NOTE

Synthesis and Detection by HPLC of 3-Oxohexadecanoyl-CoA for the Study of Peroxisomal Bifunctional Proteins

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Abstract: 3-oxohexadecanoyl-CoA was synthesized for the study of D-bifunctional protein (EC 4.2.1.107, EC 4.2.1.119, EC 1.1.1.n12) and L-bifunctional protein (EC 4.2.1.17, EC 5.3.3.8, EC 1.1.1.35). First, tetradecanal was subjected to the Reformatsky reaction with ethyl bromoacetate, and the product was then converted into ethyl 3-oxohexadecanoate. After acetylation of the 3-oxo ester with ethylene glycol, 3,3-ethlenedioxyhexadecanoic acid was obtained by alkaline hydrolysis. The acid was condensed with coenzyme A (CoA) by the mixed anhydride method, and the resulting CoA ester was deprotected with 4 M HCl to obtain 3-oxohexadecanoyl-CoA. In addition, the behavior of the CoA ester under several conditions of high-performance liquid chromatography (HPLC) was also investigated. We established separation detection of (R)-3-hydroxyhexadecanoyl-CoA, (S)-3-hydroxyhexadecanoyl-CoA, 3-oxohexadecanoyl-CoA, and trans-2-hexadecenoyl-CoA.

Key words: high-performance liquid chromatography, 3-oxoacyl-CoA, peroxisomal β-oxidation, peroxisomal bifunctional protein

1 INTRODUCTION

Peroxisomal β-oxidation involves degradation of very long chain fatty acids, branched chain fatty acids1-8. In addition, it is required for biosynthesis of bile acids9. The multifunctional enzyme type 2 (MFE2) or 17β-hydroxysteroid dehydrogenase type 4 (HSD17B4), EC 4.2.1. 107, EC 4.2.1.119, EC 1.1.1.n12) and L-bifunctional protein (L-BP), also known as multifunctional enzyme type 1 (MFE1) or enolyl coenzyme A (CoA) hydratase/3-hydroxyacyl-CoA dehydrogenase (EHHADH), EC 4.2.1.17, EC 5.3.3.8, EC 1.1.1.35 exist in peroxisome. Both enzyme have hydratase activity and dehydrogenase activity, and catalyze the second and third step of peroxisomal β-oxidation. When a water molecule is added to trans-2-enoyl-CoA by hydratase activity, D- and L-BP yield (R)-3- and (S)-3-hydroxyacyl-CoA, respectively. Subsequently, both enzymes produce 3-oxoacyl-CoA by dehydrogenase activity (Fig. 1)9. Although details of this convergence in function and the physiological significance of the contrasting stereospecificity between D- and L-BP have not yet been clarified, it has been shown that congenital malfunction of lipid metabolism is caused by defects or mutations of D-BP6-7. However, no L-BP-associated diseases have been reported yet.

To investigate the physiological significance of the stereospecificity of D- and L-BP, we have previously established a method for measurement of the enzymes’ hydratase activities using high-performance liquid chromatography (HPLC) equipped with a chiral separation column10-12. However, this method is only able to measure the hydratase activity. To improve analysis, procurement of the dehydrogenase’s CoA ester product is necessary. Therefore we aimed to chemically synthesize 3-oxohexadecanoyl-CoA. And the behavior of the CoA ester under several conditions of HPLC was also investigated. At last, we established a separation detection method of (R)-3-hydroxyhexadecanoyl-CoA, (S)-3-hydroxyhexadecanoyl-CoA, 3-oxohexadecanoyl-CoA, 3-oxohexadecanoyl-CoA, and trans-2-hexadecenoyl-CoA.
CoA. The separation method is useful for the study of peroxisomal bifunctional proteins.

