12,13,16-Trihydroxy-9(*Z*)-octadecenoic Acid, A Possible Intermediate in the Bioconversion of Linoleic Acid to Tetrahydrofuranyl Fatty Acids by *Clavibacter* sp. ALA2

Sir:

Microbial systems convert unsaturated fatty acids to monohydoxy-, dihydroxy-, and trihydroxy fatty acids (1). Some of these hydroxy fatty acid products were reported to have antimicrobial activities (2–4). In our previous work (5), biotransformation of linoleic acid by *Clavibacter* sp. ALA2 produced a number of products, among which 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid (THOA) was the predominant metabolite. Two tetrahydrofuranyl fatty acids (THFA), 12-hydroxy-13,16-epoxy-9(*Z*)-octadecenoic acid (12-hydroxy-THFA) and 7,12-dihydroxy-13,16-epoxy-9(*Z*)-octadecenoic acid (7,12-dihydroxy-THFA), were tentatively identified by gas chromatography/mass spectrometry (GC/MS) and microchemical techniques (6) and later confirmed by nuclear magnetic resonance (NMR) analyses (7). It is interesting to note that the structure of THOA resembles those of the plant self-defense substance (4). THFA were also known to have anticancer activity (8). We also identified 12,13-dihydroxy-9(*Z*)-octadecenoic acid (12,13-DOA) in the reaction mixture (7). For the biosynthetic pathway of linoleic acid to THFA, it appears that we have missed an intermediate between 12,13-DOA and THFA. Now, we have found this missing intermediate in the reaction mixture. We report here the identification by GC/MS of this missing intermediate as 12,13,16-trihydroxy-9(*Z*)-octadecenoic acid (12,13,16-TOA). We also report the optimal conditions for the production of THFA and discuss a possible biotransformation pathway.

Strain *Clavibacter* sp. ALA2 (NRRL B-21660) was grown at 30°C aerobically in a 125-mL Erlenmeyer flask (shaker at 200 rpm) containing 50 mL of medium as previously reported (5). Bioconversions were carried out by adding 0.35 mL linoleic acid to a 24-h-old culture, and the flasks were shaken again at 200 rpm at 30°C for 2–3 d. At the end of this time, the culture broth was acidified to pH 2 with 6 N hydrochloric acid. The culture broth was then extracted with an equal volume of ethyl acetate and then diethyl ether. The solvent was removed from the combined extracts with a rotary evaporator. Crude extracts containing reaction products were subjected to high-performance liquid chromatography following the method described earlier (7) to isolate pure material for structure identification. The reaction products were analyzed by GC and MS as described previously (5,6). GC/MS of methyl esters or methyl esters/trimethylsilyloxy ethers (OTMSi) was completed as described previously (6).

We found a small amount of new product, 12,13,16-TOA, existed in the reaction mixture. In our GC analysis of methyl esters of products, this compound was overlapped with the main product, THOA, and could not be separated. Recently, we analyzed GC/MS of the methyl ester and trimethylsilane ether derivatives of the products and found a small peak before the main product, THOA. Earlier, we dismissed this small peak as a stereoisomer of THOA. Now, we have found that this is a new compound, the missing link of the bioconversion pathway between linoleic acid and THFA. The mass spectrum (Fig. 1) with a molecular ion of 560 was interpreted as follows; electron ionization mass spectrometry *m/z* (relative intensity): 529 $[M - OCH_3]^+$ (1), 441 $[M - CH_2CH_3 - TMSOH]^+$ (1), 363 $[M - C_{11}H_{18}O_2CH_3]$ (5), 299 $[M - C_6H_{11}2(OTMS)]^+$ (15), 273 $[363 - TMSOH]^+$ (10), 270 (7), 261 $[C_6H_{11}2\text{-}(OTMS)]^+$ (9), 217 (5), 171 $[261 - TMSOH]^+$ (100), 159 (4), 147 (18), 131 $[C_3H_6OTMS]^+$ (25), 129 (28), 117 (5), 73 (100), 55 (9). The mass spectrum was largely identical to the spectrum of the main product, THOA, except the 131 fragment, which represents a hydroxy group at C16. Therefore the structure of the new product is identified as 12,13,16-trihydroxy-9(*Z*)-octadecenoic acid.

