Cetyl Myristoleate Isolated from Swiss Albino Mice: An Apparent Protective Agent against Adjuvant Arthritis in Rats

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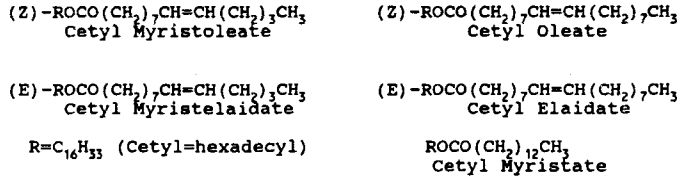
Received February 12, 1993, from the [§]*Department of Pharmacology, Medical College of Virginia, McGuire Hall, Box 613, Richmond, VA 23298.* Accepted for publication May 20, 1993[®]. [‡]Present address: 4424 Oak Hill Road, Rockville, MD 20853.

Abstract □ Cetyl myristoleate was isolated from National Institutes of Health, general purpose, Swiss albino mice that were immune to the polyarthritis induced in rats with Freund's adjuvant. This substance, or material synthesized from cetyl alcohol and myristoleic acid, afforded good protection against adjuvant-induced arthritic states in rats. In limited comparisons, cetyl oleate, also found in Swiss albino mice, gave lesser protection, whereas cetyl myristate and cetyl elaidate, the *trans*-isomer of cetyl oleate, appeared to be virtually ineffective. Dosage of the protective compound as well as the site of injection of Freund's adjuvant was important.

Although Harwick et al.[1] have produced arthritic mice by intravenous injection of *Mycobacterium pulmonis*, repeated attempts by us to induce polyarthritis in National Institutes of Health (NIH), general purpose, Swiss albino mice by subcutaneous (sc) administration of Freund's adjuvant (heat-killed, desiccated *Mycobacterium butyricum*), to which rats and certain other rodents are susceptible,[2] were unsuccessful. These failures prompted a search for a substance in these mice that might confer immunity to artificially induced, generalized arthritis. We report herein the isolation and identification of such a substance, cetyl myristoleate, as well as its limited testing against adjuvant arthritis in rats. Also tested, in limited trials, were three related esters; that is, cetyl myristate, cetyl oleate, and cetyl elaidate, the *trans*-isomer corresponding to cetyl oleate. [Because this work was carried out by a private individual (H.W.D.) with limited financial and space resources, only a relatively few experimental trials were possible and some desirable controls were omitted. It is to be hoped that our very promising but preliminary results will stimulate other investigators to repeat and extend our studies with larger test groups and more exact protocols with respect to dosages and length of trials and, particularly, more extensive tests of cetyl myristoleate analogues.]



The putative "protective factor", cetyl myristoleate, was extracted from Swiss albino mice with methylene chloride and purified by silica gel column chromatography. It was identified by alkaline hydrolysis to cetyl (hexadecyl) alcohol and myristoleic (*cis*-9-tetradecenoic) acid. The only other report[3] of cetyl myristoleate in nature is its occurrence as one component in a complex mixture of waxes in the anal gland of male beavers; that is, *Castor fiber*. Fatty acid cetyl esters are known to occur in sperm whale oil.[4]