2 EXPERIMENTAL

2.1 Materials and instruments
The lithium salt of CoA was purchased from Oriental Yeast (Tokyo, Japan). All other materials and solvents were of analytical grade, and deionized water was used throughout the study. Proton nuclear magnetic resonance (1H-NMR) spectrometry was recorded at 400 MHz on a JNM-EX 400 spectrometer (JEOL Ltd., Tokyo, Japan). HPLC was carried out using a Shimadzu LC 6-A system equipped with SPD-10 vp (Shimadzu Co., Kyoto, Japan); detection was performed at 260 nm. A CHIRALPAK AD-RH (4.6 × 150 mm, 5 μm, Daicel Chemical Ind. Ltd., Tokyo, Japan) was used as the separation column. (R)-3-hydroxyhexadecanoyl-CoA, (S)-3-hydroxyhexadecanoyl-CoA and trans-2-hexadecenoyl-CoA were prepared using our procedures described in previous reports.

2.2 3-oxohexadecanoyl-CoA
3-oxohexadecanoyl-CoA (7) was synthesized using a slight modified method of our previous report. The scheme is shown in Fig. 2. Ethyl 3-hydroxyhexadecanoate (2) was synthesized from tetradecanal (1) and ethyl bromoacetate using the Reformatsky reaction as described in a previous study. The acetone solution of the 3-hydroxylated ester (2) was completely oxidized to ethyl 3-oxohexadecanooate (3). After the reaction, methanol was added to the mixture to remove excess reagent. The mixture was evaporated, and the residue was extracted with ether. The organic layer was washed with water, saturated sodium bicarbonate, and saturated sodium chloride. The organic layer was then dried with anhydrous sodium sulfate. The solvent was evaporated and the residue was purified by column chromatography (20 g of silica gel, hexane/ethyl acetate (20/1 v/v) as a mobile phase), resulting in a needle crystal of ethyl 3-oxohexadecanoate (3) (1.34 mmol, 89.3%). The 3-oxo ester (3, 1.34 mmol) was dissolved in 12 mL of anhydrous toluene/ethylene glycol (5/1 v/v). A catalytic amount of methane sulfonic acid was added to the mixture and refluxed for 1.5 day using a Dean-Stark apparatus. After the reaction, the mixture was evaporated, and the residue was extracted with ethyl acetate, and the organic layer was washed with water and a saturated sodium chloride aqueous solution. The organic layer was then dried with anhydrous sodium sulfate. The solvent was evaporated and the residue was purified by silica gel column chromatography (20 g of silica gel, hexane/ethyl acetate (20/1 v/v) as a mobile phase), resulting in 3,3-ethylenedioxyester (4) as an oil (0.31 mmol, 23.1%). This ester (4, 0.30 mmol) was dissolved in 2 mL of a 2 M potassium hydroxide methanol solution and refluxed for 1.5 h. The mixture was then cooled on ice, neutralized with 2 M HCl, evaporated, and extracted with ethyl acetate. The organic layer was washed with water and saturated sodium chloride aqueous solution. The organic layer was
then dried with anhydrous sodium sulfate. This solvent was evaporated and the residue was purified by silica gel column chromatography (7 g of silica gel, hexane/ethyl acetate (5:1 v/v) as a mobile phase) resulting in 3,3-ethylendioxyhexadecanoic acid (5, 0.24 mmol, 80%) as a white crude crystal. This crude crystal was recrystallized by diethyl ether and hexane to produce a needle crystal. This carboxylic acid (5, 0.1 mmol) was converted to a CoA ester (6) by the mixed anhydride method according to a previously reported method. The CoA ester (6, 0.05 mmol) was suspended in 4 M HCl (0.5 mL) and tetrahydrofuran (THF) was added to the mixture until the CoA ester was dissolved. The mixture was then stirred for 1 day at room temperature. After checking the deprotection using HPLC, THF was removed with N₂ gas, and water (1 mL) was added to the residue, resulting in an oily precipitate. The precipitate was treated with 10⁻⁴ sodium perchlorate as previously reported, resulting in a white powder of 3-oxohexadecanoyl-CoA (7, 0.03 mmol).

