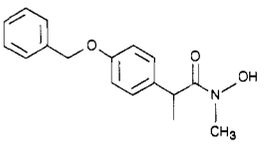
Summary

Hydroxamic acid containing molecules rank among the most potent inhibitors of 5-lipoxygenase known. Despite their in vitro activity, many hydroxamates exhibit limited ability to block leukotriene synthesis in vivo following oral administration. This low activity can be attributed to the rapid metabolism of the hydroxmate group to the corresponding inactive carboxylic acid. However, 2-aryl propionyl hydroxamic acids are less prone to metabolic hydrolysis. Several members of this class of hydroxamates are orally active inhibitors of leukotriene formation.

Experimental Section

Determination of 5-Lipoxygenase Inhibitory Potencies. Assays to determine 5-lipoxygenase activity were performed in

Table III. Inhibitory and Plasma Concentration Data for Selected 2-Arylpropionohydroxamic Acids Bearing a $CHCH_3$ Spacer Unit

structure	no.	in vitro 5-LO inhibn IC_{50}, μM[a]	plasma concentrations,[b] μM after 100 mg/kg po				in vivo LT inhibn % at 100 mg/kg po[c]
			hydroxamate		carboxylate		
			20 min	60 min	20 min	60 min	
	6	4.8 (4.4–5.2)	49 (48–50)	27 (4–62)	114 (113–115)	146 (89–244)	NS[d]
	7	0.98 (0.89–1.1)	29 (3–67)	14 (1–27)	41 (18–60)	36 (8–71)	65 ± 6%
	16	0.73 (0.61–0.85)	38 (17–52)	13 (8–23)	40 (24–54)	50 (32–77)	62 ± 5%
	17	0.41 (0.34–0.50)	1.7 (0.4–3.9)	1.3 (0.8–1.9)	3 (2.3–4.3)	4 (3.2–4.8)	52 ± 5%
	18	3.7 (3.5–3.8)	*e*	38 (26–51)	*e*	*e*	67 ± 5%
	19	0.28 (0.27–0.28)	8.4 (7.5–9.3)	7.9 (7.4–8.5)	16 (14–17)	51 (45–57)	62 ± 8%[f]

[a] IC_{50} with 95% confidence limits in parentheses calculated by the method of Finney.[10] [b] Average plasma concentration from four determinations. The range of experimental values is in parentheses. [c] Percent inhibition values ± SEM in the rat peritoneal anaphylaxis model. [d] NS = not significantly different from control values ($p > 0.05$). Typically 30–40% inhibition was required for statistical significance. [e] Value not determined. [f] Determined at 86 mg/kg.

200-μL incubations containing the 20000*g* supernatant from 6×10^4 homogenized RBL-1 cells, 2% DMSO vehicle, and various concentrations of the test compound. Reactions were initiated by addition of radiolabeled arachidonic acid and terminated by acidification and ether extraction. Reaction products were separated from nonconverted substrate by thin-layer chromatography and measured by lipid-scintillation spectroscopy. All treatments were evaluated in triplicate incubations. Inhibition of 5-lipoxygenase activity was computed by comparison of the quantity of products formed in the treatment incubations to the mean product formation in vehicle control groups ($n = 8$). IC_{50} values and 95% confidence limits were computed by linear regression analysis of percentage inhibition versus log inhibitor concentration plots.[10]

Rat Peritoneal Anaphylaxis Model. Fasted male Sprague–Dawley derived rats (SASCO Inc., Oregon, WI) were passively sensitized by ip injection of rabbit antibovine serum albumin (anti-BSA) in phosphate buffered saline (PBS), pH 7.1. Three hours after sensitization, the rats were injected ip with BSA (4 mg) in PBS (5 mL) containing 30 mM l-cysteine. Test compounds suspended in 0.2% methylcellulose or vehicle controls were administered by gavage 1 h prior to the antigen challenge. Compounds administered ip were given 15 min prior to antigen challenge. Typically six to eight rats were included in both the control and treatment groups.

The rats were sacrificed by carbon dioxide asphyxiation 15 min after challenge, the peritoneal catvities opened, and the fluid contents collected with a plastic trocar. The cavitities were rinsed with cold PBS (5 mL), pH 7.4, containing gelatin (5 mg), sodium azide (5 mg), EDTA (18.8 mg), and l-cysteine (30 mM). These fluids were transferred to ice-cold methanol, incubated for about 20 min, vortexed, and then centrifuged at 1000*g* for 15 min. Fluid volumes were recorded, and the samples were stored frozen until radioimmunoassay (New England Nuclear, Boston, MA) for LTC_4 equivalents was conducted. For the results reported here, leukotriene levels in control groups ranged from a minimum of 132 (SEM = 20) to a maximum of 329 (SEM = 43) ng LTC_4 equivalents per rat.

