9(Z)-Octadecenamide and Fatty Amides by *Bacillus megaterium* (B-3437) Conversion of Oleic Acid

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9(Z)-Octadecenamide, hexadecenamide, tetradecenamide and tetradecanamide were produced by a novel bioconversion of oleic acid with *Bacillus megaterium* NRRL B-3437. Although chemical synthesis is more practical, the bioconversion to fatty amides (5–7% of total recovered lipids) was unique for its requirement of both enzymatic catalysis and equimolar oleic acid/ammonium salt substrates. Purified octadecenamide was obtained by silica gel and high-pressure liquid chromatographic procedures and was characterized by gas chromatography, mass spectrometry, infrared and nuclear magnetic resonance.

KEY WORDS: *Bacillus megaterium* (B-3437), bioconversion, fatty amides, hexadecenamide, 9(Z)- octadecenamide, oleic acid.

Oleic [9(Z)- octadecenoic] acid is a valuable industrial product retrievable from many natural sources, including corn and soybean oils (1,2). Moreover, microbial conversions of oleic acid have produced a variety of value-added hydroxylated fatty acids that may be utilized commercially (1–4). Bacilli (5,6) are particularly useful for oxidizing the distal ω-ends of oleic and other aliphatic acids to produce unsaturated and hydroxylated fatty acids. A limited survey of bacterial cultures has now uncovered a *Bacillus megaterium* (B-3437) that converts oleic acid to octadecenamide and shorter chained fatty amides.

Analogous to oleic acid, erucic [13(Z)-docosenoic] acid is a valuable component extracted from crambe and rapeseed (7). Random selection of a few patents on erucamide (8–11), a 13(Z)-docosenamide chemically derived (12) from erucic acid, discloses diverse applications as antifriction lubricants, chemical additives, nonsticky and protective coatings, and as an angiogenic agent. Though synthesis of specific fatty amides is more practical and efficient by chemical processes, the biocatalysis by B-3437 discloses a novel alternative. Accordingly, octadecenamide and other fatty amides derived from seed oil may be potential alternatives to erucamide. This report indicates that octadecenamide has physicochemical properties similar to erucamide and can be produced by a biotransformation of oleic acid.

EXPERIMENTAL PROCEDURES

Microorganisms. Bacillus megaterium strains NRRL B-3437, BD-297, B-1369 and B-14308 (syn. ATCC 14581) and *Pseudomonas* sp. B-2994 were obtained from L.K. Nakamura (Agricultural Research Service Culture Collection, Peoria, IL). Inocula were incubated at 25°C in 125-mL flasks containing 50 mL of tryptone/glucose/yeast extract (TGY) medium at pH 7.0 and shaken aerobically at 200 rpm.

Biochemicals. Technical-grade oleic acid (95% purity), purchased from Fisher Scientific Co. (Fairlawn, NJ) and Sigma Chemical Co. (St. Louis, MO), and all other reagent-grade chemicals were used without further purification. Erucamide and stearamide (octadecanamide) were purchased from Aldrich Chemical Co. (Milwaukee, WI). All other fatty acid compounds were purchased from Sigma Chemical Co.

Cell production, bioconversion and extraction. The complete growth medium contained (per L): 4 g dextrose, 2 g yeast extract, 4 g K_2HPO_4, 0.2 g NH_4Cl and 0.25 g $MgSO_4 \cdot 7H_2O$. All media were adjusted to pH 7.2 with 5N H_2SO_4. A medium with twice the level of Mg salt and other trace minerals (13) was found to be ineffective. Other parameters of growth were also varied to obtain suitable amide-synthesizing conditions. A fresh, fully-grown culture in TGY medium that gave maximum turbidity within 24 h was inoculated (1% vol/vol) into 100 mL of fermentation medium contained in a 500-mL Erlenmeyer flask and incubated at 28°C. After 24 h growth, cultures were readjusted to approximately pH 7 with sterilized 2N NaOH. Oleic acid (0.7 mL or 0.63 g) and ammonium chloride (0.14 g) were then added to each flask. The 1:1 molar ratio of oleic/ammonium was changed to 0:1, 2:1 and 1:0 to determine the effect on amide production. Each set of the compared bioconversions was harvested on the same day, two to six days following oleic acid additions.

