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ORIGINAL PAPER

Production of 14-Oxo-*cis*-11-eicosenoic Acid from Lesquerolic Acid by *Sphingobacterium multivorum* NRRL B-23212

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Abstract The objective of this study was to explore the extent of microbial conversion of lesquerolic acid (14-hydroxy-*cis*-11-eicosenoic acid; LQA) by whole cell catalysis and to identify the newly converted products. Among compost isolates including NRRL strains B-23212 (*Sphingobacterium multivorum*), B-23213 (*Acinetobacter* sp.), B-23257 (*Enterobacter cloacae* B), B-23259 (*Escherichia* sp.) and B-23260 (*Pseudomonas aeruginosa*) the *S. multivorum* strain was the only microorganism that converted LQA to produce a new product identified as 14-oxo-*cis*-11-eicosenoic acid by GC-MS and NMR analyses. The conversion yield was 47.4% in 48 h at 200 rpm and 28°C in small shake flask experiments. In comparison, both *Acinetobacter* and *Pseudomonas* strains failed to convert LQA to major new products but used LQA apparently as an energy source during fermentation. For structural analysis, 6.88 g of 14-oxo-*cis*-11-eicosenoic acid was produced from converting 11 g LQA (a 62% yield) in 72 h at 200 rpm and 28 °C in Fernbach flasks using 18-h-old NRRL B-23212 cultures and an improved medium that also contained EDTA and glycerol in lieu of glucose as carbon source. NRRL B-23212 was further identified by 16S rRNA gene sequence analysis as a unique strain of *S. multivorum*. Therefore, *S. multivorum* NRRL B-23212 possesses an enzymatic activity presumably a secondary alcohol dehydrogenase for converting LQA to produce 14-oxo-*cis*-11-eicosenoic acid, a first report that demonstrates the functional modification of LQA by whole cell catalysis.

Keywords Bioconversion · Lesquerolic acid · 14-oxo-*cis*-11-eicosenoic acid · *Sphingobacterium multivorum* · Whole-cell catalysis

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Introduction

A number of microbial systems have been examined for the conversion of fatty acids in vegetable oil to products with enhanced functionality for potential new uses [1]. Use of whole microbial cells or enzymes as biocatalysts in bioconversion offers greater specificity and less environmental concerns than many chemical reactions. In our laboratory, we applied an enrichment culture technique to allow selection of microorganisms that would transform unsaturated fatty acids from a compost mixture amended with soybean oil [2]. Among the selected reactive microbial species, several strains of *Sphingobacterium thalpophilum* possessed the biological activity to convert oleic acid mainly to 10-ketostearic acid in addition to small amounts of 10-hydroxystearic acid [3]. These oxygenated fatty acids are industrial chemicals useful in plasticizers, surfactants, lubricants, and detergents formulations. Various keto fatty acids or derivatives of keto fatty acids are patented ingredients proposed for use in multipurpose greases [4, 5].

Several strains of *Pseudomonas aeruginosa* are known to convert ricinoleic acid to produce an antifungal compound, 7,10,12-trihydroxy-8(*E*)-octadecenoic acid (TOD) in shake flasks [6] and in a scale-up reactor process [7].

A homolog of ricinoleic acid, lesquerolic acid (14-hydroxy-*cis*-11-eicosenoic acid; LQA) is the major fatty acid component (45%) of *Lesquerella fendleri* seed oil being developed in the US as a source of hydroxy fatty acids to replace the imported castor oil [8]. In this study, we investigated the microbial conversion reactions of LQA with the objective of producing new products by the selected microorganisms. This communication describes the result of a dehydrogenation reaction carried out by *S. multivorum* NRRL B-23212 on LQA to produce a new compound that was identified as 14-oxo-*cis*-11-eicosenoic acid (14-OEA).

Experimental Procedures

Chemicals

Yeast extract and tryptone were from Difco Laboratories (Detroit, MI, USA). High purity (>99.9%) palmitic acid (C16:0) and margaric acid (C17:0) used as reference were from Nu-Chek Prep (Elysian, MN, USA). All other chemicals were reagent-grade and used without further purification. *L. fendleri* oil was supplied by International Flora Technologies, Ltd. (Apache Junction, AZ, USA). LQA was prepared from lesquerella oil by saponification and crystallization as described previously [9]. A total of 150 g LQA was produced from 400 ml *L. fendleri* oil with a purity of about 98% based on GC analyses.