Analysis of variance followed by Duncan's multiple range test was used to determine the statistical significance of treatment effects. Percent inhibition values were determined by comparing the treatment values to the mean of the control group. Typically, mean inhibition values of less than 30–40% were not significantly different from control values ($p > 0.05$).

Determination of Plasma Concentrations. Compounds were suspended in 0.2% methylcellulose and administered to rats by oral gavage at a volume dose of 4 mL/kg. For intravenous dosing, the compounds were prepared in 20% DMSO, 40% ethanol, and 40% PBS and administered at a volume dose of 1.7 mL/kg through a jugular cannula.

Multiple blood samples were collected from a lateral tail vein by using heparinized capillary tubes. Larger blood samples were collected by cardiac puncture after CO_2 asphyxiation.

Plasma was separated from whole blood by centrifugation. Prior to reversed-phase HPLC, plasma proteins were precipitated by addition of 2 volumes of methanol and centrifugation at 13000*g* for 10 min. When normal-phase chromatography was used, plasma was acidified and extracted with ether. The ether was evaporated under a stream of nitrogen, and samples were reconstituted in the mobile phase for injection.

The reversed-phase HPLC system consisted of a C_{18} Adsorbosphere 7 μm column (Alltech Associates, Deerfield, IL) with a mobile phase of 50% CH_3CN, 8 mM triethylamine acetate (pH 6.5), and 10 mM acetohydroxamic acid. Normal-phase HPLC was conducted on 5-μm silica gel columns (Alltech Associates, Deerfield, IL) with an 2-propanol/methylene chloride mobile phase containing 10 mM acetohydroxamic acid. In both systems, flow rates were typically 1 mL/min and compounds were detected by UV spectroscopy at their absorbance maximum. The amounts of compounds were calculated by use of external standards.

Synthesis. Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. NMR spectra were re-

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Table IV. Summary of Experimental Data for Selected Hydroxamic Acids Prepared from the Corresponding Carboxylic Acid As Described for **7**

no.	yield, %[a]	mp, °C	formula[b]
8	35	85–86	$C_{16}H_{25}NO_2$
9	38	75–77	$C_{19}H_{23}NO_2$
10[c]	74	oil	$C_{13}H_{19}NO_2$
14[d]	73	oil	$C_{14}H_{21}NO_2$
17[c]	59	oil	$C_{15}H_{17}NO_3$
18[e]	64	oil	$C_{13}H_{19}NO_3$
19[e]	42	121–125	$C_{17}H_{19}NO_3$

[a] Unoptimized yields for the conversion of carboxylic acid to hydroxamic acid. [b] Elemental analysis (C, H, N) within ±0.4 of the theoretical value. [c] Corresponding carboxylic acid prepared as previously described for **10**[11] and **17**.[12] [d] Corresponding carboxylic acid prepared by catalytic hydrogen of 4-isobutylcinnamic acid (prepared as described for **15**) with 20% palladium on charcoal in methanol. [e] Corresponding carboxylic acid prepared by the method described for **16**.

corded at 300 MHz on a GE QE300 instrument with $(CH_3)_4Si$ as the internal standard and are reported in units of δ. Mass spectra were obtained on a Kratos MS-50 instrument with EI ion source (70 eV). Analytical data indicated by elemental symbols were within ±0.4% theoretical values unless noted.

The purity of compounds was checked by TLC analysis on silica gel F_{254} (Merck), and compounds were visualized with UV fluorescence inhibition or $Ce(SO_4)_3$ spray. Flash column chromatography was conducted on silica gel 60 (Merck, 40–60 μm) under 10–20 psi of pressure.

Compounds **1**–**5**[2] and **6**[8] were prepared as previously described. Hydroxamates **8**–**10**, **14**, and **17**–**19** were prepared from the corresponding carboxylic acid by using the method described for **7**. Physical data for these compounds are summarized in Table IV.