After readjustment to approximately pH 7, the fermentation broths were harvested by extracting twice with equal volumes of either 10% methanol in ethyl acetate (1) or diethyl ether. Broths were not acidified because extractions for amides were not intended for simultaneous recovery of free fatty acids. The solvent phase and fermentation broth often formed emulsions that were broken by centrifugation. The upper solvent phases were then carefully decanted or pipetted off and concentrated in a rotary vacuum evaporator.

Chromatographic separation. Bioconversions in fermentation extracts were detected by gas (GC), thin-layer (TLC) and silica gel chromatographies. Aliquots of lipid samples were dried, esterified with diazomethane and analyzed (2 mg/mL of 20% methanol in ethyl acetate) by GC as mixtures of volatile amides and methyl esters (SPB-1 capillary column, 15 m × 0.32 mm i.d.). Purity of amide samples was estimated from the area % of total peak areas; yields were estimated from area % × mg dry wt of sample.

Initially, amides were detected by TLC on Silica Gel 60 plates (EM Science, Cherry Hill, NJ) developed with the solvent mixture hexane/ethyl acetate/acetic acid (50:50:1, vol/vol/vol). The respective R_f values of standard oleic acid, 12-hydroxystearic acid, erucamide and stearamide were 0.54, 0.40, 0.20 and 0.20, respectively. Unsaturated compounds were made visible by exposure to iodine vapor; all other spots were detected by charring at 140°C after spraying with sulfuric acid.

The crude, dried extracts (approximately 1 g) were mixed with small volumes of hexane/ethyl acetate (90:10, vol/vol) and separated by column chromatography over 40 mL packed-volume of Silica Gel 60 (230–400 mesh; EM Science, Gibbstown, NJ). Samples were eluted with six column volumes of solvent sequentially: (i) 90:10, (ii) 80:20 and (iii) 50:50 mixtures of hexane/ethyl acetate; (iv) pure ethyl acetate; and (v) 90:10 (vol/vol) ethyl acetate/methanol. Although the amides dissolved readily in 80:20 hexane/ethyl acetate, they did not elute in fraction (ii), but

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eluted only in fractions (iii) and (iv). Samples of each eluent fraction were methyl-esterified and analyzed by GC with flame-ionization detection (FID).

Mixed amides fractionated over silica gel (100–600 mg) were separated further into specific amides through preparative high-pressure liquid chromatography (HPLC). Samples in ethyl acetate/hexane/trifluoroacetic acid (75:25:0.1, vol/vol/vol) were chromatographed isocratically through a Dynamax-60A silica column (25 cm × 41 mm i.d.) (Rainen, Woburn, MA), and the eluent was monitored with a refractive index detector. Amide-containing eluents detected by the change of refractive index were collected as separate peak fractions and concentrated to dryness before weighing.

Fatty amide analysis. Standard erucamide and stearamide in 20:80 methanol/ethyl acetate solutions were analyzed directly by GC. Separations were conducted with a GC temperature program: initial temperature of 185°C for 20 min, then a 5°C/min gradient to 220°C, which was held for 15 min. For GC–mass spectrometry (MS) (70 eV), a fused-silica capillary column (25 m × 0.32 mm i.d.) coated with methyl silicone was utilized for similar temperature-programmed separation (160–250°C) of amides.

Samples, fractionated by HPLC and giving GC peak areas better than 90% as a single component, were characterized further. Amide bonds and *cis-trans* configurations were deduced from infrared (IR) spectra of samples that were prepared as dry films on a KRS-5 plate (Wilks Scientific Corp., Southwalk, CT). The ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra of samples in $CDCl_3$ solutions were obtained with a Bruker ARX400 Spectrometer (Burlington, Ontario, Canada).

RESULTS

Bioconversion required for amide production. When equimolar oleic acid and ammonium chloride were added to fully grown *B. megaterium* (B-3437) cultures, approximately 5% of the recovered lipids were detectable as fatty amides after two- to six-day bioconversions. In the fermented broths of 17 aerobic cultures, the preponderant recovered lipid (73% mean and 65–86% range by GC) was unconverted oleic acid. For oleic acid with >95% purity, the recovered oleic acid content was 93% of the peak areas. Total lipids recovered averaged either 62 wt% (diethyl ether extracts) or 44 wt% (ethyl acetate extracts) of the added oleic acid. The batch fermentations ranged from pH 5.1–7.0, ending usually at about pH 6. The GC analyses indicated formation of a product with the same retention time as octadecenamide in yields from 0.9–3.0% (1.4% mean, ether extraction) or 2.0–3.4% (2.5% mean, ethyl acetate extraction). If other amide peaks detectable in the fermentation broths (range 0–3.8%, e.g., see Fig. 1) were taken into account, the overall conversion of oleic acid was estimated to be 5–7%.