This study and previous work (5,6) showed that *Clavibacter* ALA2 is especially efficient at oxidizing C-12, -13, and -17 with hydroxyl groups. To a lesser extent, hydroxyls also occurred at C-7 and -16. Although not proved, it would appear that linoleic acid is converted by strain ALA2 into 12,13-DOA first, and then further oxidized to 12,13,16-TOA. (Scheme 1). In a subsequent step, 12,13,16-TOA is cyclized into THFA. In an analogous mechanism, cyclization of the main product THOA leads to the formation of diepoxy bicyclic fatty acid (7). It remains interesting to resolve the linoleic acid biotransformation pathway by strain ALA2.

In order to find an optimal condition to produce these THFA, the following variables of the bioconversion of linoleic acid by strain ALA2 were studied. The effect of pH on the production of THFA and THOA was studied using 0.1 M buffer

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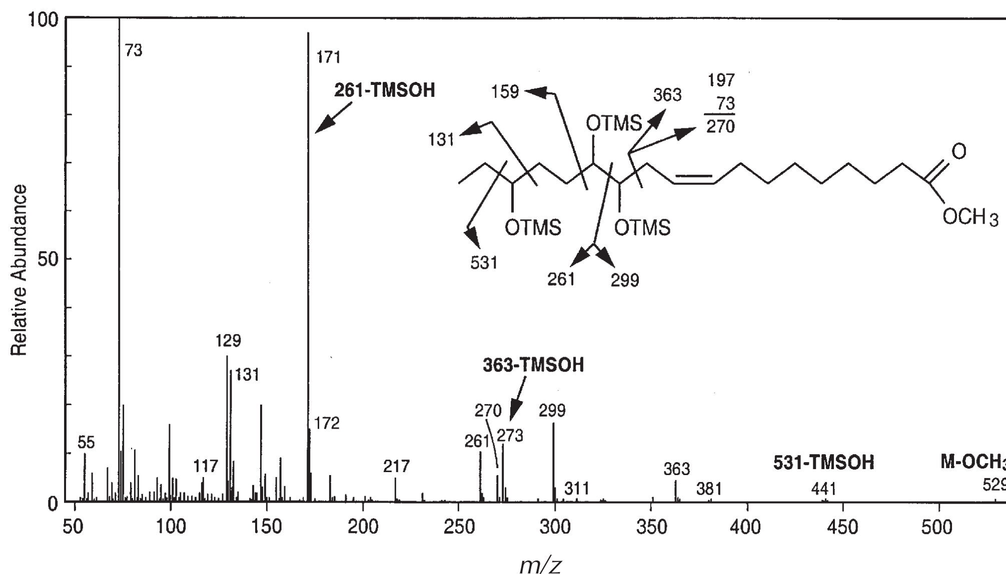
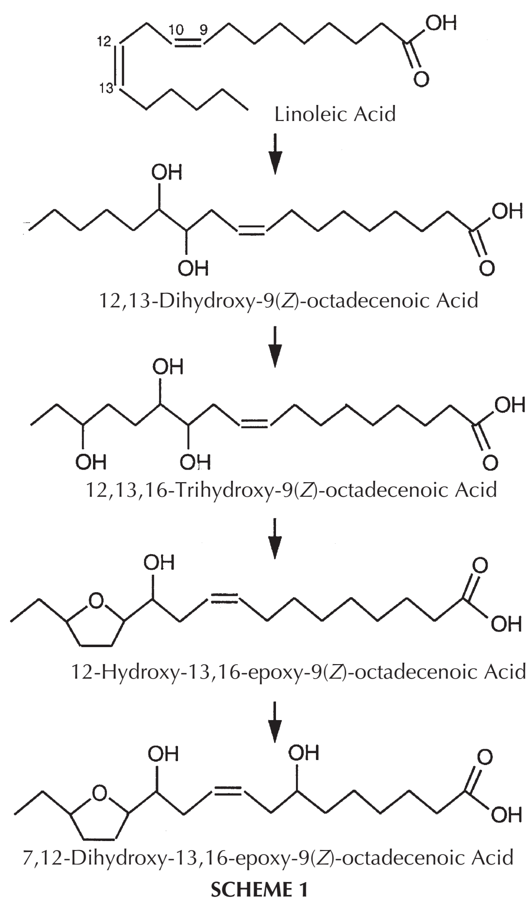


