of I were identified as rat liver S9 *in vitro* metabolites, and this current paper reports the identification of a dozen metabolites of I in which hydroxylation occurred at C(4) - C(7a) of the cyclohexane ring. The intact animal produces not only a greater total number of and several more-extensively hydroxylated metabolites, but also effects an entirely different and quantitatively significant metabolic conversion, *i.e.*, degradation of the nitroimidazole ring. The most common metabolic transformation both *in vivo* and *in vitro*, however, is hydroxylation to form the 5-axial hydroxy metabolite. Axial hydroxyl groups at C-5 are also found in three of the dihydroxy metabolites and both of the urinary trihydroxy metabolites.

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Acyloxyamines as Prodrugs of Anti-inflammatory Carboxylic Acids for Improved Delivery Through Skin

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Abstract □ An *N,N*-dialkylhydroxylamine derivative of indomethacin has been synthesized. It has been shown to improve the delivery of indomethacin through mouse skin (compared to indomethacin itself) by a factor of two, to be more effective than indomethacin in inhibiting thermal inflammation (two to three times) in animal models, but to be only as effective as indomethacin in inhibiting UV-B radiation erythema in human volunteers.

Keyphrases ■ Indomethacin—derivatives, erythema inhibition in humans, inflammation inhibition in rats, delivery vehicle comparison □ Erythema—inhibition by indomethacin derivatives in humans, inflammation inhibition in rats, delivery vehicle

It is well known that UV radiation on skin produces intense erythema, pain, and blistering (1). However, the proximal cause or causes of the response of skin to UV radiation is less than well understood. UV radiation is usually divided into three arbitrary regions: UV-A, 320–400 nm; UV-B, 320–290 nm, which is also known as sunburn UV radiation; UV-C, 290–200 nm. Regardless of the wavelength, there is a delay of onset of redness that is inversely proportional to the intensity of the radiation. Furthermore, the erythema will persist for hours or days depending directly on the intensity of the radiation. The intensity of the effect of the UV-B radiation has been shown to be particularly sensitive to treatment with nonsteroidal anti-inflammatory agents while UV-C radiation is less sensitive (2) and UV-A radiation is insensitive to nonsteroidal anti-inflammatory agents (3). For instance, topically administered indomethacin has been shown to decrease the redness, as determined visually, and the temperature, as determined by telethermometer readings, of sunburned or UV-B treated skin compared with controls (4).

Since the nonsteroidal anti-inflammatory agents are known to prevent inflammation by inhibiting prostaglandin synthesis, it was logical to suspect that prostaglandins (5) were the proximal cause of at least some of the effects of UV-B radiation because UV-B radiation was susceptible to treatment with nonsteroidal anti-inflammatory agents. Indeed, increased levels of arachidonic acid and prostaglandins E_2 and $F_{2\alpha}$ were found in human skin after treatment with UV-B (6) and UV-C radiation (7); the levels of prostaglandin E increased in a parallel manner with increased erythema over the first 4 h after exposure of guinea pig skin to UV-B radiation (8). However, after 4 h erythema scores stayed high but prostaglandin levels fell back to normal (8). Thus, oral or topical administration of indomethacin completely suppressed the elevation of the levels of the prostaglandins (9) but the erythema associated with the radiation damage was only partially suppressed compared with controls and then for only ~24 h, and the acute

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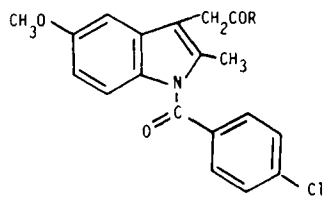
UV damage to epidermal cells was not affected (10). Moreover, the concentration of indomethacin used was fairly high (2.5%) and the vehicle used in all treatments contained a penetration enhancer (ethanol–propylene glycol–dimethylacetamide or dimethylformamide, 19:19:2 or 1:1:2).