When conditions of the bioconversion were changed, strain B- 3437 produced the amides more readily at 25–28°C and did not require metal ion supplements. Regardless of such supplements, the octadecenamide product was approximately 1%. Both *B. megaterium* B-3437 and oleic acid were specifically required to produce the amide (Table 1). Other *B. megaterium* strains and a *Pseudomonas* sp. did not produce detectable amounts of amides.

Oleic acid (6.3 g/L medium) and ammonium chloride (1.4 g/L) supplements were both necessary for enhanced amide production. Addition of 5, 10 and 15 g yeast extract per L medium, in the absence of ammonium chloride supplement, gave moderate increases of 0.1, 0.5 and 0.9% octadecenamide, respectively. Also, those concentrations of yeast extract and 630 mg oleic acid per 100 mL medium

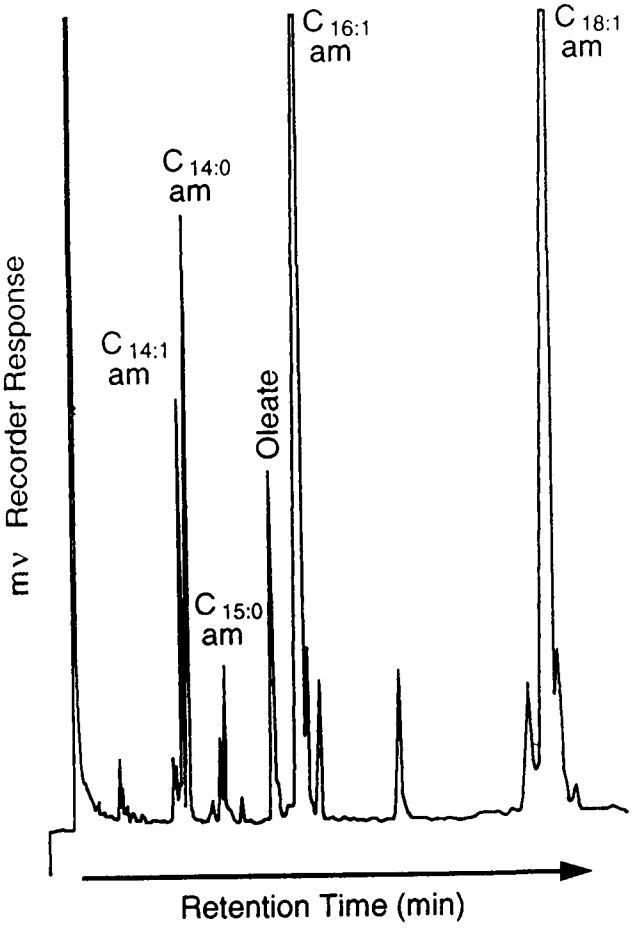

FIG. 1. Gas chromatography (GC) of mixed amides and methyl esters from bioconversion of oleic acid by *Bacillus megaterium* B-3437. Lipid extract was concentrated for amides by silica gel chromatography [fraction (iv)], and GC peaks identified by GC/mass spectrometry: 3.7 area% $C_{14:1}$ am (tetradecenamide, by conventional abbreviations), 5.0% $C_{14:0}$ am, 1.8% $C_{15:0}$ am, 4.5% Me oleate; 25.2% $C_{16:1}$ am, 39.9% $C_{18:1}$ am.

TABLE 1

Bioconversion of Oleic Acid[a] to Fatty Acid Amides

Condition	Lipid recovered (mg)	% Fatty amides[b]	
		$C_{18:1}$ am	$C_{16:1}$ am
Oleic acid omitted	5	2.6	—
Cells omitted[c] (B-3437)	388	0.0	0.0
Complete[a]	440	3.4	3.8

[a]Lipids recovered after 630 mg/L oleic acid and 140 mg ammonium chloride added to day-old cultures (1 L); subsequently incubated for additional 4 d at 28°C before extraction.
[b]Percentage of total peak areas as determined by gas chromatographic analyses. $C_{18:1}$ am, octadecenamide; $C_{16:1}$ am, hexadecenamide.
[c]*Bacillus megaterium* NRRL B-3437.