Microorganisms

Microbial strains were previously selected from aerobic composts amended with soybean oil and sub-cultured in enrichment media and selection plates containing specific unsaturated fatty acids and their identity was established with the Biolog System (Biolog Inc., Hayward, CA, USA) [2]. They were deposited in the ARS Culture Collection, US Department of Agriculture, Agricultural Research Service (USDA-ARS) in Peoria, Illinois as NRRL (Northern Regional Research Laboratory) strains B-23212 (*S. multivorum*), B-23213 (*Acinetobacter* sp.), B-23257 (*Enterobacter cloacae* B), B-23259 (*Escherichia* sp.) and B-23260 (*P. aeruginosa*). Strain NRRL B-23212, which was previously identified by the Biolog System as *S. thalpophilum*, was further subjected to 16S rRNA gene sequence analysis. Genomic DNA for sequencing of the 16S rRNA gene was isolated from growth on tryptone-glucose-yeast extract (TGY) [10] medium plates. A fragment of the 16S rRNA gene corresponding to positions 63–1387 of the *E. coli* 16S rRNA was amplified from each strain using the polymerase chain reaction (PCR) protocol of Rooney et al. [11]. The forward and reverse strands of each fragment were sequenced using the Big Dye® Kit (Applied Biosystems, Inc., Foster City, CA) on an ABI 3730 automated sequencer. The resulting DNA sequences were compared with the closest 16S matches in GenBank using the program RDP-II [12]. The GenBank accession numbers for the published 16S rRNA gene sequences included in the analysis were AB020205 (*S. multivorum*), AJ438177 (*S. thalpophilum*), AJ459411 (*S. spiritivorum*), AJ438176 (*S. faecium*) and AJ438171 (*Pedobacter africanus*). Comparison of these sequences revealed that NRRL B-23212 was a unique strain of *S. multivorum*, with levels of 16S nucleotide divergence less than 2.7%. Microbial strains were transferred from freeze-dried culture onto TGY liquid medium to grow at 28 °C for 24–48 h and subsequently maintained on TGY agar slants at 4 °C and sub-transferred monthly.

Bioconversion Reaction and Lipid Analysis

Bioconversions in shake flasks were carried out in modified Wallen fermentation (WF) medium, which contained (per liter) 4 g dextrose, 5 g yeast extract, 4 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 0.0075 g $FeSO_4 \cdot 7H_2O$, and its pH was adjusted to 7.3 with 3 N H_2SO_4 [13]. Conversion of LQA by various microbial strains was carried out according to the basic procedures described previously [14]. An aliquot of LQA (0.15–0.3 mL, 0.5–1%) was added to 20-h-old cultures in 30 mL WF medium and then shaken for an additional 2 days at 28 °C and 200 rpm. After the reaction, each sample broth was acidified and lipids were extracted twice with an equal volume of methanol/ethyl acetate (1:9; v/v). After solvent was removed with a rotary evaporator, the concentrated lipid extracts were transferred to one-dram vials and dried under a nitrogen stream for further analysis. Methyl esters were prepared with diazomethane and analyzed by GC using an HP (Hewlett Packard; Palo Alto, CA, USA) model 5890 Series II gas chromatograph, equipped with a Supelco (Bellefonte, PA, USA) SPB-1 or with an equivalent Phenomenex (Torrance, CA) ZB-1 capillary column (15 m × 0.32 mm, 0.25-μm film thickness) under the conditions described previously [14]. The product yields were calculated from GC percentages relative to C16:0, which was added as an internal standard prior to lipid extraction.

