Chemoenzymatic Synthesis of Conjugated Linoleic Acid

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Conjugated linoleic acid (CLA) refers to a mixture of positional and geometric isomers of octadecadienoic acids (18:2). It was first isolated and identified by Pariza and coworkers from a fraction of grilled beef that possessed antimutagenic activity.[1] Since then, interest in CLA has increased markedly because of reports that dietary CLA reduced carcinogenesis, atherosclerosis, and body fat in laboratory animals.[2] The major isomer, 9(*Z*),11(*E*)-octadecadienoic acid (**1a**), is believed to be the most biologically active CLA isomer[1,2] and was shown to be the first intermediate in the biohydrogenation of linoleic acid by bacteria in the rumen,[3] which may account for the high CLA content in meat from ruminants and dairy products.[4] However, commercial CLA products[5] fed to experimental animals are complex mixtures of CLA isomers, many of which have not been characterized. Analysis of this mixture using silver-ion impregnated HPLC revealed the presence of at least 12 components.[6] To date, there is no definitive evidence as to which isomer within the CLA mixture is the biologically active component or whether different isomers are responsible for different bioactivities. To further study the biological effects of CLA, it is desirable to obtain substantial amounts of the individual pure isomers, ideally by simple methods. Here, we describe a chemoenzymatic method for the synthesis of 9(*Z*),11(*E*)-(**1a**) and 10(*E*),12(*Z*)-octadecadienoic acids (**1b**), the two most abundant CLA isomers found in foods (Scheme 1).[7]

Since the superbasic mixture of *n*-butyllithium and potassium *tert*-butoxide has been successfully used for the deprotonation of allylic and 1,4-pentadienyl systems with excellent regio- and stereoselectivity,[8] we selected this organometallic reagent for the metalation of linoleic acid. In a representative experiment, 1 g of linoleic acid was treated with 3.5 equiv of this base at −78 °C in THF for 30 min. The reaction was quenched by pouring the mixture into 50 mL of 6 N HCl at 0 °C. After the usual workup, the crude product mixture was treated with diazomethane and purified via $AgNO_3$−silica gel chromatography using hexane/ethyl acetate (60:1) as the eluent to give a mixture of methyl octadecadienoate in 77% isolated yield. Analysis of this mixture using silver-ion impregnated HPLC[6] revealed the presence of only two peaks in a ratio of 4:6, corresponding to the retention times of methyl 10(*E*),12(*Z*)- and 9(*Z*),11(*E*)-octadecadienoates (**2b** and **2a**), respectively. The ^{1}H and ^{13}C NMR spectra[9] of the pure isomers were consistent with this assignment.[16]

Although the 10*E*,12*Z* and 10*Z*,12*E* isomers can be separated by silver-ion impregnated HPLC,[6] no current analytical techniques are available to separate the 9*Z*,11*E* and 9*E*,11*Z* isomers. Therefore, it was necessary to determine more definitively the position and geometric configurations of double bonds in the CLA preparation, which was carried out as follows: (1) The double bonds of the CLA isomers were partially reduced with hydrazine.[10] (2) The resulting *E* monoene fraction, isolated by silver-impregnated silica gel column chromatography, was subjected to oxidative ozonolysis followed by methylation.[11] (3) The diesters were isolated by silica gel chromatography, and their structures were verified by mass spectral analyses. When this analytical procedure was used on the synthetic CLA isomers, only dimethyl 1,11-undecanedioate and dimethyl 1,10-decanedioate were obtained.[12] These results not only confirm the absence of geometric isomers but also demonstrate the outstanding selectivity of the deprotonation−protonation procedure.

As the CLA regioisomers are difficult to separate from each other using chromatographic methods, we turned our attention to the use of enzymatic methods. It is well-known that the lipase from *Geotrichum candidum*[13] has a unique specificity for unsaturated fatty acids containing a 9*Z* double bond. That is, esters of 9*Z* unsaturated fatty acids are attacked more rapidly than other types of unsaturated fatty acids. When the synthetic CLA methyl esters (**2a** and **2b**) were exposed to this lipase at pH 7.0, the 9*Z*,11*E* isomer (**2a**) was preferentially hydrolyzed with a regioselectivity factor[14] (RS) of about 9. Since selectivity can often be enhanced by conducting the reverse reaction in organic solvents,[15] we incubated the synthetic CLA isomers with this lipase in 1-butanol for esterification. As expected, the enzyme preferentially esterified the 9*Z*,11*E* isomer (**1a**), but the regioselectivity (RS) was improved to only 12. This moderate improvement led us to examine other lipases with improved regioselectivities. After much experimentation, we discovered that the lipase of *Aspergillus niger* (APF 12, Amano) was considerably more selective. In the esterification