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Experimental Section

Isolation of Cetyl Myristoleate—Eighty male Swiss albino mice,[5] weighing a total of 2300 g, were killed using chloroform and macerated, eight at a time, in a Waring blender with 400 mL of CH_2Cl_2 per group of eight. The total suspension, in two 4-L beakers, was stirred until the CH_2Cl_2 layer separated. The total mixture was filtered through a 6-mm layer of Filter-Cel, a powdered cellulose filter aid. The residue was washed with two 100-mL portions of CH_2Cl_2. The combined filtrate and washings were transferred to a large separatory funnel, and the CH_2Cl_2 layer was withdrawn from a small volume of aqueous layer, filtered again, and concentrated under reduced pressure to a mobile oily residue (167 g), which was treated with 670 mL of acetone and left at −5 °C for 3 days with brief stirring each day. This mixture was filtered by light suction through a 3-mm layer of Filter-Cel. The residue was washed with four 25-mL portions of cold (−5 °C) acetone. The combined filtrate and washings were evaporated under reduced pressure to an oil (121 g) that was dissolved in 50 mL of pentane (bp, 20–40 °C):diethyl ether (20:1) and chromatographed on 2500 mL of 70–325 mesh silica gel in a 25 × 14-cm column with pentane: ether (20:1) as eluate. After collecting 1500 mL of eluent, eleven 100-mL and six 200-mL fractions were collected. Fractions 3–15 were combined and filtered under suction through 120 g of Darco-X decolorizing carbon, which in turn was washed with several portions of CH_2Cl_2. The combined filtrate and washings were evaporated under reduced pressure to a residue (0.8 g) that was rechromatographed with a column (129 × 7.5 cm, 350 mL) of 70–325 mesh silica gel and CCl_4: ether (40:1) as eluate. Fractions 67–79 (6 mL each) gave 0.4 g of material that was rechromatographed [43 × 2.5-cm column of 125 mL of silica gel; CCl_4: ether (60:1)] with 2.5-mL fractions collected. Fractions 117–127 gave an oil (0.15 g) that proved to be principally cetyl myristoleate, identified as described below. The preceding fractions (105–115) provided 0.07 g of cetyl oleate.[6] Bioassays in arthritic rats were used throughout to monitor the isolation and purification of the "protective factor" after it was established that the crude or partially purified mouse extract did indeed prevent adjuvant-induced arthritis in rats (see *Biological Testing of Cetyl Myristoleate* below). TLC was used to correlate this biological activity with the silica gel column fractions. Activity was found in a nonpolar fraction of three closely migrating components, with maximum activity shown by the material of intermediate retardation factor (R_f) in the TLC system cyclohexane:isopropyl ether (9:1). This proved to be a mixture of cetyl oleate and cetyl myristoleate, separable by TLC in another solvent system.[6,7] Cetyl myristoleate was also isolated from nine wild mice, and its presence in wild mice was also be demonstrated in two other experiments by TLC.[7]

Anal.—Calcd for $C_{30}H_{58}O_2$: C, 80.0; H, 12.9. Found: C, 78.6; H, 12.9.

Chemical Identification of the Protective Factor, Cetyl Myristoleate—A mixture of 0.15 g (0.33 mmol) of cetyl myristoleate, obtained as described above, 2 mL of acetone, and 3 mL of 10% aqueous NaOH was refluxed with stirring for 14 h, treated with 10 mL of H_2O and 0.7 mL of 12 M HCl, and extracted with four 5-mL portions of CH_2Cl_2. After drying with sodium sulfate, filtration through Filter-Cel, and evaporation of the CH_2Cl_2 under reduced pressure, 0.14 g of residue was obtained and was chromatographed on a column (30 × 2.5 cm, 140 mL) of silica gel with CH_2Cl_2 as eluate. One 400-mL and 110 6-mL fractions were collected. Fractions 20–55 gave 40 mg (50%) of cetyl alcohol [melting point (mp), 49–50 °C after recrystallization from ethanol]. On admixture, this material did not change the mp of authentic cetyl alcohol and had the same R_f (0.46) on TLC (70:30:2, pentane: ether:acetic acid).

Anal.—Calcd for $C_{16}H_{34}O$: C, 79.3; H 14.1. Found: C, 79.2; H 14.3.

Fractions 89–100 yielded 60 mg (80%) of an oil that was further purified by chromatography with CH_2Cl_2:ether (3:1) and identified[8] as myristoleic (*cis*-9-tetradecenoic) acid.

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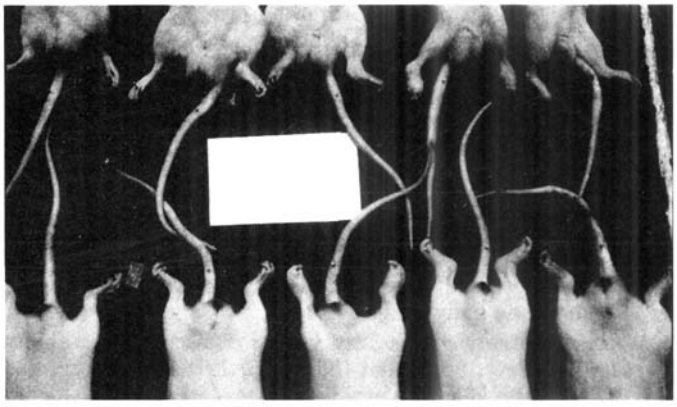

Figure 1—Sprague-Dawley rats 58 days after sc injection of Freund's adjuvant in the tail.

Figure 2—Enlarged view of several rats shown in Figure 1.