**7** H-NMR (dimethyl sulfoxide-d₆): 0.74, 0.94 (each 3H, s, dimethyl in CoA residue), 0.84 (3H, m, 16-H), 1.23 (each 2H, bs, 6-H, 7-H, 8-H, 9-H, 10-H, 11-H, 12-H, 13-H, 14-H, 15-H), 1.33 (2H, m, 5-H), 2.25 (2H, m, 5-H), 2.60 (2H, m, 4-H), 2.89 (2H, m, -SCH₂-), 3.15, 3.24 (each 1H, 3-H), 3.53, 3.75, 3.83, 4.17, 4.39, 4.71, 4.79 (ribose-H and CO-CH(OH) -C), 5.98 (1H, d, J = 5.14 Hz, anomeric H), 7.75, 8.12 (each 1H, m, NH), 8.35, 8.60 (each 1H, s, adenine H).

### 2.3 Investigation of HPLC conditions

A mixture of 50 mM phosphate buffer and methanol was used as a mobile phase. Flow rate was set to 0.5 mL/min. CoA esters were monitored at 260 nm. The potential hydrogen (pH), the composition of the mobile phase (proportion of methanol), and column temperature were changed, and the retention times of the CoA esters were measured under each condition. The retention factor (k') and separation factor (α) were calculated from the retention time of the CoA esters as the following formula, with tᵣ and t₀ indicating the retention times of CoA ester and sodium nitrate, respectively.

\[ k' = \frac{tᵣ - t₀}{t₀} \]

\[ α = \frac{k₂'}{k₁'} \] (k₂' > k₁')

The resolutions (Rₛ) were calculated from the following formula, with W₀.₅h₀ indicating the full width half maximum value.

\[ Rₛ = 1.18 \times \frac{(tᵣ₂ - tᵣ₁) / (W₀.₅h₁ + W₀.₅h₂)}{W₀.₅h₀} \]

### 3 RESULTS AND DISCUSSION

#### 3.1 Synthesis of 3-oxohexadecanoyl-CoA

Free 3-oxo-carboxylic acids are easily decarboxylated, and this property prevents obtaining 3-oxo-carboxylic acids and synthesis of 3-oxoacyl-CoAs. We overcame this problem when synthesizing 3α,7α,12α-trihydroxy- and 3α,7α-dihydroxy-24-oxo-5β-cholestanoyl-CoA. Here, 3-oxohexadecanoyl-CoA (7) was synthesized according to their simple method. First, ethyl-3-hydroxyhexadeccanoate (2) was synthesized from tetradecanal (1) and ethyl bromoacetate by the Reformatsky reaction. The 3-hydroxylated ester was obtained as a racemic compound; chiral separa-
tion of this racemate has been previously reported\(^6\). Then, ethyl-3-oxohexadecanoate(3) was obtained by oxidation using chromic acid. Prior to hydrolysis of 3-oxoester(3) into the corresponding 3-oxo carboxylic acid, it was thought that the direct alkaline hydrolysis of 3-oxoester(3) would fail, resulting in only 2-oxopentadecane. To prevent this decarboxylation, the carbonyl group was converted into a ketel group, compound 4, by treatment with ethylene glycol prior to alkaline hydrolysis. Because the 3-oxoester(3) is a keto-enol tautomeric compound and absorbs ultraviolet (UV) light, progress of the reaction was monitored by thin layer chromatography (TLC) and UV light. Despite the overlap of the 3-oxo ester(3) and 3,3-ethylenedioxy ester(4) spots on TLC, we were able to determine when to stop the reaction when the UV-absorbing spot disappeared. After alkaline hydrolysis of compound 4, the carboxylic acid(5) was converted to a CoA ester(6). To deprotect the product, the 3,3-ethylenedioxy CoA ester(6) was dissolved in a mixture of 4 M HCl/THF and stirred at room temperature. Although we carried out the deprotection in 4 M HCl\(^7\), the 3,3-ethylenedioxyhexadecanoyl-CoA(6) could not be dissolved in 4 M HCl due to its hydrophobicity. Accordingly, organic solvent (THF) was supplemented in the reaction mixture. The progress of the deprotection was monitored by HPLC, showing the appearance of a new peak and disappearance of the peak corresponding to compound 6 (Fig. 3). The ¹H-NMR spectra of this newly synthesized compound indicated the disappearance of the 4.00 ppm derived from the ketel protons.