Thus, although in general the treatment (oral) of sunburn with nonsteroidal anti-inflammatory agents appears promising from the point of view of increasing the minimal dose of light necessary to produce erythema (11), the lack of reports of topical activity by indomethacin without the use of penetration enhancers and its lack of effect on the long term effects of sunburn make it an unsatisfactory therapeutic agent at least from a topical delivery point of view. Therefore, in order to determine if increased delivery of indomethacin through skin would improve its therapeutic effectiveness, a number of prodrug derivatives have been prepared and evaluated (12). This paper describes the preparation and evaluation of an *N,N*-dialkylhydroxylamine derivative of indomethacin as one example of the prodrugs of anti-inflammatory carboxylic acids (13) designed to improve therapeutic effectiveness by increasing their delivery through skin.



Ia, R = OH
Ib, R = Cl
Ic, R = $ON(C_2H_5)_2$

EXPERIMENTAL SECTION[1]

Preparation of 1-(4′-Chlorobenzoyl)-5-methoxy-2-methyl-3-indolylacetyl Chloride (Ib)—To a suspension of indomethacin (21.9 g, 0.061 mol) in 300 mL of benzene, 10.8 g (0.085 mol) of oxalyl chloride was added with stirring. The reaction was heated at reflux for 2 h. Then the solution was concentrated to give a residue which was triturated with 300 mL of ether. The suspension was filtered and the solid material dried to give the acid chloride as a yellow powder (18.9 g, 82% yield), mp 125–128°C. IR (KBr): 1790 and 1675 cm^{-1} (s, C=O);[1] H-NMR ($CDCl_3$): δ 7.60 (ABq, 4, J = 9 Hz, $\Delta_{\nu AB}$ = 11 Hz, ArH), 7.0–6.55 (m, 3, ArH), 4.17 (s, 2, CH_2COCl), 3.83 (s, 3, OCH_3), and 2.41 ppm (s, 3, CH_3–C=C).

Anal.—Calc. for $C_{19}H_{15}Cl_2NO_3$: C, 60.65; H, 4.02; N, 3.72. Found: C, 60.59; H, 4.08; N, 3.50.

Preparation of *N*-[1-(4′-Chlorobenzoyl)-5-methoxy-2-methyl-3-indolylacetyloxy]-*N,N*-diethylamine (Ic)—To a suspension of Ib (18.9 g, 0.048 mol) in 500 mL of ether was added *N,N*-diethylhydroxylamine (8.88 g, 0.1 mol). The mixture was stirred for 1 h at room temperature and then was filtered. The filtrate was concentrated to give solid material which was extracted with 200 mL of dichloromethane. The organic phase was washed successively with 100 mL of 0.5 M NaOH and 100 mL of water. The organic layer was separated, dried over Na_2SO_4, and concentrated to a viscous mass which was triturated with 100 mL of ether. The crystals were removed by filtration and then recrystallized from dichloromethane–ether to give 14.4 g (70% yield) of the desired product as transparent yellow prisms, mp 100–101°C. IR (KBr): 1747 cm^{-1} (s, O—C=O) and 1650 cm^{-1} (s, N—C=O); ^{1}H-NMR ($CDCl_3$): δ 7.8–7.3 (m, 4, ArH), 7.0–6.6 (m, 3, ArH), 3.83 (s, 3, OCH_3), 3.67 (s, 2, O=CCH_2), 2.89 (q, 4, J = 7 Hz, NCH_2), 2.39 (s, 3, CH_3C=C), and 1.06 ppm (t, 6, J = 7 Hz, NCH_2CH_3).

Anal.—Calc. for $C_{23}H_{25}ClN_2O_4$: C, 64.40; H, 5.88; N, 6.53. Found: C, 64.39; H, 5.86; N, 6.29.