FIG. 1. Mass spectral analyses of methyl ester and trimethylsilane ether of new product, 12,13,16-trihydroxy-9(*Z*)-octadecenoic acid. OTMS, trimethylsilane ether; TMSOH, hydroxytrimethylsilyl.

solutions. A potassium phosphate buffer was used for pH 5.0 to 7.0, and a Tris-HCl buffer was used for pH 7.5 to 8.5. We found that the optimal pH for the production of 12-hydroxy-THFA was between 6.0 and 7.5 and for 7,12-dihydroxy-THFA was between 6.0 and 7.0. For comparison, the optimal pH for the production of THOA was between 6.5 and 7.5 with a peak at 7.0. The effect of temperature for the production of THFA was studied between 15 and 45°C. The optimal temperature for the production of 12-hydroxy-THFA was found to be between 30 and 35°C. The production of 7,12-dihydroxy-THFA was detected only between 20 and 30°C and could not be detected at temperatures below 15°C or above 35°C. The optimal temperature for THOA production was found to be between 25 and 35°C with a peak at 30°C. The reaction time course was studied at 30°C (Fig. 2). 12-Hydroxy-THFA was detected after about 10 h of incubation and reached a maximum at 20 h. After that, its amount did not decrease. The 7,12-dihydroxy-THFA was detected after 15 h of incubation, and its amount was maintained the same throughout the study period. Similarly, the main product,



SCHEME 1

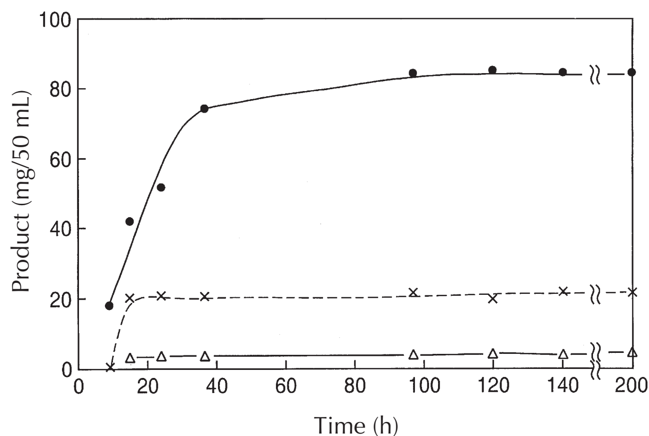


FIG. 2. Time course of the production of tetrahydroxyfuranyl fatty acids and 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid from linoleic acid by strain ALA2. ●, 12,13,17-Trihydroxy-9(*Z*)-octadecenoic acid; X, 12-hydroxy-13,16-epoxy-9(*Z*)-octadecenoic acid; △, 7,12-dihydroxy-13,16-epoxy-9(*Z*)-octadecenoic acid.

THOA, was detected after 10 h of incubation, and its amount in the culture media increased with time and reached a maximum after 5–6 d of reaction. Further incubation did not reduce THOA content in the medium.

This is the first report of the production of these compounds by microbial transformation.

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