Production and Identification of 14-OEA

Bioconversion of LQA was scaled up in batch cultures using Fernbach flasks. NRRL B-23212 inoculum was grown in TGY medium in 50-mL Erlenmeyer flask at 28 °C and 200 rpm for 24 h. This culture was used to inoculate (1% by volume) 500 mL reaction medium at pH 7.0 containing (g/L) 6.0 glycerol, 4.0 yeast extract, 6.0

K_2HPO_4, 2.0 KH_2PO_4, 0.3 $MgSO_4 \cdot 7H_2O$, 0.02 $FeSO_4 \cdot 7H_2O$, 0.02 $MnSO_4 \cdot H_2O$, 0.02 $CuSO_4 \cdot 5H_2O$, and 0.4 EDTA·$Na_2 \cdot H_2O$ in the flasks that were incubated at 28 °C and 200 rpm for 18 h. Thereafter, the conversion was initiated by adding 3.75 mL LQA to the culture broth for 72 h under the same conditions. The reaction was terminated by adding 6 M HCl to pH about 2.0 prior to lipid extraction. Aliquots of culture (10 mL) were analyzed by GC as described above to monitor the reaction. The dried lipid extract after solvent removal was dissolved in ethyl acetate and the desired new product was isolated from the solvent after repeated crystallization at –20 °C. The purified material was analyzed GC for determination of purity and by GC-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) for structural identification. Electron-impact GC-MS was obtained for the methylated sample with an HP model 5890 gas chromatograph coupled to an HP model 5972 mass selective detector as described previously [3]. Proton, carbon, DEPT, COSY, and HSQC NMR spectra of the underivatized sample was analyzed by a Bruker, Avance 500 spectrometer (Billerica, MA, USA) equipped with a 5-mm broadband inverse Z gradient probe (^{13}C NMR, 125 MHz; ^{1}H NMR, 500 MHz). The solvent was deuterated chloroform, which served as the internal standard.

Results and Discussion

Specific compost microbial isolates are known to convert ricinoleic acid to 7,10,12-trihydroxyoctadecenoate possessing antifungal activity and oleic acid to 10-ketostearic and 10-hydroxystearic acids potentially useful in lubricant and detergent formulations [2]. These microbial strains were examined for their ability to carry out the transformation of LQA in small shake flask fermentation experiments. Among the NRRL strains examined (Table 1), strains B-23259 (*Escherichia* sp.) and B-23257 (*E. cloacae* B) did not use LQA because most, or nearly all, of the lipid recovered from the fermentation broth was LQA. Strain B-23260 (*P. aeruginosa*) was the best LQA user, followed by B-23212 (*S. multivorum*) and B-23213 (*Acinetobacter* sp.). Strains B-23213 and B-23260 had degraded LQA for use as an energy source during metabolism leaving 28.8 and 45.5%, respectively, of the extractable lipid that was devoid of any LQA or the converted product. Strain NRRL B-23260 was the best *P. aeruginosa* producer of TOD from the conversion of ricinoleic acid, a homolog of LQA [6]. However, it failed to convert LQA to a product homologous to TOD. Among the examined species, *S. multivorum* NRRL B-23212 was the only strain that exhibited the ability of LQA conversion into producing 14-OEA. There were 69.7 mg 14-OEA and 36.2 mg LQA present in the fermentation broth in small shake flasks after 48 h (Table 1). This represented a product yield of 47.4% based on the amount of LQA substrate (147 mg) originally added to the culture medium. The microbial culture also consumed 11.4% of the added LQA as energy source most likely by β-oxidation reaction. The LQA converted product migrated closely with LQA on GC (Fig. 1). The close migration of these two compounds highly resembled the GC pattern observed for 10-ketostearic and 10-hydroxystearic acids [15]. It was indicative that the LQA converted product might be a dehydrogenated derivative of LQA.

Bioconversion of LQA was carried out in batch cultures using Fernbach flasks to produce gram quantity of 14-OEA for its chemical structure determination. A new conversion culture medium that included yeast extract and ethylenediamine-tetraacetic acid (sodium salt) was shown effective in preliminary experiments for the bioconversion reaction. Inclusion of glycerol in lieu of glucose in the culture medium was also effective for use as carbon source for the conversion reaction. The reaction was proceeded for 72 h to minimize the amount of LQA substrate left in the fermentation broth in order to facilitate the downstream processing of the newly converted product. The final product was 6.88 g after crystallization, which represented a conversion yield of greater than 62% from three Fernbach flask

