Analytical Biochemistry 401 (2010) 114–124

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Synthesis and characterization of *cis*-4-decenoyl-CoA, 3-phenylpropionyl-CoA, and 2,6-dimethylheptanoyl-CoA

Hany F. Sobhi [a,b], Paul E. Minkler [a,b], Charles L. Hoppel [a,b,c,*]

[a] Center for Mitochondrial Diseases, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA
[b] Department of Pharmacology, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA
[c] Department of Medicine, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA

ARTICLE INFO

Article history:
Received 28 December 2009
Received in revised form 4 February 2010
Accepted 19 February 2010
Available online 23 February 2010

Keywords:
Acyl-CoA dehydrogenase activity
Semipreparative HPLC
Short-chain acyl-CoA dehydrogenase (SCAD)
Medium-chain acyl-CoA dehydrogenase (MCAD)
Long-chain acyl-CoA dehydrogenase (LCAD)
Very-long-chain acyl-CoA dehydrogenase (VLCAD)

ABSTRACT

The measurement of acyl-CoA dehydrogenase activities is an essential part of the investigation of patients with suspected defects in fatty acid oxidation. Multiple methods are available for the synthesis of the substrates used for measuring acyl-CoA dehydrogenase activities; however, the yields are low and the products are used without purification. In addition, the reported characterization of acyl-CoAs focuses on the CoA moiety, not on the acyl group. Here we describe the synthesis of three medium-chain acyl-CoAs from mixed anhydrides of the fatty acids using an aqueous–organic solvent mixture optimized to obtain the highest yield. First, *cis*-4-decenoic acid and 2,6-dimethylheptanoic acid were prepared (3-phenylpropionic acid is commercially available). These were characterized by gas chromatography/mass spectrometry (GC/MS), ^{1}H nuclear magnetic resonance (NMR), and ^{13}C NMR. Then *cis*-4-decenoyl-CoA, 3-phenylpropionyl-CoA, and 2,6-dimethylheptanoyl-CoA were synthesized. These were then purified by ion exchange solid-phase extraction using 2-(2-pyridyl)ethyl-functionalized silica gel, followed by reversed-phase semipreparative high-performance liquid chromatography with ultraviolet detection (HPLC–UV). The purified acyl-CoAs were characterized by analytical HPLC–UV followed by data-dependent tandem mass spectrometry (MS/MS) analysis on the largest responding MS mass (HPLC–UV–MS–MS/MS) and ^{13}C NMR. The yields of the purified acyl-CoAs were between 75% and 78% based on coenzyme A trilithium salt (CoASH). Acyl-CoA dehydrogenase activities were measured in rat skeletal muscle mitochondria using, as substrates, the synthesized *cis*-4-decenoyl-CoA, 3-phenylpropionyl-CoA, and 2,6-dimethylheptanoyl-CoA. These results were compared with the results using our standard substrates butyryl-CoA, octanoyl-CoA, and palmitoyl-CoA.

Medium-chain acyl-CoA dehydrogenase (MCAD)[1] deficiency is recognized as a potentially lethal inborn error of metabolism during childhood [1]. The use of metabolic data, such as elevation of blood octanoylcarnitine coupled with enzymatic and mutational analysis, is used for the diagnosis of MCAD deficiency [2]. From a metabolic viewpoint, 3-phenylpropionyl-CoA has been identified as a specific substrate for MCAD [3] and the glycine conjugate 3-phenylpropionylglycine has been established as a pathognomonic marker in urine from patients affected with MCAD deficiency [4]. *cis*-4-Decenoic acid also is a marker for MCAD deficiency [5,6]. Metabolically, MCAD effectively accepts *cis*-4-decenoyl-CoA as a substrate, whereas long-chain acyl-CoA dehydrogenase (LCAD) does not [7]. 2,6-Dimethylheptanoyl-CoA is a selective substrate for LCAD but not for very-long-chain acyl-CoA dehydrogenase (VLCAD) [8].

Several methods have been described to synthesize fatty acyl-CoA substrates for use in the enzymatic assessment of acyl-CoA dehydrogenase activity. These synthetic approaches involve acylating free coenzyme A trilithium salt (CoASH) using commercially available fatty acid derivatives such as symmetric anhydrides [9], mixed anhydrides of ethylhydrogen carbonate [10], acid chlorides [11], *N*-hydroxysuccinimide esters [12,13], and acyl imidazoles [14,15]. Due to the low solubility of CoASH in most organic solvents, the acylation has been performed in aqueous solvents at pH 7.5 to 8.0 [16]. However, under these conditions, poor solubility of the acylating reagents or the occurrence of various side reactions

* Corresponding author. Address: Department of Pharmacology Medicine, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA. Fax: +1 216 368 5162.
E-mail address: charles.hoppel@case.edu (C.L. Hoppel).

[1] *Abbreviations used:* MCAD, medium-chain acyl-CoA dehydrogenase; LCAD, long-chain acyl-CoA dehydrogenase; VLCAD, very-long-chain acyl-CoA dehydrogenase; CoASH, coenzyme A trilithium salt; SPE, solid-phase extraction; HPLC–UV, high-performance liquid chromatography with ultraviolet detection; HPLC–UV–MS–MS/MS, HPLC–UV followed by data-dependent tandem mass spectrometry analysis on the largest responding mass spectrometry mass; NMR, nuclear magnetic resonance; GC/MS, gas chromatography/mass spectrometry; RT, room temperature; DMF, dimethylformamide; BSTFA/TMCS, bis(trimethylsilyl) trifluoroacetamide/trimethylchlorosilane; TMS, trimethylsilane; THF, tetrahydrofuran; TIC, total ion current; SCAD, short-chain acyl-CoA dehydrogenase.

0003-2697/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2010.02.026

often results in low yields. The current article describes a method for solubilization of CoASH in an aqueous–organic solvent mixture followed by acylation using an ethylchloroformate mixed anhydride. By this procedure, we synthesized *cis*-4-decenoyl-CoA, 3-phenylpropionyl-CoA, and 2,6-dimethylheptanoyl-CoA and used them as substrates for MCAD and LCAD activity measurements. A significant feature of this study is that the synthesized acyl-CoAs were purified by solid-phase extraction (SPE) and semipreparative high-performance liquid chromatography with ultraviolet detection (HPLC–UV). The overall procedure results in excellent yields with high recovery of the acyl-CoAs. Furthermore, the purified acyl-CoAs are characterized by analytical HPLC–UV followed by data-dependent tandem mass spectrometry (MS/MS) analysis on the largest responding mass spectrometry (MS) mass (HPLC–UV–MS–MS/MS) and nuclear magnetic resonance (NMR).

Materials and methods

Chemicals and solvents

CoASH, *cis*-4-decen-1-al (97% pure), hydrocinnamic acid (3-phenylpropionic acid), anhydrous ethylchloroformate, ammonium formate, silver nitrate, thionyl chloride, 6-methyl-2-heptanol, *p*-toluene sulfonyl chloride, potassium phosphate, cytochrome *c*, potassium cyanide, phenazine ethosulfate, *N*-ethylmaleimide, and rotenone were purchased from Sigma–Aldrich (St. Louis, MO, USA). Butyryl-CoA, octanoyl-CoA, and palmitoyl-CoA trilithium salts also were purchased from Sigma–Aldrich. Methanol (HPLC grade) was purchased from Fisher Scientific (Cleveland, OH, USA). Acetonitrile (Burdick & Jackson brand, HPLC grade) was purchased from Jade Scientific (Canton, MI, USA). The $CDCl_3$ and D_2O used in NMR experiments were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Reagent-grade water was prepared using a Milli-Q system (Millipore, Bedford, MA, USA). 2-(2-Pyridyl)ethyl-functionalized silica gel and empty polypropylene 6-ml SPE tubes with polyethylene frits were purchased from Sigma–Aldrich.