***N*-Hydroxy-*N*-methyl-2-[4-(2-methylpropyl)phenyl]-propionamide (7).** Ibuprofen[11] (2.0 g, 9.7 mmol) and DMF (0.71 g, 9.6 mmol) were dissolved in CH_2Cl_2 (100 mL) and cooled to 0 °C. Oxalyl chloride (2.17 mL, 24 mmol) was added slowly. Vigorous gas evolution was noted. After being stirred for 40 min, this solution was added to a solution of *N*-methylhydroxylamine hydrochloride (3.24 g, 38.7 mmol) and triethylamine (5.89 g, 58.1 mmol) in THF (50 mL)/H_2O (5 mL). After being stirred an additional 30 min, the mixture was poured into 2 N HCl and extracted with CH_2Cl_2. The organic phase was dried over $MgSO_4$ and evaporated in vacuo. The residue was chromatographed on 50 g of silica gel, eluting with 60% ether in hexanes (v/v) to yield 1.5 g (66%) of a colorless oil: ^{1}H NMR (Me_2SO-d_6) δ 0.85 (d, 6 H, J = 6 Hz), 1.27 (d, 3 H, J = 8 Hz), 1.80 (m, 1 H, J = 6 Hz), 2.39 (d, 2 H, J = 6 Hz), 3.07 (s, 3 H), 4.36 (m, 1 H, J = 8 Hz), 7.07 (d, 2 H, J = 7 Hz), 7.17 (d, 2 H, J = 7 Hz), 9.83 (s, 1 H); MS, m/e 235, 220, 161, 145. Anal. ($C_{14}H_{21}NO_2$) C, H, N.

***N*-Hydroxy-*N*-methyl-3-methyl-2-[4-(2-methylpropyl)-phenyl]butanamide (11).** Sodium bis(trimethylsilyl)amide (12.6 mL, 1.0 M in THF) was added to a solution of methyl 4-(2-methylpropyl)phenylacetate[11] (2.0 g, 9.7 mmol) in THF (15 mL) at 0 °C. Five minutes later, isopropyl iodide (1.65 g, 9.7 mmol) was added. After the mixture was stirred for 30 min, the reaction was quenched with 2 N HCl (5 mL). The desired material was extracted into ether, which was dried with $MgSO_4$ and evaporated to give 1.5 g of crude ester. Without purification this residue was dissolved in 2-propanol (20 mL) and lithium hydroxide (1.14 g, 27 mmol) in H_2O (10 mL) was added. This mixture was heated at reflux for 12 h, cooled to room temperature, and made acidic with concentrated HCl. The product was extracted into ether, which was dried with $MgSO_4$ and evaporated. The residue was chromatographed on 30 g of silica gel, eluting with 50% ether in hexanes (v/v), to afford 3-methyl-2-[4-(2-methylpropyl)-phenyl]butanoic acid (900 mg, 40%).

This material was converted to **11** by using the method described above for **7** (oil, 74%): ^{1}H NMR (Me_2SO-d_6) δ 0.56 (d, 3 H, J = 7 Hz), 0.86 (d, 6 H, J = 6 Hz), 0.92 (d, 3 H, J = 7 Hz), 1.80 (m, 1 H, J = 6 Hz), 2.20 (br m, 1 H), 2.39 (d, 2 H, J = 6 Hz), 3.05 (s, 3 H), 3.78 (d, 1 H, J = 7 Hz), 7.05 (d, 2 H, J = 7 Hz), 7.18 (d, 2 H, J = 7 Hz), 9.84 (s, 1 H); MS, m/e 263, 189, 147. Anal. ($C_{16}H_{25}NO_2$) C, H, N.

***N*-Hydroxy-*N*-methyl-2-methyl-2-[4-(2-methylpropyl)-phenyl]propionamide (12).** The corresponding carboxylic acid was prepared as follows: *n*-butyl lithium (5.3 mL, 2.5 M in hexanes) was added to a solution of ibuprofen[11] (1.3 g, 6.3 mmol) in THF (15 mL) at −78 °C. Thirty minutes later, methyl iodide (1.1 g, 7.6 mmol) was added. After an additional 30 min, the mixture was warmed to room temperature and 2 N HCl was added (5 mL). The desired material was extracted into ether, which was dried over $MgSO_4$ and evaporated to give 1.4 g of a solid.