Anal.—Calcd. for $C_{14}H_{28}O_2$: C, 74.3; H, 11.6 (neutralization equivalent, 226.4). Found: C, 74.1; H, 11.7 (neutralization equiv., 225.5).

Synthesis of Cetyl Myristoleate—A mixture of cetyl alcohol (150 mg, 0.62 mmol), 140 mg (0.62 mmol) of myristoleic acid, 50 mg (0.3 mmol) of *p*-toluenesulfonic acid, and 20 mL of benzene was refluxed (Dean-Stark water trap) for 4 h. The solution was washed with 10% aqueous NaOH, dried with Na_2SO_4, and evaporated under reduced pressure to give 300 mg of mobile oil, identical to cetyl myristoleate[9] isolated as described above. Cetyl myristoleate was also prepared from cetyl alcohol and myristoleic acid obtained from the hydrolysis of the ester isolated from Swiss albino mice.

Anal.—Calcd for $C_{30}H_{58}O_2$: C, 80.0; H, 12.9. Found: C, 79.3; H, 13.2.

Attempts to Induce Arthritis in Swiss Albino Mice—Ten normal NIH, general purpose, male Swiss Albino mice, weighing 19–22 g, were injected sc with 200 μg of heat-killed, desiccated *Mycobacterium butyricum* [Bacto M. Butyricum (Difco 0640-33), Freund's adjuvant] in 0.1 mL of light mineral oil (Carroll Chemical Company, Baltimore, MD) in the tail 5 mm from the point of attachment to the body. In a period of 10–20 days, no noticeable swelling developed in the legs or paws. A second group of five mice were each injected with the same quantity of Freund's adjuvant in the left hind paw. Again, after 10–20 days, no swelling was detected as determined by comparison of the measurements of the paws made with a small metric tape at the time of injection.

Biological Testing of Cetyl Myristoleate—A group of 10 male rats (Sprague-Dawley strain from Hormone Research Labs., Inc., Chicago, IL), weighing 160–180 g, were each injected (1.3 cm, #26 needle) parenterally at the top of the rump with 440 mg of a nonpolar fraction containing cetyl myristoleate (fractions 67–79 obtained from Swiss albino mice as described above) in 1.0 mL of light mineral oil. Forty-eight hours later, the same rats were inoculated with Freund's adjuvant (1.0 mg of Bacto M. Butyricum in 1.0 mL of light mineral oil, slightly warmed). The sc injection was made in the tail ~1.9 cm from the point of attachment to the body. A control group of nine rats received only Freund's adjuvant. Both groups were observed for a total of 58 days with respect to weight change, hind and front leg swelling (hind legs were measured just below the knee, front legs just above the paw), and general well-being. The results after 58 days are shown in Figures 1–3 and data are summarized in Tables 1 and 2.

Synthetic cetyl myristoleate was tested similarly. Groups of four and five rats were studied for 26 and 28 days, respectively, after an injection into each animal of 75 mg of synthetic cetyl myristoleate 48 h before inoculation with Freund's adjuvant (Table 1).

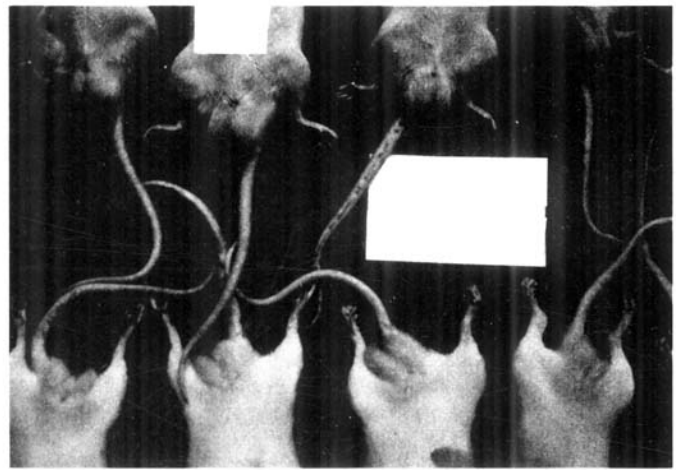

Figure 3—Sprague-Dawley rats 58 days after sc injection of Freund's adjuvant in the tail preceded 48 h earlier by sc injection with cetyl myristoleate (isolated from Swiss albino mice) in the rump.