Equipment

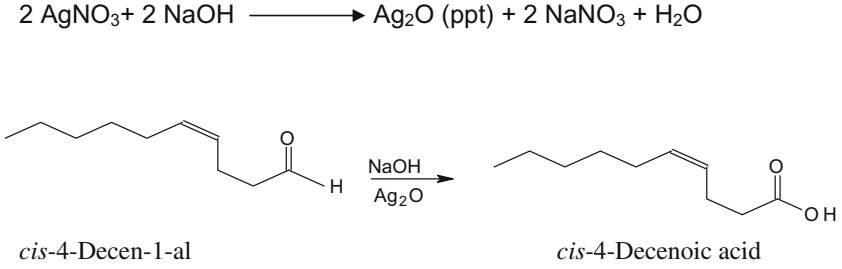
The gas chromatography/mass spectrometry (GC/MS) consisted of an HP6890 GC device, an HP5973 mass spectrometer, and an HP autosampler purchased from Agilent Technologies (Wilmington, DE, USA). A BioCAD SPRINT perfusion chromatography system (PerSeptive Biosystems, Framingham, MA, USA) was configured to perform semipreparative reversed-phase HPLC [17] and included a Gilson model FC-205 fraction collector. The column used was an Alltima C18 LL (250 × 22 mm, 5 μm) purchased from Alltech Associates (Deerfield, IL, USA), and the UV detector was set to monitor absorbance at 258 nm. For flow injection MS and MS/MS analysis of the fractions collected from the semipreparative HPLC–UV, an LC–MS–MS/MS system was used. It consisted of an HP1100 series HPLC system (quaternary pump with degasser and autosampler) from Agilent Technologies and a 3200 Q TRAP mass spectrometer from Applied Biosystems (Concord, Ontario, Canada). Both instruments were controlled and data were collected using Analyst 1.5 software (Applied Biosystems). For final characterization of the synthesized acyl-CoAs, an analytical HPLC–UV–MS–MS/MS system was used and consisted of an Agilent HPLC device and an LCQ Deca ion trap mass spectrometer as described previously [17]. The analytical column used was a Hypersil GOLD column (250 × 4.6 mm, 5 μm) purchased from Thermo Fisher Scientific (Waltham, MA, USA). NMR spectra were obtained with a 400-MHz Varian Inova spectrometer (Varian, Lake Forest, CA, USA) or a 600-MHz Varian Inova spectrometer. Fourier transform data were processed using Master Nova software (version 5.2.5-4119, Masterlab Research, Bajo, Spain). The acyl-CoA dehydrogenase activity measurements were made using a DU 800 UV/visible spectrophotometer purchased from Beckman Coulter (Fullerton, CA, USA). The concentrations of synthesized acyl-CoAs were determined by the method of Ellman [18].

Synthesis of cis-4-decenoic acid

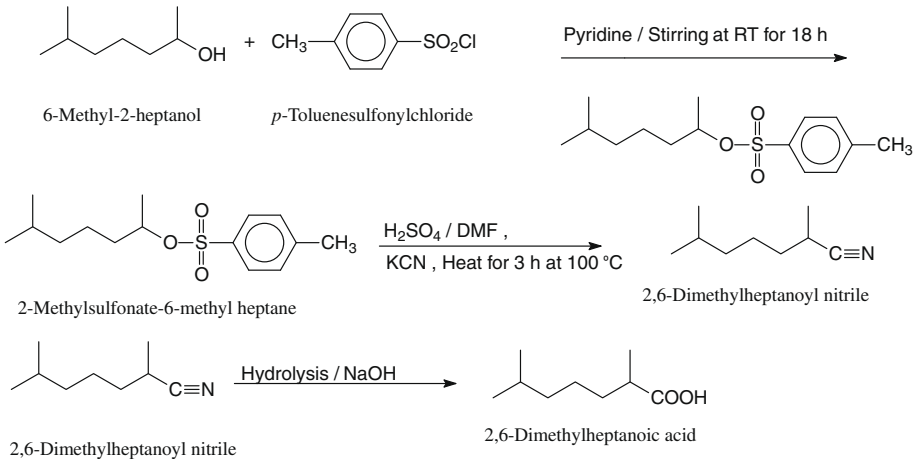
cis-4-Decenoic acid was synthesized according to Scheme 1 [19]. First, silver oxide was freshly prepared by the addition of a silver nitrate solution (3.03 g in 10 ml of water) into a solution of sodium hydroxide (2.40 g in 20 ml of water), and the mixture was stirred for 60 min. Silver oxide was precipitated and collected by filtration, and the filtrate was discarded. Second, the silver oxide was dissolved in a solution of sodium hydroxide (1.25 g in 15 ml of water), followed by the dropwise addition of 1.5 ml of *cis*-4-decen-1-al over 20 min, and the reaction mixture was stirred for 2 h. During the reaction, silver is formed and precipitates. The solution was filtered and the pellet was discarded. Third, the filtrate containing the acid was collected. The product, *cis*-4-decenoic acid, was obtained by acidification of the filtrate to pH 4.5 with 1 N HCl followed by extraction (three times with 15 ml of ethyl ether each). The ethyl ether (45 ml containing the acid) was collected and the aqueous phase was discarded. Finally, the ethyl ether was removed under reduced pressure and *cis*-4-decenoic acid was collected.

Synthesis of 2,6-dimethylheptanoic acid

2,6-Dimethylheptanoic acid was synthesized according to Scheme 2 [20,21]. First, 6-methyl-2-heptanol (5.0 g) was added to pyridine (25 ml), followed by the addition of *p*-toluene sulfonyl chloride (8.8 g), and the reaction mixture was stirred for 18 h at room temperature (RT) under nitrogen. Second, the 2-methylsulfonate-6-methyl-heptane formed was extracted with hexane (15 ml), and the hexane was removed under reduced pressure. The residue of 2-methylsulfonate-6-methyl heptane was dissolved in 100 ml of 3 N sulfuric acid, stirred for 30 min, and extracted three times with 15 ml of ethyl ether. Third, the combined ethyl



Scheme 1. Synthesis of *cis*-4-decenoic acid.



Scheme 2. Synthesis of 2,6-dimethylheptanoic acid.

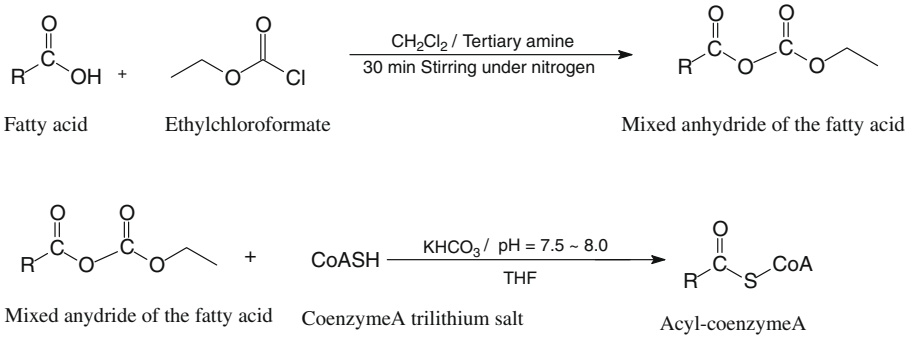
ether extracts (45 ml) were evaporated under reduced pressure, and the residue was dissolved in 30 ml of dimethylformamide (DMF). This solution was stirred with potassium cyanide (2.6 g) for 3 h in a silicone oil bath of 100 °C to form 2,6-dimethylheptanoyl nitrile. The reaction mixture was then extracted with hexane (15 ml) and the hexane was evaporated. Fourth, the residue (2,6-dimethylheptanoyl nitrile) was dissolved in 30 ml of ethanol, to which was added an NaOH solution (1 g in 25 ml of ethanol), and this solution was then refluxed for 24 h. For the final step, the ethanol was evaporated and the reaction mixture was acidified to pH 4.0 using 1 N HCl followed by extraction (three times with 10 ml of ethyl ether). The ethyl ether extract was evaporated and the product (2,6-dimethylheptanoic acid) was collected.

GC/MS

Acid samples (2×10^{-6} mol) were derivatized with 50 µl of bis-(trimethylsilyl) trifluoroacetamide and trimethylchlorosilane (BSTFA/TMCS, 99:1, Sigma–Aldrich), and 1 µl containing 10^{-8} mol of the resulting trimethylsilane (TMS) derivative was injected into a split/splitter injector set at 280 °C [22]. The capillary column was 30 × 0.25 mm and 0.25 µm (HP-5 ms). The oven temperature was programmed to change from 80 to 150 °C at a rate of 30 °C min^{-1} (2.5 min) and then from 150 to 280 °C at a rate of 5 °C min^{-1} (30.0 min), and finally it was held at 280 °C for 10.0 min. Helium was used as a carrier gas at a flow rate of 1.5 ml/min, the electron ionization setting was 70 eV, and the heating source was set at 230 °C.