This material was converted to **12** by using the method described above for **7** (oil, 65%): ^{1}H NMR (Me_2SO-d_6) δ 0.85 (d, 6 H, J = 7 Hz), 1.42 (s, 6 H), 1.82 (m, 1 H, J = 7 Hz), 2.39 (d, 2 H, J = 7 Hz), 3.02 (s, 3 H), 7.02–7.09 (m, 4 H), 9.03 (br s, 1 H); MS, m/e 249, 175, 132, 117. Anal. ($C_{15}H_{23}NO_2$) C, H, N.

***N*-Hydroxy-*N*-methyl-4-(2-methylpropyl)benzamide (13).** 4-Isobutyl acetophenone[13] (2 g, 11.3 mmol) was added to an aqueous solution of sodium hypochlorite (25 mL, 75%), and the mixture was refluxed for 12 h. The mixture was made acidic with concentrated HCl, and a white solid (750 mg, 38%) precipitated. This material was collected by filtration and dried in vacuo. It was then converted to **13** without further purification by using the method described above for **7** (44%): mp 112–115 °C; ^{1}H NMR (Me_2SO-d_6) δ 0.85 (d, 6 H, J = 7 Hz), 1.85 (m, 1 H, J = 7 Hz), 2.46 (d, 2 H, J = 7 Hz), 3.02 (s, 3 H), 7.22 (d, 2 H, J = 6 Hz), 7.66 (d, 2 H, J = 6 Hz), 9.03 (br s, 1 H); MS m/e 207, 175. Anal. ($C_{12}H_{17}NO_2$) C, H, N.

***N*-Hydroxy-*N*-methyl-3-[4-(2-methylpropyl)phenyl]-propenamide (15).** Borane (32.7 mL, 1 M in THF) was added to a solution of 4-isobutylbenzoic acid (3.88 g, 21.8 mmol, prepared as described for **13**) in THF (30 mL) at 0 °C. After the initial hydrogen evolution had ceased, the mixture was allowed to warm to room temperature and stirred for 3 h. Ether (50 mL) and 2 N HCl (50 mL) were added, and the organic layer was separated. The solvent was removed in vacuo to afford a colorless liquid, (4-isobutylphenyl)methanol.

Without further purification, the material (3.7 g, 20.8 mmol) was dissolved in CH_2Cl_2 (50 mL), and pyridinium chlorochromate (11.8 g, 54.5 mmole) was added. After the solution was stirred for 3 h, ether was added (50 mL), and the mixture was filtered through Celite. The solvent was evaporated to yield a slightly yellow colored oil (3.17 g, 89%), 4-isobutylbenzaldehyde.

A solution of the aldehyde (1.17 g, 7.2 mmol) prepared as described above, malonic acid (1.69 g, 16.3 mmol), and a few drops of morpholine was heated at reflux for 2 h. The mixture was then poured into 2 N HCl (100 mL), and a white precipitate formed. This was collected by filtration and recrystallized from aqueous ethanol to give 4-isobutylcinnamic acid as a white solid (1.26 g, 86%).

This acid was converted to **15** by using the method described above for **7** (74%): mp 125–127 °C, ^{1}H NMR (Me_2SO-d_6) δ 0.86 (d, 6 H, J = 7 Hz), 1.84 (m, 1 H, J = 7 Hz), 2.47 (d, 2 H, J = 7 Hz), 3.20 (s, 3 H), 7.18 (d, 1 H, J = 16 Hz), 7.21 (d, 2 H, J = 6 Hz), 7.47 (d, 1 H, J = 16 Hz), 7.57 (d, 2 H, J = 6 Hz), 10.09 (br s, 1 H); MS, m/e 233, 187, 144. Anal. ($C_{14}H_{19}NO_2$) C, H, N.

***N*-Hydroxy-*N*-methyl-2-(4-butoxyphenyl)propionamide (16).** The corresponding carboxylic acid was prepared as follows: Potassium *tert*-butoxide (12.9 g, 0.11 mol) was added to a solution of methyl 4-hydroxyphenylacetate (16.6 g, 0.1 mol) in DMSO (50 mL). 1-Bromobutane (17.1 g, 0.12 mol) was then added. The mixture was stirred for 30 min and poured into H_2O (150 mL). The product was extracted into ether, which was then dried with $MgSO_4$ and evaporated. The residue was distilled at 118–120 °C (0.4 mmHg) to give methyl 4-butoxyphenylacetate (15.1 g, 68%).