Biological Tests—*Rat Ear Burn Test*—These results were obtained according to the method of Bronaugh *et al.* (14) where warm (51.7°C) metal cylinders were applied to the right ear of rats to cause the burn. After 1 min the burned ears were treated with 50 μL of a 0.03–0.003 M isopropyl myristate, propylene glycol solution, or a suspension of Ia or Ic. The animals were sacrificed after 10 h, circular sections of the ears were made with a leather punch, and the weights of both the right ear and the left ear pieces were determined immediately. Inflammation was determined as the increase in weight of the burned right ears compared with the weight of unburned left ears of the same animals. The percent increase in ear weight was calculated as: (weight of right ear − weight of left ear)/(weight of left ear) × 100. The percent increase in burned ear weight of animals receiving vehicle (%C) was compared with the percent weight increase in ear weight of animals receiving vehicle containing drug (%D) to give a percent inhibition of edema as follows: (%C − %D)/%C = percent inhibition of edema. The results for the comparison of indomethacin with the indomethacin hydroxylamine derivative are given in Table I.

Sunburn Test—This procedure is slightly modified from that described by Snyder (15). The differences in this procedure were the concentrations (1.1 *versus* 2.5% for Ia), amounts applied (50 *versus* 20 μL), number of applications (1 *versus* 4–19), period of observation (6 *versus* 48 h), and the fact that occlusion was used. Briefly, the test areas (4 cm^2) on the backs of human volunteers were irradiated using a bank of sunlamps. The minimum erythemal dose was determined for each subject; subsequent test exposures were 2–3 times this amount. Test formulations of Ia or Ic (0.03 M, 50 μL) or the vehicle alone were applied immediately after exposure and were partially occluded for 5 h with 2.54-cm adhesive bandages (9 spots per subject); three applications each of Ia, Ic, and vehicle in a randomized asymmetric array. Erythemal response was evaluated each hour for 6 h after exposure and graded on a scale of 0–4 (4 being a response of maximum redness). Indomethacin and Ic in the polyethylene ointment base[2] were each tested on two volunteers with the result that no difference between indomethacin and Ic could be seen (Table II). Similar results were obtained when Ia and Ic were compared in petrolatum.

Table I—Ear Burn Test: Effect of Indomethacin and Its Derivative on Inhibition of Inflammation[a]

Compound	Increase in Ear Weight, %	Inhibition of Edema	*n*
Isopropyl myristate (control)	24.1 ± 2.0	—	10
Indomethacin (0.03 M)	18.6 ± 2.0	22.8	10
Ic (0.03 M)	12.0 ± 1.6	50.2	10
Propylene glycol (control)	36.1 ± 1.8	—	18
Indomethacin			
0.03 M[b]	30.9 ± 2.3	21.6	21
0.01 M	31.9 ± 4.3	11.6	5
0.003 M	34.7 ± 3.6	3.9	16
Ic			
0.03 M[b]	28.3 ± 2.5	28.2	20
0.01 M	24.9 ± 4.0	31.0	5
0.003 M	30.5 ± 2.5	15.5	16

[a] Suspension or solutions prepared by a brief (5 min) sonication of solvent–compound mixture. Results ± *SE*. [b] Compared with a control experiment where there was 39.4 ± 2.6% increase in ear weight.

Table II—Sunburn Test: Comparison Between Indomethacin and Ic

After UV Exposure	Evaluation of Redness[a]		
	0.03 M Ic	0.03 M Indomethacin	Control
1	0	0	0
2	0.5 ± 0.8	0.8 ± 1.0	2.0 ± 1.3
3	0.7 ± 0.8	1.1 ± 0.8	2.6 ± 1.2
4	1.1 ± 0.5	1.3 ± 0.6	3.0 ± 1.1
5	1.8 ± 0.6	1.9 ± 0.8	3.5 ± 0.8
6	2.0 ± 0.6[b]	2.0 ± 0.6[b]	3.5 ± 0.8

[a] The vehicle was the polyethylene ointment base: given as 50 μL/4 cm^2 spot (n = 6). Score ± *SD*; maximum redness = 4. [b] $p < 0.005$ compared with control.