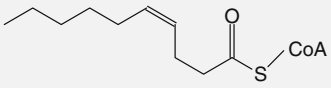
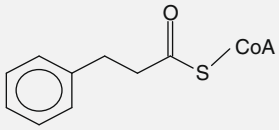
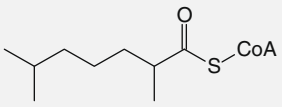
General method for synthesis of medium-chain acyl-CoAs

Medium-chain acyl-CoAs were synthesized from the mixed anhydrides of fatty acids by a modification of the procedures of Goldman and Vagelos [10] and Wieland and Rueff [23]. The general synthesis is shown in Scheme 3, and the compounds prepared in this study are shown in Table 1. First, the fatty acid (0.6 mmol) was mixed with 10 ml of CH_2Cl_2, followed by the addition of a tertiary amine (1.2 mmol, i.e., triethylamine or 2,4,6-trimethylpyridine (collidine) [see Table 1]). The reaction mixture was stirred for 30 min under nitrogen and then placed in an ice bath for 10 min. Second, anhydrous ethylchloroformate (1.2 mmol) was added dropwise over 10 min while stirring under nitrogen at RT, and stirring was continued for 2 h. Third, the solvent, CH_2Cl_2, was removed under reduced pressure until dryness. The residue (containing the mixed anhydride) was dissolved in 10 ml of anhydrous tetrahydrofuran (THF), the undissolved amine salts were precipitated and removed by filtration, and the filtrate (containing the mixed anhydride) was collected. Under nitrogen, CoASH (64 µmol) was dissolved in 2 ml water/8 ml THF and the pH was adjusted to 7.5 with 0.01 M $KHCO_3$. Fourth, the mixed anhydride of the fatty acid was added to the CoASH solution and the pH was adjusted to 8.0. This solution was stirred for 30 min under nitrogen and then acidified to pH 4.5 with 1% $HClO_4$. The THF was removed under reduced pressure. Finally, the reaction mixture was acidified with 1 ml of 10% $HClO_4$ and washed three times with 5 ml of ethyl ether to remove any acid present. The reaction mixture containing the synthesized acyl-CoAs was then subjected to SPE and semipreparative HPLC–UV.



Scheme 3. Synthesis of medium-chain acyl-CoAs.

Table 1
Synthesized medium-chain acyl-CoAs.

Acyl-CoA	Molecular structure	Tertiary amine	Yield (%)
cis-4-Decenoyl-CoA		2,4,6-Trimethylpyridine (collidine)	75.0
3-Phenylpropionyl-CoA		Triethylamine	78.0
2,6-Dimethylheptanoyl-CoA		Triethylamine	76.0

Purification of synthesized acyl-CoAs using ion exchange SPE

SPE columns (6 ml) were packed with 500 mg of 2-(2-pyridyl)ethyl-functionalized silica gel [24] and rinsed with 10 ml of the condition solvent (acetonitrile/isopropanol/water/glacial acetic acid, 9:3:4:4 by volume) using a Visiprep SPE vacuum manifold (Sigma–Aldrich). The acyl-CoA reaction mixture (~5 ml) was added to 5 ml of the conditioning solvent, and then this solution was applied to the SPE column and the flow-through was discarded. The SPE column was then washed with 10 ml of the conditioning solvent, and this wash also was discarded. The acyl-CoAs were eluted with 20 ml methanol/250 mM ammonium formate (4:1 by volume); this eluent was collected and the volume was reduced to approximately 5 ml by evaporation under reduced pressure. This entire sample was then injected into the semipreparative HPLC–UV.

Semipreparative HPLC–UV

The eluents used were 95% 0.1 M ammonium formate in water/5% methanol (eluent A) and 95% acetonitrile/5% water (eluent B). The instrument flow rate was 10 ml/min and was programmed to start with 100% eluent A for 10 min, followed by a gradient of 100% A to 100% B over 30 min, followed by 100% eluent B for 10 min. The UV detector collected data at 258 nm, and eluent fractions were collected at a rate of 1 fraction/0.5 min. Fractions were characterized by flow injection analysis.

Flow injection analysis by LC–MS–MS/MS

Aliquots (100 µl) from the semipreparative HPLC–UV fractions were evaporated to dryness in HPLC sample tubes, reconstituted in 500 µl acetonitrile/water (80:20 by volume), and at a flow of

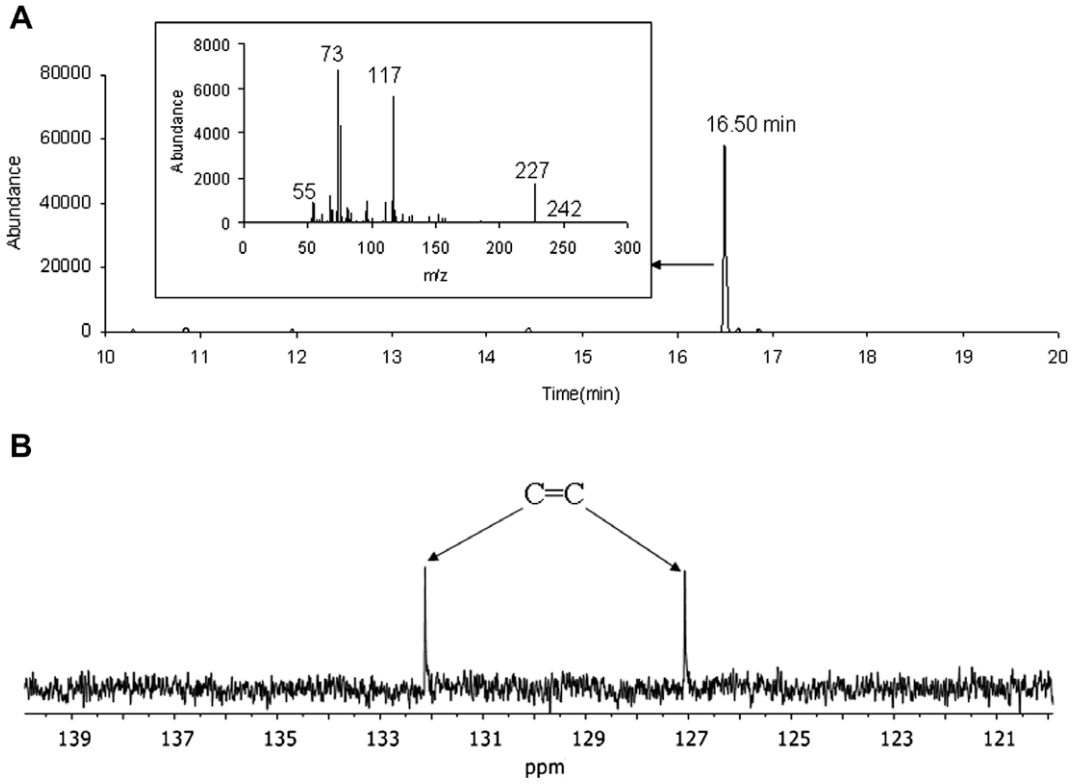


Fig. 1. Characterization of synthesized *cis*-4-decenoic acid. (A) GC/MS chromatogram of the TMS derivative of *cis*-4-decenoic acid has a single peak at 16.50 min. The MS spectrum contains the [M^+] ion at m/z 242. (B) ^{13}C NMR contains signals at δ = 132.13 and 127.07 ppm, consistent with a double bond at carbon 4.

Table 2
Key spectral features used for characterization.

Acyl-CoA species	^{13}C NMR data (feature that is observed)		Acyl-CoA MS data (feature that is observed)	
	Acid (δ, ppm)	CoA (δ, ppm)	MS (m/z)	MS/MS (m/z)
cis-4-Decenoyl-CoA	132.13,127.07 (C=C)	132.73, 127.08 (C=C)	918 ([M$^-$])	408, 426 (phosphoadenosine group)
3-Phenylpropionyl-CoA	140.37, 128.81, 128.51, 126.63 (phenyl group)	140.18, 128.73,128.47, 126.55 (phenyl group)	898 ([M$^-$])	408, 426 (phosphoadenosine group)
2,6-Dimethylheptanoyl-CoA	38.95 (C–CH_3 carbon 6) 28.01 (C–CH_3 carbon 2)	38.95 (C–CH_3 carbon 6) 30.65 (C–CH_3 carbon 2)	906 ([M$^-$])	408, 426 (phosphoadenosine group)

200 µl/min (acetonitrile/water, 80:20) were injected (50 µl) into the HP1100 HPLC/3200 Q TRAP mass spectrometer. The instrument was operated in the ion trap mode, and full-scan MS spectra (m/z 700–1000) were collected for 2 min. For MS/MS analysis, a second injection was made and the full-scan MS/MS spectra were collected, fragmenting the ion observed in the MS spectra using a collision energy of −70 V.

