Biotransformation of 12-Hydroxyoctadecanoic Acid to 12-Hydroxyoctadecanamide by *Bacillus cereus* 50

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ABSTRACT: A new microbial isolate (*Bacillus cereus* 50) transformed 12-hydroxyoctadecanoic acid to 12-hydroxyoctadecanamide when grown aerobically in 1% yeast extract medium at 30°C, shaken at 250 rpm for 2 to 5 d. The compound was purified by thin-layer chromatography and characterized by infrared, gas chromatography, mass spectrometry, and nuclear magnetic resonance. The yields of 12-hydroxyoctadecanamide were 9.1 and 21.5% after 2 and 5 d, respectively. *JAOCS 74*, 601–603 (1997).

KEY WORDS: 12-Hydroxyoctadecanamide, 12-hydroxyoctadecanoic acid, *Bacillus cereus,* biotransformation.

Fatty amides have diverse applications as chemical additives, lubricants, slip agents, and nonsticky and protective coatings (1). Fatty amides, such as erucamide, are chemically synthesized from 13(*Z*)-docosenoic acid (erucic acid) (2,3). Microbial transformation of 9(*Z*)-octadecenoic acid to 9(*Z*)-octadecenamide (6% yield on total lipids extracted) by *Bacillus cereus* B-14812 (4), and to 9(*Z*)-octadecenamide, hexadecenamide, tetradecenamide, and tetradecanamide by *B. megaterium* B-3437 (5–7% yield on total lipids extracted) (5) has been reported.

During our continuing efforts to isolate new microorganisms for biotransformation of 12-hydroxyoctadecanoic acid (HOA) (6,7), we discovered another active strain of *B. cereus* 50 (8). *Bacillus cereus* 50 produced significant amounts of 12-hydroxyoctadecanamide (12-HOAM) when grown aerobically in 1% yeast extract (1% YE) that contained 0.2% (wt/vol) of 12-HOA at 30°C when shaken at 250 rpm. In this paper, we report the conversion of 12-HOAM from 12-HOA after 2 and 5 d, and its isolation and identification by infrared (IR), gas chromatography–mass spectrometry (GC–MS), positive chemical ionization–mass spectrometry (PCI–MS), and nuclear magnetic resonance (NMR).

EXPERIMENTAL PROCEDURES

Materials. All chemicals and solvents were of ACS grade obtained from commercial sources. The acids, *R*-(+)-12-HOA and hexadecanoic acid (palmitic acid), were obtained from Sigma (St. Louis, MO) with purities greater than 98% by GC analysis. The product, 12-hydroxyoctadecanamide, was purified to better than 97% purity, as determined by GC analysis. YE was from Difco Laboratories (Detroit, MI).

Microorganisms and microbial conversion study. Strain 50 was isolated from the intestinal tract of a fish (large mouth bass, *Micropterus salmoides*) (8). A single colony was cultured in 5 mL of tryptone medium (containing 0.5% tryptone, 0.2% KH_2PO_4, 0.4% Na_2HPO_4, 0.2% $(NH_4)_2SO_4$, 0.01% $CaCl_2{\cdot}H_2O$ and 0.001% $FeSO_4{\cdot}7H_2O$, pH 7.2) at 30°C, 200 rpm for 18 h. The following day, 2-mL cultures were added to 100 mL of 1% YE medium (containing 1% yeast extract, 0.2% KH_2PO_4, 0.4% Na_2HPO_4, 0.2% $(NH_4)_2SO_4$, 0.01% $CaCl_2{\cdot}H_2O$ and 0.001% $FeSO_4{\cdot}7H_2O$, pH 7.2). Ten mL of the 1-to-50 diluted culture was transferred to each of the 125-mL capacity Erlenmeyer flasks, and 12-HOA was added to a final concentration of 0.2% to carry out the bioconversion reaction for up to 5 d. Pure dilution culture served as control. Triplicate samples were used.

Three flasks (10 mL/flask) were removed on day 2 and on day 5. The entire contents of triplicate flasks (10 mL) were extracted. Palmitic acid (2 mg) was added as internal standard, and the acidified (with 3 N HCL) sample was extracted twice with an equal volume of diethyl ether. The extracts were washed once with an equal volume of water. The washed ether was evaporated with N_2 to a white residue.