Table 1 Conversion of lesquerolic acid by compost microbial isolates

NRRL no.	Species	Total lipid (mg)	LQA (mg)	14-OEA (mg)
B-23212	*Sphingobacterium multivorum*	114.9 (88.6%)	36.2	69.7
B-23213	*Acinetobacter* sp.	37.4 (28.8%)	37.4	0.0
B-23257	*Enterobacter cloacae* B	116.7 (90.0%)	105.3	0.0
B-23259	*Escherichia* sp.	131.3 (101.2%)	129.0	0.0
B-23260	*Pseudomonas aeruginosa*	59.0 (45.5%)	0.0	0.0

Bioconversion reactions were performed for 48 h at 200 rpm and 28 °C after adding LQA (0.5%, vol/vol) substrate to 30 mL culture medium as described in Methods. Total lipid was obtained from the weight of each culture broth after lipid extraction and solvent removal. The figure in parentheses is the ratio of lipid weights between each fermentation broth and the control sample, which did not have any microbial strains present and contained 129.7 mg of total lipid under the same experimental conditions

LQA lesquerolic acid, *14-OEA* 14-oxo-*cis*-11-eicosenoic acid

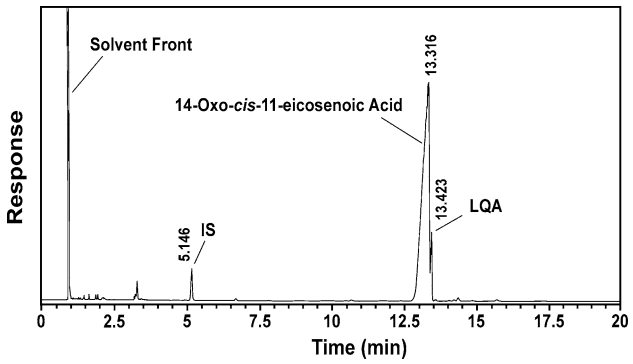

Fig. 1 Gas chromatograms of methyl esters recovered after the conversion of lesquerolic acid by *Sphingobacterium multivorum* NRRL B-23212. Peak with retention time (RT) 5.146 min is margaric acid reference standard (IS); RT of 13.316 min is 14-oxo-*cis*-11-eicosenoic acid from lesquerolic acid (LQA) conversion; RT of 13.423 min is the substrate LQA added to the culture medium

cultures containing a total of 11.25 mL of LQA substrate at about 98% purity. The crystallized product did not contain any detectable impurity upon GC analysis and it was subjected to GC-MS and NMR analyses.

The electron impact spectrum of the purified product after methylation with diazomethane gave a molecular ion of *m/z* 338 and an M-31 ion at *m/z* 307 corresponding to the loss of the methoxy group (Fig. 2). The primary fragmentation pattern appeared to be α-cleavage to the methoxy side of the keto group resulting in a base peak of *m/z* 113 from which the prominent *m/z* 85 peak may arise by loss of carbon monoxide [16]. This fragmentation pattern could also yield the *m/z* 194 ion through the loss of the methoxy group from the corresponding fragment (mol. mass 225) as resulted from the α-cleavage reaction described above [17]. Additional diagnostic peaks were seen at *m/z* 268 and 236, probably the result of McLafferty rearrangement and *β*-cleavage to the methyl side of the keto group followed by the loss of methanol [17]. These fragments determined the position of the keto group to be at C-14 and the presence of a double bond between the keto group and the methyl ester terminus. All NMR assignments were made using ^{13}C, ^{1}H, COSY, DEPT, HMSQC and HMBC pulse experiments on a 500 MHz NMR. The key functional group assignments for 14-OEA can be found in its ^{13}C-NMR spectrum (Table 2). The ketone gives a distinct signal at 209.49 ppm with no hydrogen couplings in the DEPT experiment. The ketone position was clearly assigned to carbon 14 of the chain by strong couplings to the hydrogens on carbon 13 (3.18 ppm) and 15 (2.46 ppm) in the homonuclear multiple bond coupling (HMBC) experiment. As a result of these assignments the methylene adjacent to the carbonyl of the acid functionality was easily identified at 2.38 ppm in the ^{1}H NMR. The olefin carbons (C-11 and C-12) assignments were confirmed by a positive DEPT for CH bond resonances and HSQC coupling to their protons at 5.55–5.63 ppm. Olefin carbon 12 had a weak long range HMBC coupling to the proton on carbon 15 which was absent for carbon 11. This assignment was in agreement with ACD lab calculations for the chemical shift of these two carbons. The *cis*-configuration of the olefin was assigned because of its smaller coupling constant (11.0 Hz). This assignment was consistent with a multiple bond spin calculation on the Bruker instrument for a *cis*-configuration for a molecule with this chemical structure having nearly identical splitting pattern. The calculated *trans*-configuration had a coupling constant of 15.0 Hz with a distinctly different splitting pattern.