Analytical HPLC–UV–MS–MS/MS

The eluents were the same as for the semipreparative HPLC–UV. The gradient was 100% A to 100% B over 60 min at a flow rate of 0.5 ml/min. Simultaneously, UV (259 nm), full-scan MS, and full-scan MS/MS (data-dependent) data were collected. Data-dependent MS/MS data collection was triggered on the most intense ion observed in the MS spectrum using a setting of 26 for the relative collision energy.

^{1}H NMR and ^{13}C NMR

NMR samples of the synthesized fatty acids were prepared by dissolving 100 µl (50.0 mmol) in 400 µl of $CDCl_3$. NMR analysis of the synthesized acyl-CoAs was performed by dissolving 300 µl (1.0 mmol) in 200 µl of D_2O. All NMR spectra were obtained at ambient temperature and pressure.

Acyl-CoA dehydrogenase activity studies

Acyl-CoA dehydrogenase activities were measured using, as substrates, the synthesized and purified *cis*-4-decenoyl-CoA, 3-phenylpropionyl-CoA, and 2,6-dimethylheptanoyl-CoA as well as our standard substrates butyryl-CoA, octanoyl-CoA, and palmitoyl-CoA. Rat skeletal muscle mitochondria were prepared and the acyl-CoA dehydrogenase activities were determined spectrophotometrically at 37.0 °C with 20 µg of mitochondrial protein in

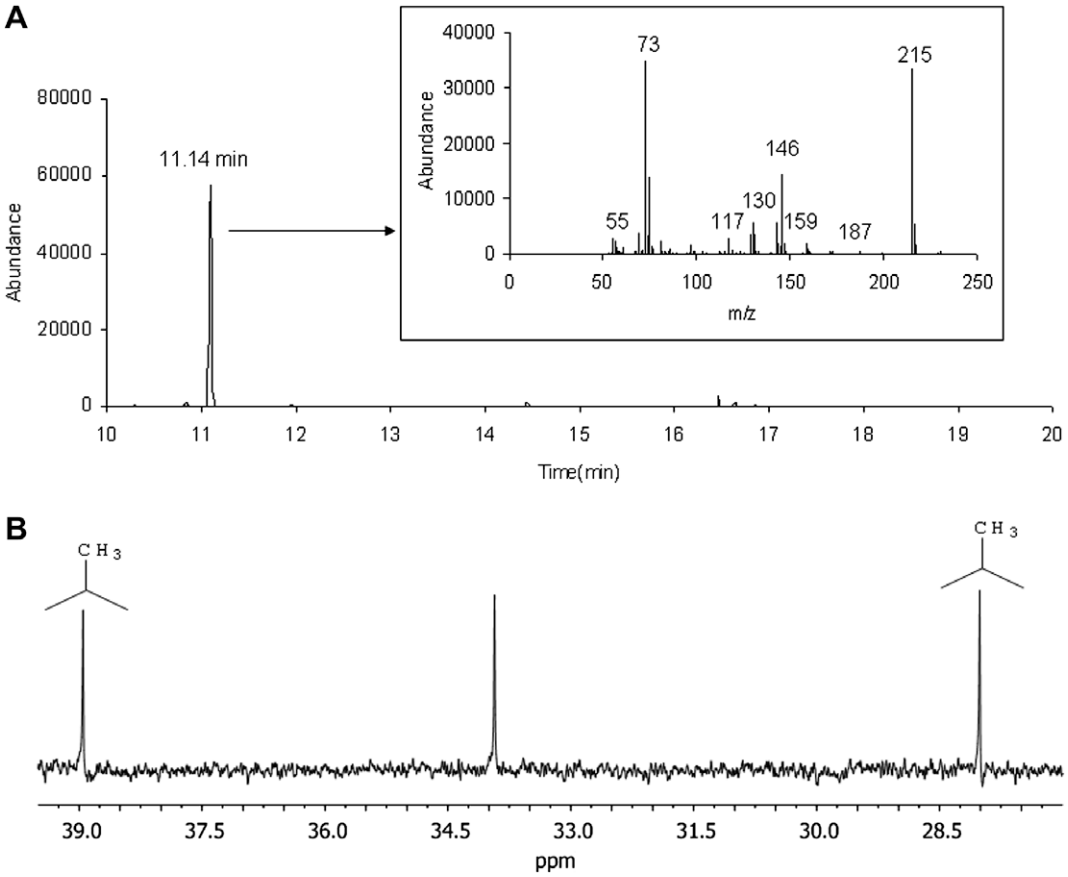


Fig. 2. Characterization of synthesized 2,6-dimethylheptanoic acid. (A) GC/MS chromatogram of the TMS derivative of 2,6-dimethylheptanoic acid with a retention time of 11.14 min. The MS spectrum contains the [M$^+$] ion at m/z 230. (B) ^{13}C NMR contains signals at δ = 38.95 and 28.01 ppm, consistent with two branched methyl groups.

a 1-ml cuvette as described by Hoppel and coworkers [25]. The reaction medium contained 34 mM potassium phosphate (pH 7.2), 0.15 mM cytochrome *c*, 1.5 mM potassium cyanide, 3.0 mM phenazine ethosulfate, 1.0 mM *N*-ethylmaleimide, and 0.004 mM rotenone. The reaction was monitored at 550 nm.

Results and discussion

The goals of this study were to develop a general method for the synthesis of medium-chain acyl-CoAs from unsaturated and less common fatty acids, which are not commercially available, and to establish a procedure for the isolation and purification of highly purified acyl-CoAs in good yields. The synthesis starts with solubilizing CoASH and the activated mixed anhydride of the medium-chain fatty acid anhydride in a solvent system of THF/water. This solvent system decreases the hydrolysis of the mixed anhydride and maintains the solubility of CoA at RT. The acylation stoichiometry is 20-fold excess of the fatty acid to CoASH; this enhances full acylation of the CoASH to acyl-CoA.

Two of the fatty acids required for this project were not commercially available, and so we synthesized these acids in our laboratory. The key step in the synthesis of *cis*-4-decenoic acid is the pH control of the reaction mixture; keeping the pH below 4.0 results in a yield of 92% and avoids saturation or isomerization. The synthesized acid was characterized by ^{13}C NMR and GC/MS (Fig. 1) (the full ^{1}H NMR and ^{13}C NMR spectra of the prepared *cis*-4-decenoic acid are contained in the supplementary material). NMR data are consistent with the presence of the double bond between carbons 4 and 5 in the *cis* conformation [26] (^{13}C NMR: δ = 132.13 and 127.07; ^{1}H NMR: δ = 5.43 and 5.35 ppm) (Table 2 and supplementary material). The GC/MS chromatogram of the TMS acid derivative shows a single peak at 16.50 min. The MS spectrum contains fragment ions consistent with *cis*-4-decenoic acid (*m*/*z* 73, 117, 147, and 157), as reported previously [16]. Likewise, 2,6-dimethylheptanoic acid was synthesized. Its yield was 39%, and it was analyzed by GC/MS. Fig. 2A shows the GC/MS chromatogram and GC/MS spectrum, and Fig. 2B contains a section of the ^{13}C NMR spectrum. The NMR data are consistent with the presence of branched methyl groups on carbons 2 and 6 [23] (^{13}C NMR: δ = 38.95 and 28.01 ppm; ^{1}H NMR: δ = 1.18 and 0.89 ppm) (Table 2 and supplementary material). The GC/MS chromatogram of the TMS derivative of the acid shows a single peak at 11.14 min, and the MS spectrum contains fragment ions consistent with 2,6-dimethylheptanoic acid (*m*/*z* 73, 130, 146, and 159), as reported previously [15]. The purchased 3-phenylpropionic acid was examined by ^{13}C NMR, and the spectrum contained the expected signals (Table 2).