Table 1—Comparison of Leg Size and Weight Gain in Cetyl Myristoleate-Treated and Control Rats

	Leg Size (circumference), cm				
	Front		Hind		
Treatment	Right	Left	Right	Left	Weight Gain, g
control[a]	2.8 (0.05)	3.1 (0.14)	5.2 (0.18)	5.3 (0.18)	25.8 (7.0)
Treated[b]		Normal			80.0 (11.6)
Treated[c]		Normal			104
Treated[d]		Normal			98

[a] Non-treated (control) rats were a group of nine injected 33 days earlier with Freund's adjuvant only. SEM in parentheses. Rats shown in Figures 1 and 2 at 58 days. [b] Treated rats were a group of 10 injected at 2500 mg/kg with an extract containing natural cetyl myristoleate isolated from Swiss albino mice, followed 48 h later by injection with Freund's adjuvant. Data obtained 33 days later. Rats shown in Figure 3 at 58 days. A one-tailed p value of <0.0001 was obtained comparing the average of right and left hind leg circumferences of nine non-treated rats with averaged hind leg circumferences of four treated rats selected at random. [c] Five rats were injected with synthetic cetyl myristoleate at 450 mg/kg, followed 48 h later by injection with Freund's adjuvant. Data obtained 28 days later. [d] Four rats were injected with synthetic cetyl myristoleate at 450 mg/kg followed 48 h later by injection with Freund's adjuvant. Data obtained 26 days later.

The same procedure was used (except that Freund's adjuvant was sometimes injected 24 h, rather than 48 h, later) with doses containing partially purified mouse extract in a bioassay. In one trial, after injection into rats at 2500 mg/kg of a partially purified extract containing all four nonpolar components, no leg swelling and normal weights were observed in seven out of 10 rats when examined 23 and 33 days after inoculation with Freund's adjuvant. Here, the amount injected per rat is roughly equivalent to 0.1 the total amount of nonpolar material obtained from 80 mice. In other trials, the substance, corresponding to the lowest R_f component of the four detected by TLC in nonpolar fractions of the mouse extract, gave no protection at 1350 mg/kg against adjuvant-induced arthritis in five rats after 15 days. Also, five rats similarly treated with the highest R_f materials in the nonpolar fraction at 600 mg/kg likewise received no protection after 15 days.

Biological Testing of Other Cetyl Esters:—The procedure was the same as above except that the dosage of cetyl oleate, cetyl elaidate, or cetyl myristate was 1.5–2.5 times higher than that used for cetyl myristoleate (Table 3).

Table 2—Comparison of Weight Gains between Cetyl Myristoleate-Treated and Non-Treated Rats

Days[a]	Treated[b]	Non-Treated[c]	P (× 10^2)[d]
10	50.0 ± 4.0	11.4 ± 6.2	0.10
13	57.3 ± 5.7	13.4 ± 6.2	0.09
16	51.7 ± 7.4	12.8 ± 5.7	0.78
23	57.9 ± 10.7	13.5 ± 7.2	1.96
33	80.0 ± 11.6	25.8 ± 7.0	0.58
46	110 ± 11.0	61.1 ± 9.6	1.80
58	125 ± 11.0	82.8 ± 10.3	5.18

[a] Days from injection of Freund's adjuvant. [b] Average weight gain (g) ± SEM for a group of 10 rats, weighing 175–180 g at the start and treated 48 h earlier with 2500 mg/kg of a partially purified mouse extract containing cetyl myristoleate (rats shown in Figure 3). [c] Average weight gain (g) ± SEM for a group of nine rats, weighing an average of 188 g at start, and given Freund's adjuvant alone (rats shown in Figures 1 and 2). [d] The two-tailed p value, resulting from a parametric T test with unpaired values.

Table 3—Comparison of Leg Size and Weight Gain in Rats Treated with Reduced Dosages of Cetyl Myristoleate and Increased Dosages of Other Cetyl Esters

Cetyl Ester	Dose, mg/kg	Number of Rats	Days[a]	Rats with Swollen Legs	Weight Gain[b]
Cetyl myristoleate	290[c,d]	5	24	3	56
			29	3	68
			32	2	75
Cetyl myristate	650	4	23	4	34
Cetyl oleate	370[c,d]	5	24	5	45
			33	4	59
	1100	5	24	3	49
	1170	4	24	3	64
Cetyl elaidate	1200	5	24	3	87
Cetyl oleate plus cetyl myristoleate (1:1)	700[c]	5	23	2	56
			26	2	69

[a] Number of days between injection of Freund's adjuvant and observations. [b] Rats were weighed (g) as a combined group at the beginning and end of the period of observation; therefore, weight gains and doses are averages. [c] Natural material used; synthetic esters were used in all other cases. [d] Freund's adjuvant injected 24 h earlier rather than the usual 48 h earlier.