[1] TLC was run on Brinkman Polygram Sil G/UV 254. Melting points (uncorrected) were taken with a Thomas-Hoover capillary apparatus. NMR spectra were recorded on a Varian T-60 spectrometer. IR spectra were obtained on a Beckman Acculab 4 infrared spectrophotometer. Microanalyses were obtained from Midwest Microlab, Ltd., Indianapolis, Ind. The indomethacin was obtained from Sigma. The *N*-hydroxydiethylamine and the remaining reagents were obtained from Aldrich except for isopropyl myristate which was obtained from Eastman Kodak. The bulk solvents were obtained from Mallinckrodt. The mice used in the diffusion cell experiments were ICR white Swiss mice (22–25 g) from Sprague–Dawley and the diffusion cells were obtained from Kersco Engineering Consultants, Palo Alto, Calif. The HPLC system was a Waters Associates instrument using a μ-Bondapak C_{18} column. The sunlamp used was a Westinghouse FS-20.

[2] Plastibase; 5 g of polyethylene and 75 g of liquid paraffin.

Table III—Diffusion Cell Test: Comparison Between Indomethacin and Ic [a]

Hours after Application	Indomethacin Delivered, μg	
	From Indomethacin	From Ic
4	0.20 ± 0.19	0.40 ± 0.12
7	0.85 ± 0.48	1.78 ± 0.23
11	1.33 ± 0.73	2.87 ± 0.32
24	4.48 ± 1.86	8.81 ± 0.66

[a] 0.03 M in polyethylene ointment base, $n = 3$; mean ± *SD*.

Table IV—Stability of Ic in Various Solvents

Solvent	Stability (40°C)[a]	
	Amount in Solvent, mg/g	Percent Remaining
Isopropyl myristate	5[b]	79.5
Propylene glycol	3[b]	0.0
Mineral oil	1[b]	98.3
Isooctane	1	99.0
Polyethylene ointment base	10[c]	90.4
Petrolatum	10[c]	99.6

[a] After 7 d except for isopropyl myristate which was 5 d. [b] A solution of Ic. [c] A suspension of Ic.

Diffusion Cell Test—The plexiglass diffusion cells have been described previously (16) but essentially consist of a lower and an upper chamber with a side arm to allow sampling of the receptor phase (lower chamber). The mice were sacrificed by cervical dislocation. Their hair was carefully clipped and the dorsal skin removed and stretched over the receptor chamber using a rubber gasket to secure it. The receptor phase (40 mL) contained 0.9% NaCl and 0.01% thimerosal. The test formulations (0.25 mL, 0.03 M) were applied to the skin, then the entire cell was incubated at 32°C. Two-mL samples were withdrawn at the appropriate times and replaced with 2 mL of fresh receptor phase. The samples were analyzed immediately by HPLC using methanol–water with 1×10^{-3} M tetrabutylammonium perchlorate as the solvent (flow = 2.0 mL/min; absorbance at 280 nm). Samples of 500 μL were injected onto the HPLC column and eluted with a convex gradient using a commercial program[3]; 50% methanol–50% water (solvent A) and 75% methanol–25% water (solvent B) starting with 20% solvent system B and concluding with 60% solvent system B after 5 min. Under those conditions, Ia (indomethacin) had a retention time of 6.7 min while Ic had a retention time of 9.0 min. Peak heights for Ia were quantitated by comparison with standards of known concentration similarly chromatographed. In every case only Ia was observed in the receptor phase on analysis.

The comparison of the ability to deliver indomethacin from the polyethylene ointment base between indomethacin and Ic is shown in Table III. The amounts of indomethacin delivered from the application of Ic in isopropyl myristate (42.7 ± 5.4 μg of indomethacin) and petrolatum (7.9 ± 2.9 μg of indomethacin) were determined; the amounts delivered after 24 h are reported.