The synthesis protocol resulted in good yields of acyl-CoAs (75–78% based on CoASH). Semipreparative HPLC–UV chromatograms are shown in Figs. 3–5. Table 2 shows the key ^{13}C NMR data used to characterize the synthesized acyl-CoA and shows that they relate directly to the ^{13}C NMR data observed in the acid. Also shown in Table 2 are the MS/MS data generated from the flow injection analysis. The presence of an acyl-CoA is indicated by the specific pattern of ions reported (*m*/*z* 408 and 426). Fig. 3A shows a semi-

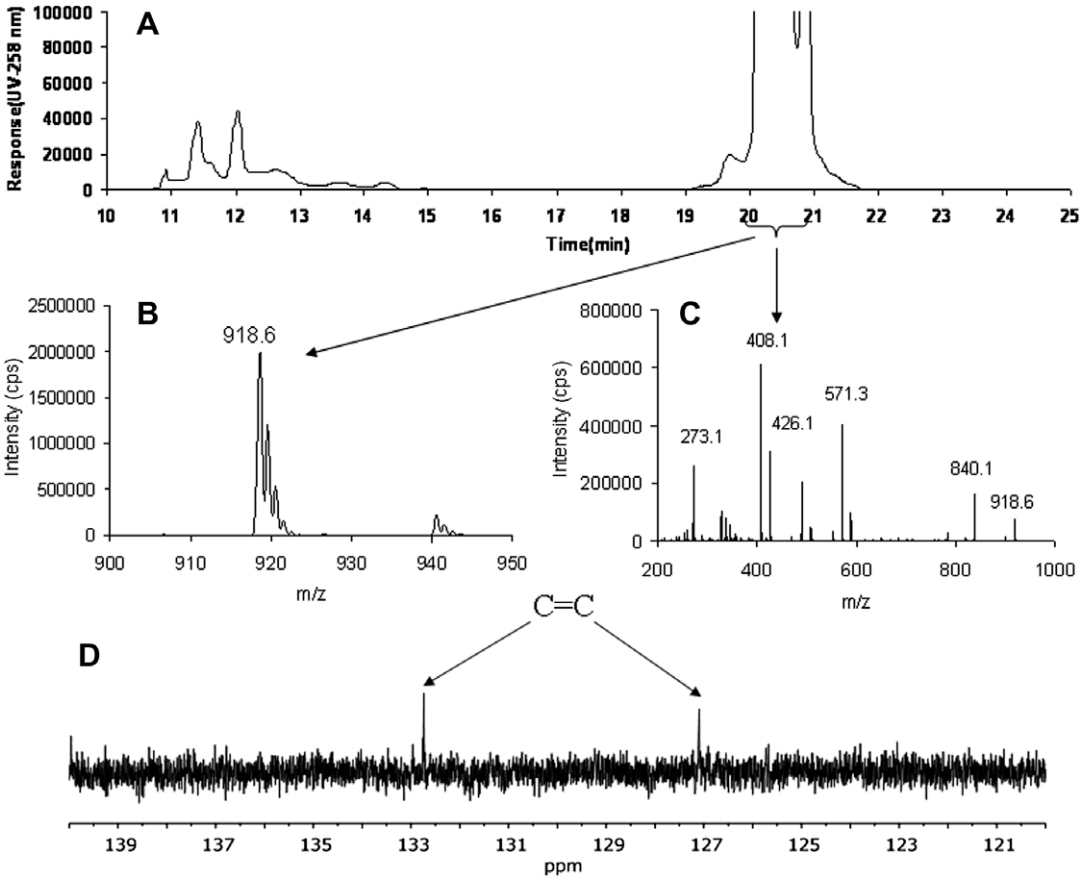


Fig. 3. Characterization of *cis*-4-decenoyl-CoA. (A) Semipreparative HPLC–UV chromatograph (258 nm). The peak between 20 and 21 min is *cis*-4-decenoyl-CoA. This peak was collected and characterized. (B) Off-line MS flow injection analysis of the collected peak gives MS response at *m*/*z* 918. (C) Full-scan MS/MS flow injection fragmentation of *m*/*z* 918 and the characteristic acyl-CoA fragments of *m*/*z* 426 and 408 are shown. (D) ^{13}C NMR spectrum of the peak collected between 20 and 21 min has signal responses at δ = 132.73 and 127.08 ppm. This is consistent with double bond peaks between carbons 4 and 5 (see also Fig. 1B and Table 2).

preparative HPLC–UV chromatogram of *cis*-4-decenoyl-CoA, which eluted at 20.2 min. Fractions 41 and 42 (20.0–21.0 min) were combined and analyzed by flow injection LC–MS–MS/MS. Fig. 3B shows the MS spectrum containing *m*/*z* 918, which is consistent with *cis*-4-decenoyl-CoA. Fig. 3C is the MS/MS spectrum generated from flow injection analysis. The fragments *m*/*z* 426 (from the phosphoadenosine group [27,28]) and *m*/*z* 408 (loss of water from the phosphoadenosine group) are consistent with CoASH. A section of ^{13}C NMR of the isolated *cis*-4-decenoyl-CoA (Fig. 3D) contains spectral peaks (δ = 132.73 and 127.08 ppm) identical to the spectral peaks observed in *cis*-4-decenoic acid (Table 2), and these peaks are consistent with the double bond between carbons 4 and 5 of the *cis*-4-decenoic acid (the full ^{13}C NMR spectrum is shown in the supplementary material).

The synthesis of 3-phenylpropionyl-CoA is greatly aided by our ability to purchase the acid (3-phenylpropionic acid) rather than undertake its synthesis (as in the case of *cis*-4-decenoic acid and 2,6-dimethylheptanoic acid). Table 2 shows the key ^{13}C NMR data used to characterize synthesized 3-phenylpropionyl-CoA and shows that the ^{13}C NMR acyl-CoA data relate directly to the ^{13}C NMR data observed in the acid. Fig. 4A shows a semipreparative HPLC–UV chromatogram of 3-phenylpropionyl-CoA, which eluted at 17.8 min. Fraction 36 (17.5–18.0 min) was analyzed by flow injection LC–MS–MS/MS. Fig. 4B shows the MS spectrum containing *m*/*z* 898, which is consistent with 3-phenylpropionyl-CoA. Fig. 4C is the MS/MS spectrum generated from flow injection analysis. The fragments *m*/*z* 408 and 426 are consistent with CoASH. Fractions 25 and 26 (12.0–13.0 min) were identified by ^{13}C NMR as containing unreacted 3-phenylpropionic acid and by flow injection MS as containing unreacted CoASH. The ^{13}C NMR of isolate 3-phenylpropionyl-CoA (Fig. 4D) contains spectral peaks (δ = 140.18 and 128.73, 128.47, and 126.55 ppm), consistent with the presence of a phenyl group. This portion of the spectra is identical to that observed in 3-phenylpropionic acid (Table 2).

Fig. 5A shows the semipreparative HPLC chromatogram of 2,6-dimethylheptanoyl-CoA, which eluted at 20.1 min. Fraction 41 (20.0–20.5 min) was analyzed by flow injection LC–MS–MS/MS. Fig. 5B shows the MS spectrum containing *m*/*z* 906, which is consistent with 2,6-dimethylheptanoyl-CoA. Fig. 5C is the MS/MS spectrum generated from flow injection analysis. The fragments *m*/*z* 408 and 426 are consistent with CoASH. These fragment ions also are present in the flow injection analysis MS/MS spectra for *cis*-4-decenoyl-CoA and 3-phenylpropionyl-CoA (Figs. 3C and 4C, respectively). Fractions 25 and 26 (12.0–13.0 min) showed unreacted CoASH. The ^{13}C NMR of the isolated 2,6-dimethylheptanoyl-CoA (Fig. 5D) contains spectral peaks (δ = 38.95 and 30.65 ppm) identical to the spectral peaks observed in 2,6-dimethylheptanoic acid (Fig. 2B). These are consistent with the presence of two branched methyl groups at carbons 2 and 6. Full NMR spectra are shown in the supplementary material.