For a larger-scale cultivation, a 1-to-50 dilution culture (from overnight culture) was grown aerobically for 5 d at 30°C in a one-liter flask with 250 mL of 1% YE medium (total 500 mL from two flasks), and 12-HOA was added (final concentration 0.2%, wt/vol). Samples (500 mL) were extracted with diethyl ether and worked up in the usual manner.

Thin-layer chromatography (TLC). The crude extracts were subjected to partial purification before applying to TLC plates. Crude extracts were dissolved in minimal amounts of methanol in a flask, and diethyl ether was added to a final concentration of 98% (vol/vol). The flask was kept at –20°C for 1 h. The precipitate was collected and dried under N_2. Dried samples were redissolved in methanol and reprecipitated once as above. The compound (in methanol) was applied unto TLC plates (20 × 20 cm Silica gel 60 plate, 0.25 mm thickness; EM Science, Cherry

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Hill, NJ) as a strip 2.5 cm from the bottom of each plate. The plates were developed with a CH_2Cl_2/MeOH (98:2, vol/vol) system. Developed plates were wrapped with Saran Wrap (Dow Brands, Indianapolis, IN) except for 1.5-cm margins on the left and right of the plate. Sulfuric acid (30%) was sprayed on the uncovered area, and color was developed by heating the sprayed area at 150°C for 3–5 min with a heat gun. Color, developed on both edges, served as marker to locate 12-hydroxyoctadecanamide. 12-Hydroxyoctadecanamide was removed from the plate, extracted with methanol, dried with N_2, and subjected to GC and GC–MS analyses.

Fourier transform infrared (FTIR) spectroscopy. IR spectra of 12-hydroxyoctadecanamide or diazomethane-treated 12-hydroxyoctadecanamide (thin film, KBr plate) were obtained with a Mattson Galaxy 6020 IR spectrometer (Mattson Instruments, Inc., Madison, WI).

GC and GC–MS. Crude extracts, partially purified samples from crude extract, and a highly purified compound from the TLC plate were analyzed by GC and GC–MS after being treated with diazomethane or diazomethane and trimethylsilane (TMS) reagent. Treated samples were analyzed by chromatography with a Hewlett-Packard 5890A series II gas chromatograph (Palo Alto, CA), equipped with a flame-ionization detector. Samples were separated on an SPB-1 column (15 m × 0.32 mm i.d. and 0.25 mm thickness), either at isothermal (200°C) or temperature-programmed conditions as previously reported (6). Peak areas were determined with a Hewlett-Packard 3396A electronic integrator. Methyl palmitate (retention time 1.6 min) was used as an internal standard, and 12-hydroxyoctadecanamide (retention time 6.8 min) was used for quantitative analyses. Electron-impact mass spectra of the compounds mentioned above were obtained on a Hewlett-Packard 5970 gas chromatograph, equipped with a DB-5ms capillary column (15 m × 0.25 mm i.d.; J&W Scientific Co., Folsom, CA) coupled to a Hewlett-Packard mass selective detector.

In the later course of these studies, a Hewlett-Packard 5890A gas chromatograph equipped with an SP-2100, methyl fluid column (2.4 m × 2 mm i.d.; Supelco, Bellefonte, PA), and a Hewlett-Packard 3396A electronic integrator was used. Esters were separated isothermally at 280°C (retention time for 12-hydroxyoctadecanamide was 14.95 min).

Low- and high-resolution MS by PCI was carried out on 12-hydroxyoctadecanamide by The Nebraska Center for Mass Spectrometry, Department of Chemistry, University of Nebraska-Lincoln (Lincoln, NE). Reagent gas was isobutane. Exact mass measurements were made by using narrow-voltage scanning and ions of perfluorokerosene as standard mass.

NMR spectra. ^1H and ^{13}C NMR spectra of 12-hydroxyoctadecanamide in $CDCl_3$ or CD_3OD were obtained with a Bruker ARX 400 Spectrometer (Burlington, Ontario, Canada).

RESULTS AND DISCUSSION

Identification of microorganism. Strain 50 was identified as *B. cereus* based on the following (8,9): It is a gram-positive rod, positive for oxidase and catalase activities, positive for nitrate reduction, degradation of gelatin and starch, and does not produce gas from glucose (9).