led to 297, 25 and 29 mg recovered lipids after 4 d bioconversions. Apparently, yeast extract, which enhanced the B-3437 oxidation of oleic acid, also contained small amounts of free ammonia available for amide bioconversions. Glucose (1–15 g/L) without ammonium supplements also gave a constant recovery of lipids (380–400 mg) without increases of octadecenamide product (0.28–0.57%). Yields of amide were also sensitive to the molar proportion of oleic/ammonium supplements. When oleic/ammonium substrate ratios fluctuated from 1:2, 1:1 and 2:1, yields of octadecenamide were 0.25, 0.8 and 0.25%, respectively.

Separation and concentration of amides. The ethyl acetate extracts of fermentation broths were pooled together, concentrated to dryness and chromatographed (1-g batches) over Silica Gel 60 columns. Five independent batches gave mean recoveries of 91 wt% total lipids and 8.2 wt% amides. The GC analyses of amide-methyl ester mixtures (Fig. 1) indicated that fraction (iii) eluents from silica gel columns contained 31% octadecenamide and 26% hexadecenamide. Fraction (iv) eluent contained both products in 18% concentrations.

The amide fractions from silica gel columns gave an intense iodine-absorbing TLC spot at R_f 0.20, similar to erucamide. By contrast, the crude extract from fermentation broths gave spots at 0.28 (tail), 0.54 (oleic acid) and 0.70. The amides were detectable only at >25% concentration in the samples. Therefore, a wide range of amides gave only a single TLC spot at R_f 0.20 and were detectable by their unsaturated bonds.

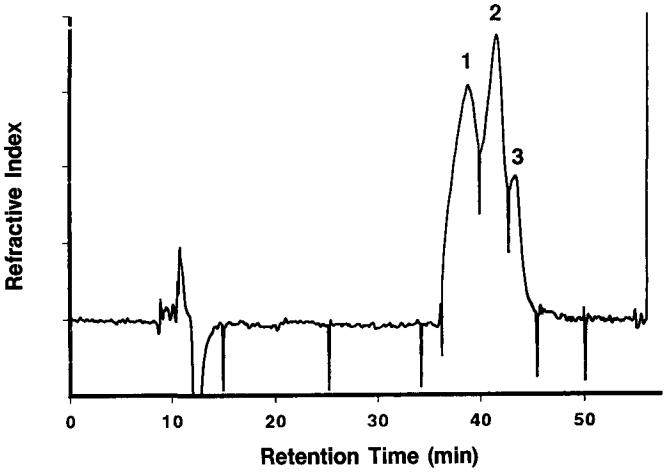

FIG. 2. High-pressure liquid chromatography of pooled fractions (iii) and (iv) obtained from silica gel chromatography; partly resolved as Peaks 1 (octadecenamide), 2 (hexadecenamide) and 3 (mixed tetradecan- and tetradecenamide).

Amide concentrates (fractions iii and iv) were pooled and purified further by HPLC (Fig. 2). Octadecenamide (Peak 1) was separated with >90% purity. Unresolved Peaks 2 and 3 were mixtures of octadecenamide (2–8%), hexadecenamide (37%), tetradecanamide (24–28%) and tetradecenamide (15–30%). Another sample of a partly resolved Peak 2 contained approximately 81% hexadecenamide, whereas Peak 3 contained a mixture of 37% tetradecenamide and 49% tetradecanamide.

Characterization of fatty amides. A concentrate of Peak 1, which contained approximately 90 area% octadecenamide (GC analysis), was passed through a 3-g Silica Gel 60 column a second time before characterization. Physicochemical properties of the purified 9(Z)-octadecenamide (wax-like solid, m.p. 33–41°C) were then compared with standard erucamide (m.p. 79–81°C). The IR spectrum indicated characteristic absorptions comparable to erucamide: 3350 and 3180 [NH_2 bonded], 3010 [CH stretching], 1650 [C=O primary amide I], 1630 [NH_2 primary amide II]. In contrast to unsaturated amides, saturated octadecanamide lacked absorption at 3010 cm^{-1}. A *trans* configuration was also unlikely because the purified compound lacked a 970 cm^{-1} absorption band.