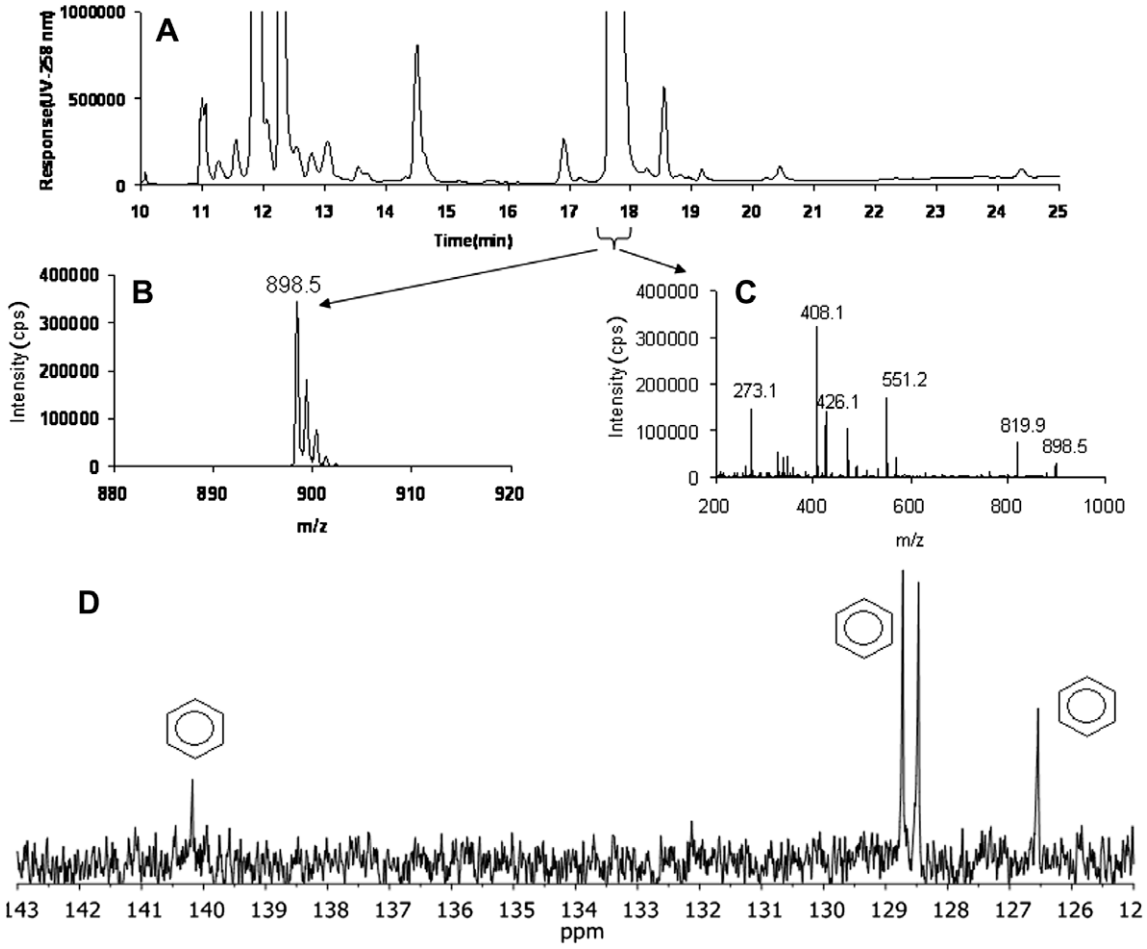


Fig. 4. Characterization of 3-phenylpropionyl-CoA. (A) Semipreparative HPLC–UV chromatogram with the 3-phenylpropionyl-CoA peak between 17.5 and 18.0 min. (B) Offline MS flow injection analysis of the peak collected between 17.5 and 18.0 min gives a response at *m*/*z* 898. (C) Full-scan MS/MS flow injection fragmentation of *m*/*z* 898, producing the characteristic acyl-CoA fragment ions. (D) ^{13}C NMR spectrum contains δ = 140.18, 128.73, 128.47, and 126.55 ppm, consistent with four characteristic peaks of a phenyl group.

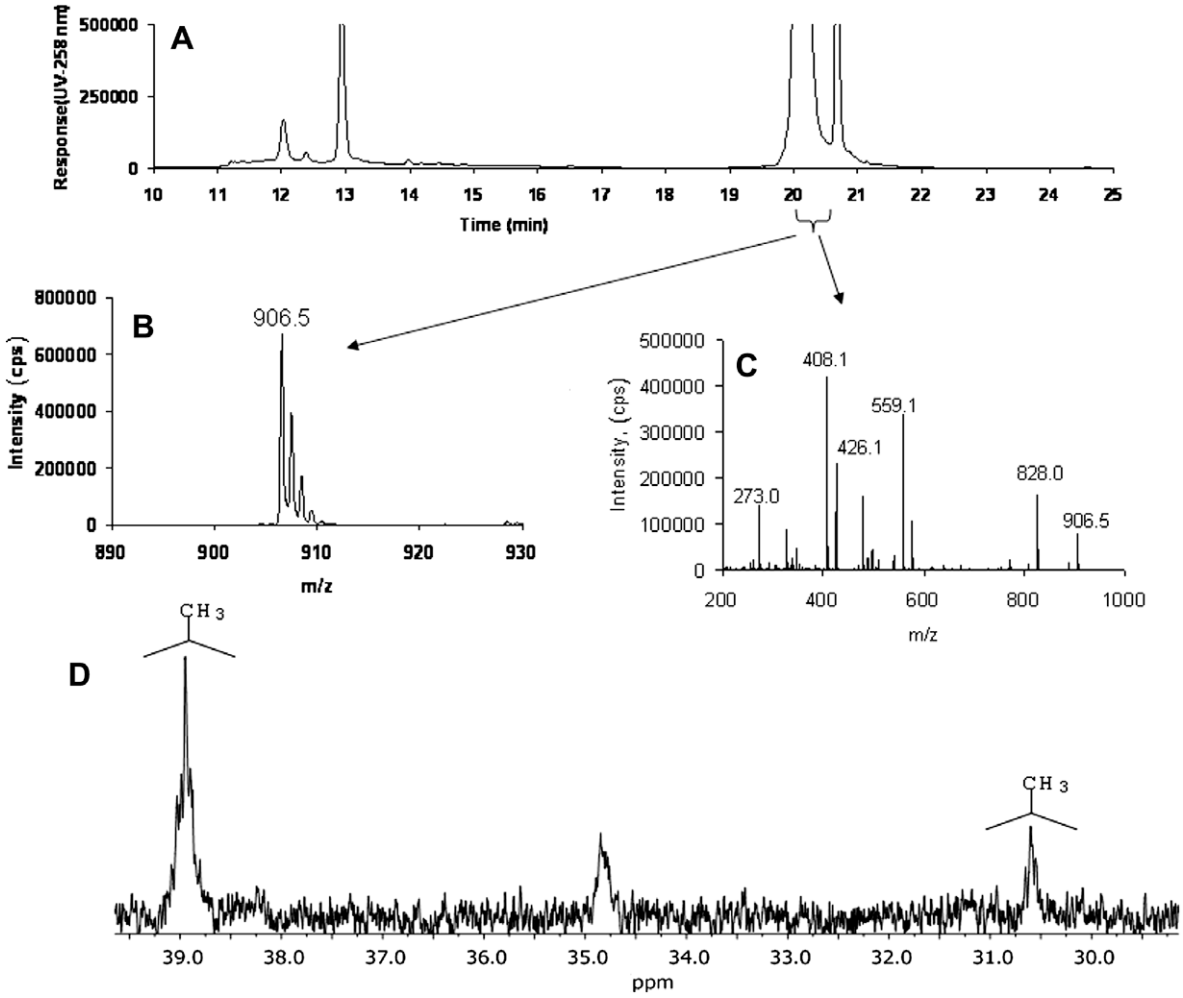


Fig. 5. Characterization of 2,6-dimethylheptanoyl-CoA. (A) Semipreparative HPLC–UV with the 2,6-dimethylheptanoyl-CoA peak between 20.0 and 20.5 min. (B) Off-line MS flow injection analysis of the peak collected between 20.0 and 20.5 min gives a response at *m*/*z* 906. (C) Full-scan MS/MS flow injection fragmentation of *m*/*z* 906, producing the characteristic acyl-CoA fragment ions. (D) ^{13}C NMR spectrum contains δ = 38.95 and 30.65 ppm, consistent with two branched methyl groups.