3.2 Separation of the CoA esters on HPLC

The (R)-3-hdroxyhexadecanoyl-CoA(8), (S)-3-hydroxyhexadecanoyl-CoA(9), and trans-2-hexadecenoyl-CoA(10) were separated under HPLC conditions that have been described in previous article\(^5\). Additionally, the 3-hydroxyhexadecanoyl-CoA(7) was mixed with other CoA esters and subjected to HPLC under the reported conditions. The elution order was (R)-3-hydroxyhexadecanoyl-CoA(8), 3-hydroxyhexadecanoyl-CoA(7), (S)-3-hydroxyhexadecanoyl-CoA(9), and trans-2-hexadecenoyl-CoA(10). Because, the peak of 3-hydroxyhexadecanoyl-CoA(7) overlapped with one of the peaks of (S)-3-hydroxyhexadecanoyl-CoA(9), the composition of the mobile phase (proportion of methanol) was investigated for better separation. When the proportion of methanol was 60 % (v/v), the retention times of the CoA esters were drastically increased, and striking peak tailings were observed. When the proportion of methanol was 70 % (v/v), all peaks overlapped one another. Therefore, the proportion of methanol was set to 65 % (v/v) (Fig. 4A). The pH of the mobile phase was also investigated, and when within the range of 3.0–7.0, the 3-hydroxyhexadecanoyl-CoA(7) overlapped with (S)-3-hydroxyhexadecanoyl-CoA(9). When the pH was 8.0, 3-hydroxyhexadecanoyl-CoA(7) overlapped with (R)-3-hydroxyhexadecanoyl-CoA(8). It was considered that 3-hydroxyhexadecanoyl-CoA converted to enol-form and keto-form at pH 7 and 8, respectively. Keto-form might be retained stronger onto the reverse-stationary phase rather than enol-form. When the pH was 7.5, all CoA esters were separated (Fig. 4B and 4C). Last, the column temperature was investigated, and at temperatures higher than 21°C, the 3-hydroxyhexadecanoyl-CoA(4) overlapped with (R)-3-hydroxyhexadecanoyl-CoA(1). At 20°C, all CoA esters could be observed separately with good reproducibility (Fig. 4D). The HPLC chromatogram obtained under the conditions described above is shown in Fig. 5. The separation factor (α) of(R)-3-hydroxyhexadecanoyl-CoA(8) and 3-hydroxyhexadecanoyl-CoA(7) as well as that of 3-hydroxyhexadecanoyl-CoA(9) and (S)-3-hydroxyhexadecanoyl-CoA(8) was 5.5. HPLC chromatograms of 3,3-ethylenedioxyhexadecanoyl-CoA(6) before deprotection(A), 3-oxohexadecanoyl-CoA(7) obtained after deprotection(B), and mixture of 3-oxohexadecanoyl-CoA(7) and 3,3-ethylenedioxyhexadecanoyl-CoA(6)(C). HPLC conditions were as follows: column, Inertsil C-8(4.6 × 250 mm, φ = 5 mm); mobile phase, 50 mM phosphate buffer (pH 5.0)/acetonitrile = 50/50 (v/v); flow rate, 1 mL/min; detection, 260 nm; column temperature, ambient.
droxyhexadecanoyl-CoA \((\text{10})\) were 1.30 and 1.26, respectively. In addition, the resolution \((R_s)\) between \((R)-3\)-hydroxyhexadecanoyl-CoA and \((S)-3\)-hydroxyhexadecanoyl-CoA were 1.48 and 1.24, respectively. Thus, we achieved separation and detection of \((R)-3\)-hydroxyhexadecanoyl-CoA, \((S)-3\)-hydroxyhexadecanoyl-CoA, and \(\text{trans-2-hexadecenoyl-CoA}\) (10). This result suggests that the method allows us to measure the hydratase activity and subsequent dehydrogenase activity peroxisomal bifunctional proteins separately at once. Advantageously, \(\text{D-}\) and \(\text{L-}\)-BP activities in a reaction mixture can be measured with this method. For example, the changes of \(\text{D-}\) or \(\text{L-}\)-BP activities in any cells or tissues against any kind of stimulation can be known by using this method. The method is useful for not only the study of physiological roles of \(\text{L-}\) and \(\text{D-}\)-BP in peroxisomal \(\beta\)-oxidation, but also for the classification of \(\text{D-}\)-BP (or \(\text{L-}\)-BP) deficiency. There are three types of \(\text{D-}\)-BP deficiency: i) Type I deficiency, of both the hydratase and dehydrogenase units; ii) Type II deficiency, of the hydratase unit; and iii) Type III deficiency, of the dehydrogenase unit \(^{12}\). Use of HPLC detection for classification