Sodium bis(trimethylsilyl)amide (22.1 mL, 1.0 M in THF) was added to a solution of the ester prepared as described above (3.5 g, 15.8 mmol) in THF (50 mL) at 0 °C. Methyl iodide (2.23 g, 15.8 mmol) was then stirred for 30 min. The solvent was evaporated, and the residue was chromatographed on silica gel (100

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(12) Harrison, I. T.; Lewis, B.; Rooks, W.; Roskowski, A.; Tomolonis, A.; Fried, J. H. *J. Med. Chem.* **1970**, *13*, 203.

(13) Braddeley, G.; Wrench, E. *J. Chem. Soc.* **1956**, 4943.

g), eluting with 3% ether in hexanes (v/v), to yield methyl 2-(4-butoxyphenyl)propionate (1.5 g, 40%).

A solution of this ester (1.5 g, 6.3 mmol) in 2-propanol (20 mL) was added to lithium hydroxide monohydrate (1.5 g) in H_2O (10 mL). After the mixture was heated for 1 h at reflux, the solvent was evaporated and the residue was partitioned between ether and 2 N HCl. The ether layer was dried with $MgSO_4$, and the solvent was evaporated to give 2-(4-butoxyphenyl)propionic acid as a white solid (1.4 g, 98%).

This was converted to **16** by using the method described above for **7** (oil, 52%): ^{1}H NMR (Me_2SO-d_6) δ 0.92 (t, 3 H, J = 8 Hz), 1.24 (d, 3 H, J = 7 Hz), 1.42 (m, 2 H, J = 8 Hz), 1.67 (m, 2 H, J = 8 Hz), 3.06 (s, 3 H), 3.92 (t, 2 H, J = 8 Hz), 4.22 (q, 1 H, J = 7 Hz), 6.83 (d, 2 H, J = 9 Hz), 7.15 (d, 2 H, J = 9 Hz), 9.80 (s, 1 H); MS, m/e 251, 234, 177, 121. Anal. ($C_{14}H_{21}NO_3$) C, H, N.

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Registry No. **1**, 106359-63-1; **2**, 106359-60-8; **3**, 105847-10-7; **4**, 106359-59-5; **5**, 76790-15-3; **6**, 53648-05-8; **7**, 110330-36-4; **8**, 110319-71-6; **9**, 110319-72-7; **10**, 110319-73-8; **11**, 110319-74-9; **12**, 110319-75-0; **12** (corresponding acid), 95499-72-2; **13**, 110319-76-1; **13** (corresponding acid), 38861-88-0; **14**, 110319-77-2; **15**, 110319-78-3; **16**, 110319-79-4; **17**, 110319-80-7; **18**, 110319-81-8; **19**, 110319-82-9; MeNHOH·HCl, 4229-44-1; 4-*i*-$BuC_6H_4CH_2CO_2Me$, 61566-33-4; i-PrI, 75-30-9; 4-*i*-$BuC_6H_4CH(CO_2Me)Pr$-*i*, 110319-83-0; 4-*i*-$BuC_6H_4CH(CO_2H)Pr$-*i*, 110319-84-1; MeI, 74-88-4; 4-*i*-BuC_6H_4Ac, 38861-78-8; 4-*i*-$BuC_6H_4CO_2H$, 38861-88-0; 4-*i*-$BuC_6H_4CH_2OH$, 110319-85-2; 4-*i*-BuC_6H_4CHO, 40150-98-9; 4-*i*-$BuC_6H_4CH{=}CHCO_2H$, 66734-95-0; 4-$OHC_6H_4CH_2CO_2Me$, 14199-15-6; $Br(CH_2)_3Me$, 109-65-9; 4-$BuOC_6H_4OAc$, 110319-86-3; 4-$BuOC_6H_4CH(Me)CO_2Me$, 110319-87-4; 4-$BuOC_6H_4CH(Me)CO_2H$, 3585-71-5; Ibuprofen, 15687-27-1; malonic acid, 141-82-2; arachidonate-5-lipoxygenase, 80619-02-9.