Analytical HPLC–UV–MS–MS/MS

The purification of the synthesized acyl-CoAs resulted in purity greater than 95% (based on the UV response of the analytical HPLC–UV–MS–MS/MS analysis [Figs. 6–8]). Documentation of the purity of our synthesized acyl-CoA products was performed by analytical HPLC–UV–MS–MS/MS. Figs. 6–8 show the UV chromatograms, MS chromatograms, MS spectra of the acyl-CoA chromatographic peaks, and MS/MS spectra of those same peaks. Fig. 6A shows the UV chromatogram (at 259 nm) of our purified *cis*-4-decenoyl-CoA, which eluted at 34.0 min. There is a second peak at 35.8 min, which is only 3.2% of the total peak area. There is a very small peak (0.3% of the total peak area) at 34.6 min. By mass and chromatographic behavior, this small peak is consistent with *cis*-4-decenoyl-iso-CoA [17]. Fig. 6B shows the MS chromatogram (total ion current [TIC] = *m*/*z* 200–1000) that was collected simultaneously. The MS spectrum of the chromatographic peak eluting at 34.0 min (Fig. 6C) shows a mass signal at *m*/*z* 918 (the expected mass of *cis*-4-decenoyl-CoA). The full-scan MS/MS spectrum on *m*/*z* 918 (*m*/*z* 200–1000 at a relative collision energy of 25 [Fig. 6D]) contains fragment ion consistent with CoASH: *m*/*z* 408 and 426. Fig. 7A shows the 3-phenylpropionyl-CoA UV chromatogram with the 3-phenylpropionyl-CoA peak (retention time = 25.8 min), representing 99.2% of the UV peak areas. The MS spectrum (Fig. 7C) shows a mass signal at *m*/*z* 898. The full-scan MS/MS spectrum on *m*/*z* 898 (Fig. 7D) shows the CoA fragment ions *m*/*z* 408 and 426. Fig. 8A shows the 2,6-dimethylheptanoyl-CoA UV chromatogram with the peak eluting at 32.4 min; this peak represents 96.5% of the total UV peak area. Fig. 8B shows the MS chromatogram that was collected simultaneously. The MS spectrum (Fig. 8C) shows a mass signal at *m*/*z* 906. The full-scan MS/MS spectrum on *m*/*z* 906 (Fig. 8D) shows the CoA fragment ions *m*/*z* 408 and 426.

Acyl-CoA dehydrogenase activity studies

The dehydrogenation of acyl-CoAs is catalyzed by enzymes with different chain length specificities. Our standard assay procedure uses three substrates: butyryl-CoA (predominantly measures short-chain acyl-CoA dehydrogenase [SCAD]), octanoyl-CoA (predominantly measures MCAD), and palmitoyl-CoA (measures both LCAD and VLCAD). The quality control samples for our determination of acyl-CoA dehydrogenase activities are frozen isolated rat skeletal muscle mitochondria. We used five different aliquots of the quality control material to evaluate activities using our current regular substrates (butyryl-CoA, octanoyl-CoA, and palmitoyl-CoA) as well as the three acyl-CoAs prepared above (*cis*-4-decenoyl-CoA, 3-phenylpropionyl-CoA, and 2,6-dimethylheptanoyl-CoA) (see Fig. 9). MCAD and LCAD are significantly different in their substrate specificity toward *cis*-4-decenoyl-CoA (LCAD oxidizes *cis*-4-dece-

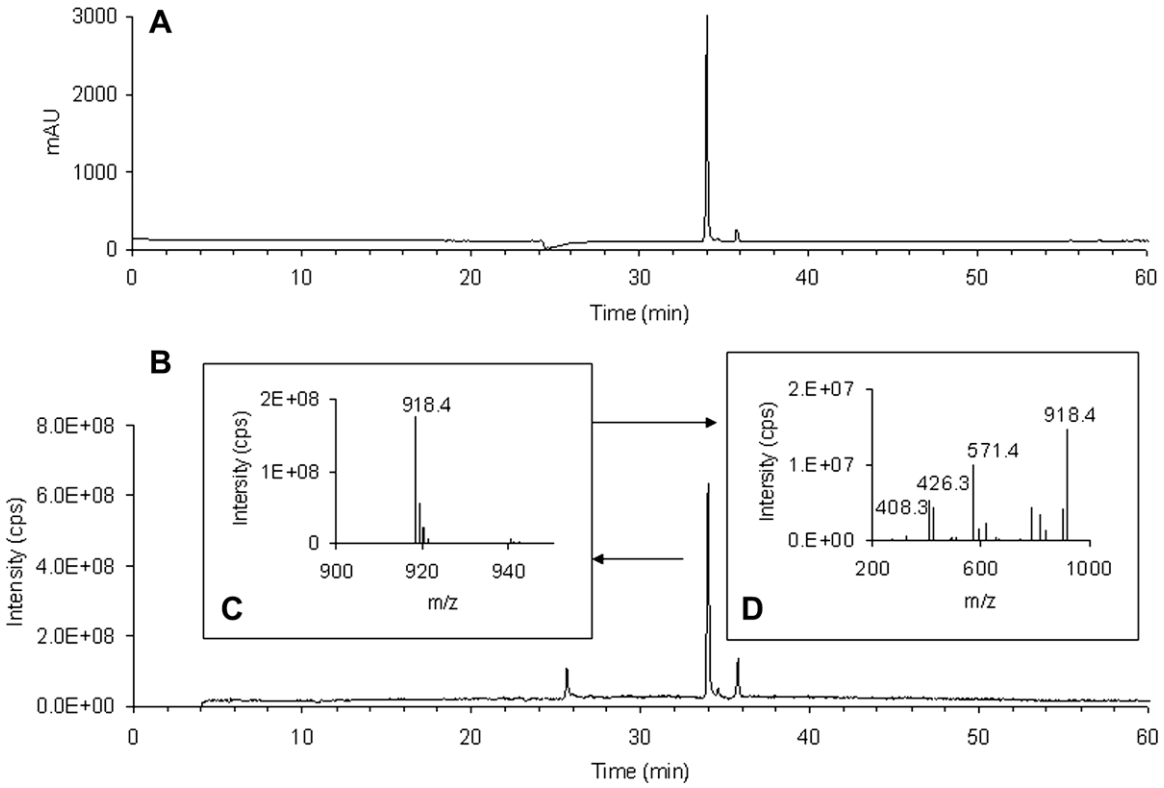


Fig. 6. Analytical HPLC–UV–MS–MS/MS of *cis*-4-decenoyl-CoA. (A) UV chromatogram at 259 nm. (B) TIC–MS chromatogram (*m*/*z* 700–1000). (C) MS spectrum of *cis*-4-decenoyl-CoA (*m*/*z* 918). (D) Full-scan MS/MS fragmentation of *m*/*z* 918.

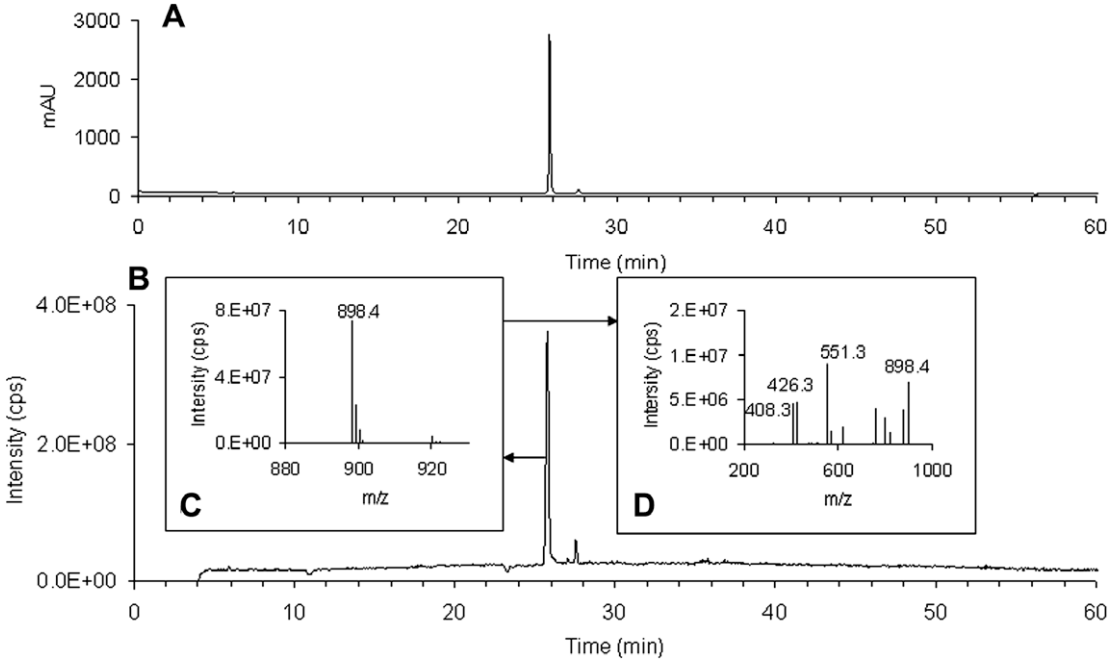


Fig. 7. Analytical HPLC–UV–MS–MS/MS of 3-phenylpropionyl-CoA. (A) UV chromatogram. (B) TIC–MS chromatogram. (C) MS spectrum of 3-phenylpropionyl-CoA (*m*/*z* 898). (D) Full-scan MS/MS fragmentation of *m*/*z* 898.