\[ \text{synthesis and HPLC of 3-oxoacyl-CoA} \]

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\[ \text{Fig. 4 Effect of methanol proportion (A), pH (B and C), and column temperature (D) on } k' \text{ value of CoA esters. Each symbol indicates the CoA ester as follows: closed circle (●), (R)-3-hydroxyhexadecanoyl-CoA (8); open circle (○), 3-oxohexadecanoyl-CoA (7); closed triangle (▲), (S)-3-hydroxyhexadecanoyl-CoA (9); open triangle (△), \text{trans-2-hexadecenoyl-CoA (10). HPLC conditions were as follows: A, mobile phase, 50 mM phosphate buffer (pH 7.5)/methanol; column temperature, 25°C. B, mobile phase, 50 mM phosphate buffer/methanol = 35/65 (v/v); column temperature, 20°C. C, mobile phase, 50 mM phosphate buffer/methanol = 35/65 (v/v).} \]
cation of D-BP deficiency, using 3-hydroxyacyl-CoA as a substrate and the lysate from cells such as fibroblasts, should show the following results: Type I deficiency should show no product peaks, Type II deficiency should show a peak for 3-oxoacyl-CoA, and Type III deficiency should show a peak for trans-2-enoyl-CoA. In contrast, healthy controls should show peaks for 3-oxoacyl-CoA and trans-2-enoyl-CoA.

To avoid overlooking the discovery of peroxisomal bifunctional protein deficiency at the clinic site, we developed the analytical method using a UV detector in consideration of the versatility of the instruments. The detection limit of CoA esters using a UV detector was 5 pmol (signal-to-noise ratio = 6) and quantification limit was estimated 15 pmol. Currently, we have not verified yet whether the analytical method can be applied to specimens such as blood or cells. Henceforth we intend to test the usefulness of the analytical method using human cultured cells and report the results in next article.

4 CONCLUSION

We chemically synthesized 3-oxohexadecanoyl-CoA for the study of D- and L-BP and also investigated the behavior of the CoA ester under several conditions of HPLC. Finally we established a separation detection of (R)-3-hydroxyhexadecanoyl-CoA, (S)-3-hydroxyhexadecanoyl-CoA, 3-oxohexadecanoyl-CoA, and trans-2-hexadecenoyl-CoA using HPLC apparatus equipped with a chiral separation column. We intend to test the usefulness of the analytical method for the study of D- and L-BP.

REFERENCES