Results and Discussion

As seen in Figures 1 and 2, all rats receiving Freund's adjuvant alone developed severe swelling of the hind legs. This is first observed 10–13 days following the injection of Freund's adjuvant. The legs averaged 5.2 cm in circumference compared with 2.3 cm for normal rats (Table 1). These rats gained only an average of 14 g each during a 32-day period of observation and were lethargic and morbid (Table 2). In several trials (data not shown), rats lost weight steadily between days 8 and 21, then began to gain at a slowly increasing rate. Those receiving Freund's adjuvant plus cetyl myristoyleate isolated from Swiss albino mice gained an average of 80 g each in the same 32-day period and in almost every instance, there was little, if any, evidence of swelling or other symptoms of polyarthritis (Figure 3, Table 1). The weight gain difference per rat between the two groups averaged 40–50 g, reached a maximum at 33 days, and then declined slightly up to the 58th or last day of observation (Table 2). It was consistently found that rats with swollen legs were invariably of lower weight, typically 50 g lighter, than those with no swelling.

Synthetic cetyl myristoleate prevented swelling at 430–450 mg/kg in all rats challenged with Freund's adjuvant. Here even greater weight gains were observed after a 26–28-day period (Table 1).

In separate experiments, livers of two of the arthritic rats were excised 30 days after injection of Freund's adjuvant and were found increased in weight by 32% over livers of untreated rats of the same body weights.

Analogues of cetyl myristoleate were also tested for their ability to retard or prevent adjuvant-induced arthritis in rats. Tested were the saturated ester, cetyl myristate, and two homologues [cetyl oleate, having the same 9–10 position of its *cis* (*Z*)-double bond as present in cetyl myristoleate; and cetyl elaidate, the *trans* (E)-isomer corresponding to cetyl oleate]. In very limited preliminary trials, only cetyl oleate had any significant activity, though much less than cetyl myristoleate (Table 3). A 1:1 mixture of cetyl oleate and cetyl myristoleate (700 mg/kg total dose) gave results not greatly different from cetyl myristoleate alone at 350 mg/kg.

That the protection given by cetyl myristoleate was dose related is suggested by the fact that at 250–300 mg/kg, only two of five treated rats were free of symptoms. In the case of cetyl oleate, 1 g/kg gave protection to two of five treated rats, whereas 370 mg/kg gave little or no protection. Additional work with larger test groups is necessary to confirm these dose-related responses.

Two sets of five-rat trials with no mineral oil were performed to rule out any effect of the vehicle. At doses of 500 mg/kg of neat synthetic cetyl myristoleate followed 48 h later by inoculation with Freund's adjuvant, all five rats in one trial appeared normal at 32 days and had gained an average of 119 g each. In another trial at 400 mg/kg, two rats had slightly swollen hind legs at 32 days and the other three were normal.

In conclusion, it is apparent that cetyl myristoleate alone, of the four fatty acid esters tested, gave virtually complete protection against adjuvant-induced arthritis in rats when administered sc neat or in mineral oil, at a dose of 450–500 mg/kg, 48 h before inoculation with Freund's adjuvant. It seems significant that cetyl myristoleate can be isolated from Swiss albino mice immune to adjuvant arthritis but not from susceptible rats.[10] The minimum levels of cetyl myristoleate in a mouse can be estimated at 350 mg/kg.

The elapsed time between administration of cetyl myristoleate and Freund's adjuvant is evidently not very critical nor is the order of these injections. In one experiment, a partially purified extract containing cetyl myristoleate was administered at 2200 mg/kg to a group of nine rats, four days after the injection of Freund's adjuvant. It still provided complete protection to five of the nine when observed 29 days later.

Cetyl myristoleate injected at the top of the rump does not reach either the hind or front paws in amounts detectable by TLC during 10 days, although significant amounts of mineral oil do. Cetyl myristoleate was accumulated in other parts of the rat, with highest concentrations, along with mineral oil, in the liver. This may be significant in the observed biological function of cetyl myristoleate in preventing adjuvant-induced arthritis in the rat.