α-Melanotropin: The Minimal Active Sequence in the Frog Skin Bioassay

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The minimal sequence required for biological activity of α-MSH (α-melanotropin, α-melanocyte stimulating hormone) was determined in the frog (*Rana pipiens*) skin bioassay. The sequence required to elicit measurable biological activity was the central tetrapeptide sequence, Ac-His-Phe-Arg-Trp-NH_2 (Ac-α-MSH_{6-9}-NH_2), which was about 6 orders of magnitude less potent than the native tridecapeptide. Smaller fragments of this sequence (Ac-His-Phe-NH_2, Ac-Phe-Arg-NH_2, Ac-His-Phe-Arg-NH_2) were devoid of melanotropic activity at concentrations as high as 10^{-4} M. We were unable to demonstrate biological activity for the tetrapeptide, Ac-Phe-Arg-Trp-Gly-NH_2 (Ac-α-MSH_{7-10}-NH_2), and for several carboxy terminal analogues including Ac-Lys-Pro-Val-NH_2 (Ac-α-MSH_{11-13}-NH_2). We prepared a series of fragment analogues of α-MSH in an attempt to determine the contribution of each individual amino acid to the biological activity of the native hormone. The minimal potency of Ac-α-MSH_{6-9}-NH_2 could be enhanced about a factor of 16 by the addition of glycine to the C-terminus, yielding Ac-α-MSH_{6-10}-NH_2 (Ac-His-Phe-Arg-Trp-Gly-NH_2). Addition of glutamic acid to the N-terminus provided the peptide, Ac-α-MSH_{5-10}-NH_2, which was only slightly more potent than Ac-α-MSH_{6-10}-NH_2, indicating that position 5 contributes little to the biological potency of α-MSH in this assay. Addition of methionine to the N-terminus of Ac-α-MSH_{5-10}-NH_2 resulted in the heptapeptide, Ac-α-MSH_{4-10}-NH_2, which was only about 4-fold more potent than Ac-α-MSH_{5-10}-NH_2. Addition of lysine and proline to the C-terminal of the Ac-α-MSH_{4-10}-NH_2 sequence yielded the peptide, Ac-α-MSH_{4-12}-NH_2 with a 360-fold increase in potency relative to Ac-α-MSH_{4-10}-NH_2. This peptide was only about 6-fold less potent than α-MSH. A series of Nle-4-substituted analogues also were prepared. Ac-[Nle^4]-α-MSH_{4-10}-NH_2 was about 4 times more potent than Ac-α-MSH_{4-10}-NH_2. Ac-[Nle^4]-α-MSH_{4-11}-NH_2 also was about 4 times more potent than Ac-α-MSH_{4-10}-NH_2, demonstrating that lysine-11 contributes somewhat to the biological activity of α-MSH on the frog skin melanocyte receptor. However, addition of proline-12 to this fragment, yielding Ac-[Nle^4]-α-MSH_{4-12}-NH_2, resulted in about a 90-fold increase in relative potency of the melanotropin. Addition of the final C-terminal amino acid, valine-13, provided the decapeptide, Ac-[Nle^4]-α-MSH_{4-13}-NH_2, which showed only a small further increase in potency. This analogue was, however, only about 2–3-fold less active than α-MSH. Addition of the N-terminal tripeptide Ac-Ser-Tyr-Ser to yield the tridecapeptide [Nle^4]-α-MSH resulted in an analogue that was 3 times more potent than α-MSH. The importance of the amino acids in the primary structure of α-MSH in contributing to the biological activity of α-MSH in the frog skin bioassay can be summarized as follows: (1) the central tetrapeptide sequence, Ac-His-Phe-Arg-Trp-NH_2, represents the minimum chain length for observable biological activity; (2) the active sequence of α-MSH is contiguous in that no two structurally noncontiguous fragment sequences were found to have biological activity; (3) Met-4, Gly-10, and Pro-12 are important potentiating amino acids and contribute significantly to the biopotency of α-MSH; and (4) Ser-1 and -3, Tyr-2, Glu-5, Lys-11, and Val-13 apparently contribute only minimally to the biological potency of α-MSH at the frog melanocyte skin receptor.

α-Melanotropin (α-melanocyte stimulating hormone, α-MSH),[1] Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2, is a tridecapeptide that is synthesized, stored, and secreted by the pars intermedia of

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(1) Symbols and abbreviations are in accord with recommendations of the IUPAC-IUB Commision on Biochemical Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977). Other abbreviations include: α-MSH, α-melanotropin; Nle, norleucine; TLC, thin-layer chromatography; DCC, dicyclohexylcarbodiimide; HOAc, acetic acid; 2,4-Cl_2-Z, 2,4-dichlorobenzyloxycarbonyl; For, formyl; Tos, tosyl; NH_4OAc, ammonium acetate; OR, optical rotation; NMR, nuclear magnetic resonance. All optically active amino acids are of the L configuration unless otherwise noted.

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