Identification of conversion products. *Bacillus cereus* 50 transformed 12-HOA to several compounds. One of these compounds was purified by TLC. In our initial studies, methylation of the purified compound had no effect on GC retention time (6.8 min for the SPB-1 capillary column at isothermal temperature and 17.6 min for the DB-5MS capillary column with temperature programming). Therefore, the purified compound is not a free fatty acid. The IR spectrum indicated the presence of a hydroxy (3426 cm^{-1}) group, and absorptions at 3320 and 3214 cm^{-1} were indicative of the antisymmetric and symmetric stretch, respectively, of an amide group $(C{=}O)NH_2$. An absorption at 1659 cm^{-1} was also consistent with the C=O stretch of a primary amide. Therefore, the compound is a hydroxy fatty amide.

Low-resolution MS by PCI (reagent gas, isobutane) was carried out on the purified compound. Results indicated the presence of a parent ion (*m/z* 299+H^+) and an ion mass of *m/z* 282 (300 − 18). High-resolution PCI–MS of the parent peak indicated the presence of a compound with a chemical formula of $C_{18}H_{37}NO_2$ (*m/z* 300.2899, observed; calculated 300.290255). Because 12-HOA was used as substrate for the conversion reaction, it is reasonable to believe that the purified compound is 12-hydroxyoctadecanamide. The location of the hydroxyl group was determined by the presence of prominent ions from α-cleavage of the sigma bonds on the left and right of the TMS groups (10). Electron-impact mass spectra of 12-hydroxyoctadecanamide and its TMS derivative indicated that the purified compound contains one hydroxyl group. For GC–MS of 12-hydroxyoctadecanamide, the prominent mass ions were *m/z* 214(37%) and 59(100%). The *m/z* 214 ion results from α-cleavage of the sigma bond between C_{12}–C_{13}; the *m/z* 59 ion is the base peak and corresponds to the McLafferty rearrangement product of the straight-chain monoamide (11). For 12-trimethyl-silyloxyoctadecanamide, the prominent fragment ions *m/z* 187(40%), 286(3%), and 358(100%). The mass ions of 187 and 286 resulted from α-cleavage of the sigma bond between C_{11}–C_{12} and C_{12}–C_{13}, respectively. The mass ion of 358(359 − 1) resulted from the mass ion fragment of 286 with an additional TMS attached to a carbonyl group (10). Therefore, the hydroxyl group is on carbon 12.

The proton NMR spectrum of the compound in $CDCl_3$ solution indicated NH_2 protons of the primary amide group (5.43 ppm, *d, br.*; 2H), a hydroxyl proton (3.57 ppm, *m*, 1H), protons α and β to an acyl group, CH_2-C=O (2.18 ppm, *m*; 2H) and CH_2-CH_2-C=O (1.65 ppm, *m*; 2H), methylene protons, -CH_2- (1.27 ppm, *m*; 27 H), and a CH_3 (0.85 ppm, *t*, 3H). ^{13}C NMR data indicated that the purified compound contained 18 carbons and confirmed the presence of -*C*=O at C_1 (176 ppm); one -*C*-OH at 72.4 (C_{12}); one CH_2-C-O- at 33.1 ppm and fourteen -CH_2-, ranging from 23 ppm to 39 ppm ($C_{3–11,13–17}$); and -CH_3 at 14.0 ppm (C_{18}). It is a white solid that melts at 82–85°C.

Production of hydroxy fatty amide. Diluted culture (10 mL

of a 1-to-50 dilution from an overnight culture (1% YE medium) in a 125-mL Erlenmeyer flask that contained 0.2% 12-HOA was grown aerobically at 30°C, 250 rpm for 5 d. Triplicate samples were prepared. Three flasks were removed on day 2 and on day 5 for sample extractions and GC analyses. The entire contents of the triplicate flasks were extracted. The yields of 12-hydroxyoctadecanamide on day 2 and day 5 were 9.1% (from two experimental data: 10 and 8.2%) and 21.5% (from two experimental data: 23 and 20%), respectively. The yield of 12-hydroxyoctadecanamide from a larger cultivation (500 mL), after incubation for 5 d, was 24%.

Although the yield was low, enzymatic conversion of the carboxylic group to an amide group provides a milder alternative way to alter the functionality of fatty acids.

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