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(9) **1a**: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.88 (3H, t, J = 6.1 Hz), 1.20−1.50 (16H, m), 1.63 (2H, m), 2.12 (4H, m), 2.34 (2H, t, J = 7.6 Hz), 5.28 (1H, m), 5.65 (1H, m), 5.94 (1H, t, J = 10.8 Hz), 6.29 (1H, dd, J = 15 Hz, 11 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 180.5 (C1), 134.8 (C12), 129.8 (C9), 128.7 (C10), 125.6 (C11), 34.1 (C2), 32.9 (C13), 31.8 (C16), 29.7, 29.4, 29.1, 29.0, 28.9 (C4, C5, C6, C7, C14, C15), 27.6 (C8), 24.6 (C3), 22.6 (C17), 14.1 (C18). **1b**: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.89 (3H, t, J = 6.3 Hz), 1.20−1.50 (16H, m), 1.63 (2H, m), 2.12 (4H, m), 2.34 (2H, t, J = 7.5 Hz), 5.30 (1H, m), 5.65 (1H, m), 5.94 (1H, t, J = 10.9 Hz), 6.30 (1H, dd, J = 15 Hz, 11 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 180.5, 134.5, 130.1, 128.6, 125.7, 34.1, 32.9, 31.5, 29.4, 29.3(8), 29.3, 29.1(8), 29.1(5), 29.0, 27.7, 24.7, 22.6, 14.1.
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(14) In systems containing two competing regioisomers, the regioselectivity (RS) may be calculated using the following expression:

$$\mathrm{RS} = \frac{\ln[(1 - C)(1 - SE)]}{\ln[(1 - C)(1 + SE)]}$$

C denotes the extent of conversion, and SE is the excess of one remaining substrate divided by the total remaining substrate at conversion C. See: Chen, C. S.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294 and also ref 13. A 1:1 mixture of **1a** and **1b** was used for the determination of RS.
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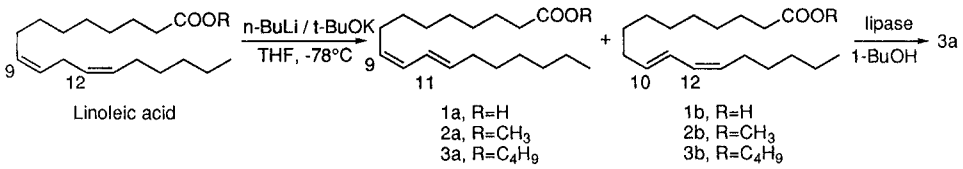
Scheme 1



mode in 1-butanol, this lipase also esterified preferentially the 9*Z*,11*E* isomer (**1a**) with an RS of 25. In a typical experiment, 1 g of CLA consisting of a 60:40 mixture of **1a** and **1b** was incubated with 100 mg of lipase APF-12 (*A. niger*, Amano) in 36 mL of 1-butanol and 14 mL of water. After being stirred at 25 °C for 18 h, the reaction was terminated. Silicic acid column chromatography [ethyl acetate/hexane (1:30) followed by methanol/methylene chloride (5:95)] of the mixture afforded 510 mg of enriched (9*Z*,11*E*)-butyl ester **3a** (SE = 0.92)[14] and 610 mg of enriched **1b** (SE = 0.26). To secure **1a**, the enriched **3a** fraction was hydrolyzed with 1 M KOH back to **1a** and subjected again to regioselective esterification using the same lipase to yield 340 mg of pure **3a** (SE = 1.0), which upon base hydrolysis afforded pure **1a** (265 mg) in 44% overall yield. Further enrichment of the **1b** fraction was achieved via the selective removal of **1a** by repeating the regioselective esterification two times. Highly enriched **1b** (SE = 0.91) was obtained in 75% overall yield.

In summary, we have shown that the superbase (*n*-butyllithium/potassium *tert*-butoxide) reacted smoothly with linoleic acid to give only the 9*Z*,11*E* and 10*E*,12*Z* CLA isomers, **1a** and **1b**, which were conveniently separated from each other using the lipase from *A. niger* via regioselective esterification. This enzyme has a preference for the 9*Z*,11*E* isomer, **1a**, and has excellent regioselectivity. This chemoenzymatic method has allowed the ready preparation of the 9*Z*,11*E* and 10*E*,12*Z* CLA isomers in their highly purified forms, which are not readily accessible by current methods.[16] Further refinement of this technology for large-scale synthesis is in progress, and the results of these experiments will be reported at a later date.

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