Physical Properties—The stability of Ic was determined by preparing solutions or suspensions of Ic in the solvents listed in Table IV. At the appropriate time samples were dissolved in or diluted with ethyl acetate and those solutions were analyzed by HPLC using the same conditions used in the diffusion cell experiments. Only Ia was observed as a decomposition product.

RESULTS AND DISCUSSION

The development of a prodrug derivative of a drug containing a specific functional group requires that the derivative of that functional group exhibit specific characteristics. The derivative should be stable enough to have a useful shelf life, yet revert quickly to the parent compound *in vivo;* the derivatizing agent should be nontoxic and impart the desired change in physicochemical properties to the parent compound. The last characteristic, in many cases, is the most difficult to predict.

N,N-Dialkylhydroxylamines appear to be attractive candidates as derivatizing agents for carboxylic acid based on the above criteria. Acyl-*N,N*-dialkylhydroxylamines are relatively stable derivatives of carboxylic acids yet are sufficiently labile to serve as activated esters in amination reactions when the reaction is catalyzed by a weak acid (17). Thus, they should be stable as long as they are kept out of contact with protic solvents, but, because they are chemically labile, they also should be labile *in vivo*. In addition, the derivatizing agents exhibit a low order of acute toxicity. *N,N*-Diethylhydroxylamine exhibits an oral LD_{LO}[4] of 1600 mg/kg in rats and a topical LD_{LO}[4] of 2000 mg/kg in rabbits (18), compared with diethylamine which exhibits an oral LD_{50} of 540 mg/kg in rats and a topical LD_{50} of 820 mg/kg in rabbits. Finally, although amines have previously been employed successfully as penetration enhancers in formulations (19, 20), derivatizing agents containing low pK_a amines [the pK_a of *N,N*-dimethylhydroxylamine is 5.2 (21) and the *O*-acylated hydroxylamine should be even less basic] have not been employed to prepare prodrugs of carboxylic acids for the purpose of improving the ability of the carboxylic acid to penetrate biological membranes. Such derivatizing agents containing a low pK_a amine group offer several potential advantages. First, the amine group generally confers to the molecule a greater ability to partition from an aqueous environment into lipids than does a carboxylic acid group (22); although it is not clear whether this tendency is due to a more favorable free energy of adsorption or dehydration contribution to the free energy of transfer, previous work (23) suggests that it is probably the latter. Second, the low pK_a amine is present in its unprotonated form (99% at pH 7.4) which is the form that undergoes partitioning (22), compared with other tertiary amines which are ~0.1% unprotonated at pH 7.4. Thus, it seemed likely that the conversion of the carboxylic acid group in nonsteroidal anti-inflammatory agents to low pK_a amine derivatives would impart the desired physical chemical properties to the derivatives.

The acylated hydroxylamine derivative (Ic) was synthesized from the reaction between Ib and two equivalents of hydroxylamine (17). Various other bases such as triethylamine or potassium carbonate were tried as substitutes for the second equivalent of hydroxylamine but the best yields were obtained with the second equivalent of hydroxylamine acting as the acid scavenger. A second route involving the use of a dehydrating agent (1,3-dicyclohexylcarbodiimide) to form the ester bond (17) has also been used to prepare these acylated hydroxylamine derivatives (13). The yields of product were generally lower using this last approach but such an approach should be more generally acceptable for carboxylic acids with sensitive functional groups.