In addition to ^1H NMR (Table 2), the carbon assignments for the ^{13}C NMR signals are as follows: 176.1 ppm, C-1[C=O]; 35.9, C-2[CH_2-C=O]; 25.5, C-3[CH_2CH_2C=O]; 29.1–29.7, C-4 to C-7 and C-12 and C- 15[CH_2]; 27.1, C-8 and C-11[CH_2CH=CH]; 129.7, C-9[CH=CH]; 129.9, C-10[CH=CH]; 31.8, C-16[$CH_2CH_2CH_3$]; 22.6, C-17[CH_2CH_3]; 14.0, C-18[CH_3]. The proton resonances were consistent with a 9,10-double bond position. Coupling constants suggest a retention of *cis* unsaturation.

In MS analyses, octadecenamide gave both the expected molecular ion (281 in contrast to octadecanamide molecular ion 283), and distinct *m/z* fragment ions: 264 [M − 16 − 1] and 222[M − 58 − 1]. Analogous to octadecenamide, distinctive ion fragments observed with standard erucamide [molecular weight (MW) 337] included *m/z* 320 and 277. The long chained amides gave a most prominent fragment at *m/z* 59, whereas methyl oleate had a 100% relative intensity fragment at *m/z* 55.

Other shorter chained homologs of octadecenamide, hexadecenamide and tetradecenamide were detected in

TABLE 2

Signals of ^1H Nuclear Magnetic Resonance (400 MHz) Resonances in $CDCl_3$

(18) (17) (16) (11) (10) (9) (8) (3) (2) (1)
$CH_3 - CH_2 - CH_2 - (CH_2)_4 - CH_2 - CH = CH - CH_2 - (CH_2)_4 - CH_2 - CH_2 - CO - NH_2$

δ (ppm)	Signal	Integration	*J* Hz	Functional group
0.88	Triplet	(3H)	7	CH_3 terminal (18)
1.24–1.28	Multiplet	(18H)		$(CH_2)_x$ chain
1.60	Multiplet	(4H)	7	CH_2 (3)
1.98	Multiplet	(4H)	6	CH_2 (8) and (11)
2.18	Triplet	(2H)	7.5	CH_2 (2)
5.31	Multiplet	(2H)	11	CH (9) and (10)
5.59–6.06	Broad singlet	(2H)		Primary NH_2

fractions (iii) and (iv) from silica gel columns (Fig. 1). When the GC retention time of $C_{18:1}$ amide (octadecenamide) was 16.2 min, the retention times of $C_{16:1}$, $C_{15:0}$, $C_{14:0}$ and $C_{14:1}$ amides were 8.18, 5.73, 4.52 and 4.32 min, respectively. Hexadecenamide (MW 253), tetradecanamide (MW 227) and tetradecenamide (MW 225) were identified by GC/MS from their characteristic molecular and fragment ions. Small amounts of pentadecanamide were also detected. The IR spectrum of these amide mixtures (Peaks 2 and 3 of the HPLC fractionation), devoid of octadecenamide, gave characteristic 3350, 3190, 1650 and 1625 cm^{-1} absorption bands, indicative of their apparent amide structures.

DISCUSSION

Hydroxylating enzyme from *B. megaterium* ATCC 14581 is found to convert a myristamide substrate from its ω-end (5).

Alternatively, we find that *B. megaterium* B-3437 produces fatty amides by converting 5–10% of an oleic acid substrate, if equimolar ammonium chloride is also provided. Although enzymatic amidations have yet to be studied, precedence of analogous glutamine synthetase (EC 6.3.1.2) from *Klebsiella aerogenes* (14) and fatty hydroxamic synthesizing lipase from *Mucor miehei* (15) equivocally suggests either a unique or multifunctional enzyme.

Although specific fatty amides are synthesized more readily by physicochemical processes (12,16), the bioconversion by B-3437 appears to be unique as an enzymatic alternative and for its equimolar oleic acid/ammonium salt requirements. It is likely that long chained fatty acids can be amidated by B-3437 or another more efficient strain to produce an array of erucamide substitutes. Such possibilities are being assessed.

The octadecenamide has a 9,10-positioned double bond in *cis* configuration, consistent with NMR (Table 2) and IR spectra. The double bond positions of $C_{16:1}$ and $C_{14:1}$ homologs are undetermined as yet, but that is now possibly from HPLC-separated fractions (Fig. 2). These and other unresolved problems give a new perspective to C-N biosyntheses (17–19) involving fatty acids and esters.

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