The results indicate that *S. multivorum* NRRL B-23212 can carry out a dehydrogenation reaction presumably by a secondary alcohol dehydrogenase that converts LQA to produce 14-OEA. This is the first report to explore the biological modification of LQA by whole cell catalysis. Such an oxo-compound may find new applications particularly useful in lubricant industry as patents have been issued describing use of keto-acid derivatives as grease thickeners [4, 5]. The new oxo-compound is also more stable than LQA to avoid forming estolides that are composed mostly of hydroxy fatty acids [18].

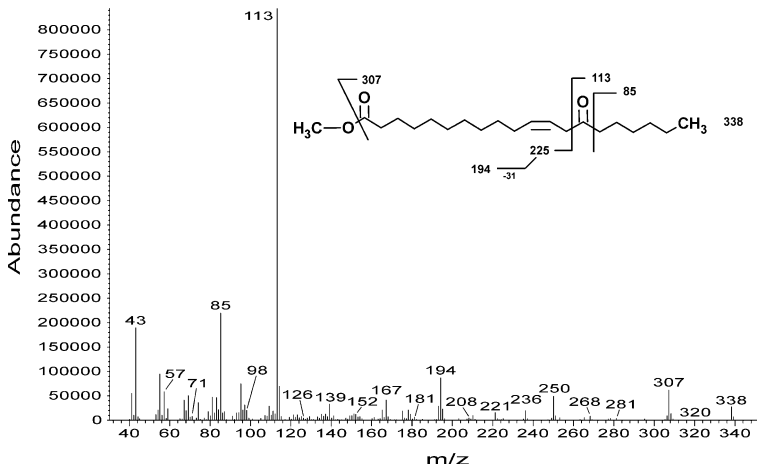

Fig. 2 Mass spectrum of the methylated bioconversion product, 14-oxo-*cis*-11-eicosenoic acid, with fragmentation pattern corresponding to specific ions

Table 2 Proton and ^{13}C NMR signals and assignments for the proposed bioconversion product from lesquerolic acid as 14-oxo-*cis*-11-eicosenoic acid

Carbon number	Proton resonance (ppm)	Hydrogens	^{13}C resonance (ppm)
1	None	0	179.47
2	2.38 (*t*) *J* = 7.46 Hz	2	33.96
3	1.66 (*m*)	2	24.68
4	1.31 (*m*)	2	29.36, 29.33, 29.24 or 29.20
5	1.31 (*m*)	2	29.36, 29.33, 29.24 or 29.20
6	1.31 (*m*)	2	29.36, 29.33, 29.24 or 29.20
7	1.31 (*m*)	2	29.36, 29.33, 29.24 or 29.20
8	1.31 (*m*)	2	29.36, 29.33, 29.24 or 29.20
9	1.31 (*m*)	2	29.42
10	2.05 (*dd*) J = 6.91 Hz and 14.14 Hz	2	27.51
11	5.61–5.63 (*m*)	1	133.67
12	5.55–5.57 (*m*)	1	120.96
13	3.18 (*d*) *J* = 6.87 Hz	2	41.69
14	None	0	209.49
15	2.46 (*t*) *J* = 7.37 Hz	2	42.38
16	1.59 (*m*)	2	23.80
17	1.31 (*m*)	2	28.91
18	1.31 (*m*)	2	31.61
19	1.31 (*m*)	2	22.50
20	0.91 (*t*) *J* = 6.78	2	14.03

t triplet; *J* coupling constant; *m* multiplet; *d* doublet

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