To determine if the substitution of a carboxylic acid group by a low basicity amine group had an effect on the ability of the parent compound to penetrate biological membranes, the diethylhydroxylamine derivative of indomethacin (Ic) was compared with indomethacin in diffusion cell tests using the polyethylene ointment base as the vehicle. Almost twice as much indomethacin was delivered by the derivative Ic than by indomethacin itself (Table III, 0.03 M = ~1% Ia). In addition, only indomethacin was observed on the receptor side; no indole-chlorobenzoyl cleavage was observed. The effect of other vehicles on the delivery of indomethacin by Ic was also determined. A vehicle of petrolatum gave diffusion results that were comparable with the polyethylene ointment base (8 μg) while a vehicle of isopropyl myristate containing Ic delivered almost five times as much indomethacin as did Ic in the polyethylene ointment base. In separate experiments, ≤6% dimethylacetamide in petrolatum was used as a vehicle for the delivery of indomethacin from Ic. However, only ~25 μg of indomethacin was delivered to the receptor phase in 24 h under the best conditions using the vehicle containing the penetration enhancer compared with 42 μg of indomethacin delivered from Ic using isopropyl myristate[5]. These results show that Ic delivers indomethacin through skin better than does indomethacin.

A comparison of the stability properties of Ic in various solvents are given in Table IV. It is apparent that Ic is stable in aprotic vehicles. The relative instability of Ic in the polyethylene ointment base was attributed to acidic impurities in the ointment. The addition of an organic base such as 1-methylimidazole or inorganic bases such as magnesium oxide or zinc oxide resulted in little or no decomposition of Ic in the polyethylene ointment base being detected using the same experimental conditions[5]. In separate diffusion cell experiments zinc oxide was shown to have no effect on the delivery of indomethacin by Ic from the polyethylene ointment base vehicle[5].

The effects of Ic on inhibiting inflammation in animal models is shown in Table I. In the ear burn test, Ic was about three times as potent as indomethacin if the compounds were tested in a propylene glycol vehicle while Ic was twice as potent as indomethacin if the two compared in an isopropyl myristate vehicle. A croton oil irritation test was also used (24, 25). In that test, Ic was only marginally better than indomethacin[6], but this may have been due to the partial decomposition of Ic by the croton oil which contains an acidic component. The advantages of the ear burn model in these tests are that a comparison between vehicles can be obtained and that labile derivatives can be compared under conditions where their stability can be assured. Thus, Ic was clearly more active than indomethacin in inhibiting inflammation in animal models.

The effectiveness of Ic and indomethacin in inhibiting inflammation due

[3] The HPLC program was program 4 on a Waters Associates M660 programmer.

[4] Lowest dose at which toxic effects are observed.

[5] K.B. Sloan, unpublished results.

[6] Kanebo Company, Japan, unpublished data.

to UV-B light is shown in Table II. There was no significant difference between the two compounds at 0.03 M in petrolatum or at 0.03 M in the polyethylene ointment base. Only the results from the latter experiment are shown but the petrolatum results were essentially identical including the lag time for development of redness, although the inhibitory effect of indomethacin and Ic was apparent after 2 h, using the polyethylene ointment base formulation, instead of 3 h for the petrolatum formulation. Thus, no difference between Ic and indomethacin was observed under conditions where the diffusion cell experiments had shown that Ic delivered twice as much indomethacin through the skin as indomethacin itself (see Table III). This effect may be the result of the delivery of a maximum effective dose of indomethacin from the application of indomethacin in the polyethylene ointment base so that there was no observable effect of the excess indomethacin delivered by Ic in the polyethylene ointment base.

The initial suppression of the redness score, then the gradual approach of the redness score for the indomethacin or Ic treated areas to the redness score for the control areas after 6 h, is similar to the observations of Snyder (8) in guinea pigs and of Eaglstein *et al.* (26) in human volunteers. Thus, although an *N,N*-dialkylhydroxylamine derivative of indomethacin exhibited improved delivery of indomethacin through skin and was more effective than indomethacin in inhibiting thermal and chemical inflammation, it was no more effective than indomethacin in inhibiting the long term erythema of UV-B radiation. However, these derivatives of nonsteroidal anti-inflammatory agents may be useful in treating other inflammatory skin reactions such as allergic eczematous contact dermatitis (27) where prostaglandins have also been implicated as causative factors and insufficient nonsteroidal anti-inflammatory agent is delivered using conventional formulations and the underivatized nonsteroidal anti-inflammatory agent.

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