It should be emphasized that the site of administration of Fruend's adjuvant appears to be critical. Positive results were obtained by us on administration of cetyl myristoleate at the top of the rat's rump 48 h before injection of Freund's adjuvant in the tail -2 cm from the body. No protection was observed when cetyl myristoleate was administered in the rump 48 h before injection of the adjuvant to the foot.[11]

References and Notes

1. Harwick, H. J.; Kalamanson, G. M.; Fox, M. A.; Guyre, L. B. *Proc. Soc. Exp. Biol. Med.* **1973**, *144*, 561–563.
2. Winter, C. A. *Fortschritte der Arzneimittelforschung* **1966**, *10*, 139–203.
3. Groenneberg, T. O. *Chem. Scr.* **1979**, *13*, 56–58; *Chem. Abstr. 91* 190204u.
4. A sample of spermaceti oil from the case in the head of the sperm whale was provided by Dr. G. R. Peppit, Cancer Research Institute, Arizona State University, Tempe, AZ) and was examined by GC and direct-probe, chemical-ionization MS and found to contain ~14% cetyl myristoleate or an isomer [identification by retention time and M+1 (451) intensity only]. It should be noted that *cis*-5-tetradecenoic acid, physteric acid, has been reported in a sperm whale head oil hydrolyzate. At the present time we have not ruled out a cetyl ester of this acid in our sample of spermaceti oil.
5. The Swiss albino mice were raised by the National Institutes of Health Animal Laboratory and had been used in the testing of analgesic compounds before being sacrificed.
6. The total amount of cetyl oleate in the CH_2Cl_2 extract is estimated by TLC to be roughly one-half that of cetyl myristoleate. It was likewise identified by alkaline hydrolysis to cetyl alcohol and oleic acid: TLC R_f of cetyl oleate is 0.41 (CH_2Cl_2:CCl_4; 5:1) or 0.73 (1:3).
7. Silica gel TLC plates (Analtech, 0.25 mm) were developed with 1:5 or 3:1 CH_2Cl_2:CCl_4. Cetyl myristoleate had R_fs of 0.31 and 0.65, respectively, in these systems. Spots were detected with a 3% sulfuric acid spray and heat.
8. The IR spectrum of this acid [υ_{max} (neat): 1712, 1722 (shoulder) cm^{-1}] was identical to authentic material purchased from Nu Chek Prep., Inc., Elysian, MN.
9. Purified cetyl myristoleate from mouse has a retention time of 7.66 min on a 25-m fused silica capillary GC column (HP-1), a value that is identical (within experimental error) to that (7.63 min) found for synthetic cetyl myristoleate. The electron-impact mass spectrum (VG 7070 instrument) for the mouse ester [450(42), 273(3), 269(5), 227(28), 208(100), 190(12), 180(4), 179(4), 166(22), 164(14), 152(8), 151(8)] was likewise identical to that of the synthetic material. Chemical-ionization mass spectrometry with ammonia gave a molecular ion of m/z 468, $M + NH_4^+$, indicating a molecular weight of 450 for both the natural and synthetic ester. A synthetic sample of cetyl myristelaidate (i.e., the *trans*-isomer) was prepared from cetyl alcohol and myristelaidatic acid obtained from Nu Check Prep., Inc. It was inseparable from cetyl myristoleate on the above capillary GC column. It does however have a slightly different electron-impact mass spectrum showing additional weak ions at m/z 297(1), 257(2), and 173(5), which are missing in the spectrum of cetyl myristoleate. A Fourier transform IR (FT-IR) spectrum (neat, Biorad FTS-45 instrument) also indicated differences between cetyl myristelaidate and cetyl myristoleate (e.g., a strong *trans* vinyl $\delta_{CH=}$ of 964 cm^{-1} and a weak band at 667 cm^{-1} are present in cetyl myristelaidate but absent in cetyl myristoleate). The FT-IR spectra of cetyl myristoleate and the mouse factor were, however, identical (e.g., υ_{max} = 1740, 1465, 1246, 1178, 1123, 1089, and 720 cm^{-1}). An ^1H NMR ($CDCl_3$, 300 MHz) spectrum of the mouse ester is entirely consistent with the cetyl myristoleate structure. In particular, two vinyl protons at δ5.24–5.28 (seven-line multiplet, $J = 1.8 \pm 0.1$ Hz) are seen that fit a *cis* olefin structure only.
10. Attempts to obtain cetyl myristoleate from Sprague-Dawley rats by CH_2Cl_2 extractions were unsuccessful. Cetyl myristoleate could not be detected by TLC.
11. Private communication, A. H. Robbins Company, Richmond, VA.

Acknowledgments

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