noyl-CoA at a rate of ~30% of that observed with MCAD in bovine liver [29]). In our study, the reaction rate of *cis*-4-decenoyl-CoA is slightly higher than that of octanoyl-CoA. 3-Phenylpropionyl-CoA (specific substrate for MCAD), unlike octanoyl-CoA, shows zero activity with SCAD in rat liver [4]. 2,6-Dimethylheptanoyl-CoA (a specific substrate for LCAD) has zero activity with VLCAD in human fibroblasts [8]. Because palmitoyl-CoA is a substrate for both LCAD and VLCAD, and in our study the activity of palmitoyl-CoA is seven times higher than that of 2,6-dimethylheptanoyl-CoA, 2,6-dimethylheptanoyl-CoA can be used to differentiate between LCAD and

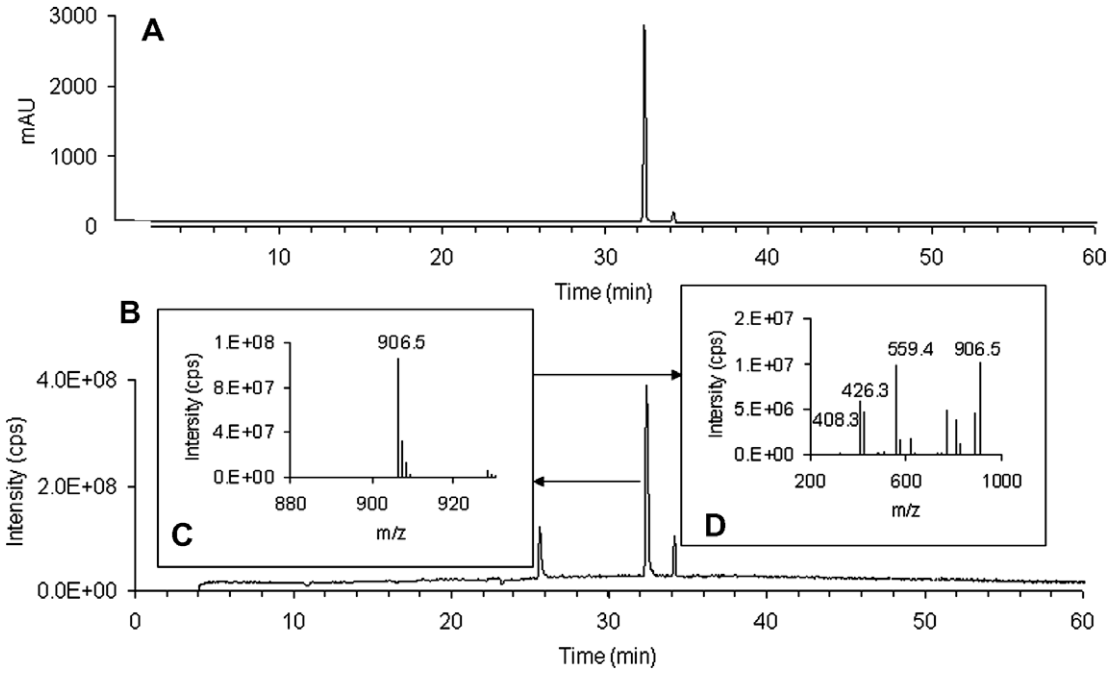


Fig. 8. Analytical HPLC–UV–MS–MS/MS of 2,6-dimethylheptanoyl-CoA. (A) UV chromatogram. (B) TIC–MS chromatogram. (C) MS spectrum of *m*/*z* 906. (D) Full-scan MS/MS fragmentation of *m*/*z* 906.

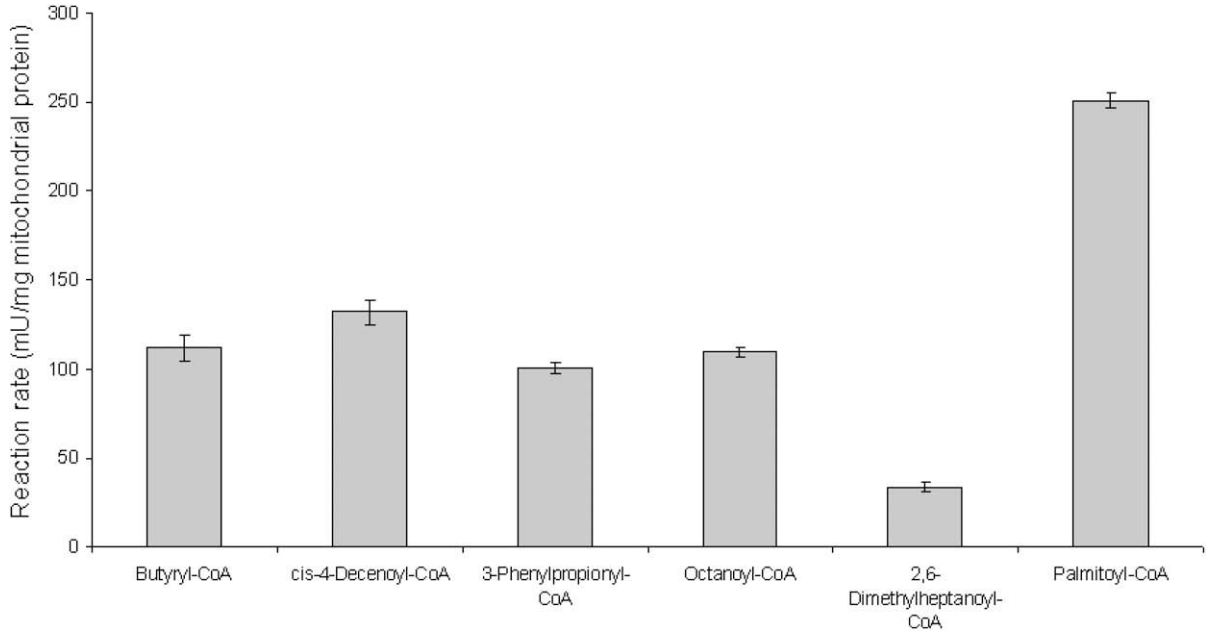


Fig. 9. Acyl-CoA dehydrogenase activity in rat skeletal muscle mitochondria. The graph shows the results generated from five different aliquots of isolated rat skeletal muscle mitochondria. Error bars represent standard deviations.

VLCAD activities (Fig. 9). With these good quality control results, we have added the new substrates to our assays for acyl-CoA dehydrogenase in studies involving human and experimental samples.

Acknowledgments

The authors thank Mariana Rosca for preparing the rat skeletal muscle mitochondria, Hiral Patel and Colin Naples for performing the acyl-CoA dehydrogenase activity studies, and Janos Kerner for engaging in helpful discussions. This work was supported by the Center for Mitochondrial Diseases.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2010.02.026.

References

[1] R.J. Pollitt, Inherited disorders of straight chain fatty acid oxidation, Arch. Dis. Child. 62 (1987) 6–7.
[2] R. Ensenauer, J.L. Winters, P.A. Parton, D.F. Kronn, J.W. Kim, D. Matern, P. Rinaldo, S.H. Hahan, Genotypic differences of MCAD deficiency in the Asian population: novel genotype and clinical symptoms preceding newborn screening notification, Genet. Med. 7 (2005) 339–343.

[3] P. Rinaldo, J.J. O'Shea, P.M. Coates, D.E. Hale, C.A. Stanley, K. Tanaka, Medium-chain acyl-CoA dehydrogenase deficiency: diagnosis by stable-isotope dilution measurement of urinary *n*-hexanoylglycine and 3-phenylpropionylglycine, N. Engl. J. Med. 319 (1988) 1308–1313.
[4] P. Rinaldo, J. Oshea, R. Welch, K. Tanaka, The enzymatic basis for the dehydrogenation of 3-phenylpropionic acid: In vitro reaction of 3-phenylpropionyl-CoA with various acyl-CoA dehydrogenases, Pediatr. Res. 27 (1990) 501–506.
[5] M. Duran, L. Brulnvis, D. Ketting, J.B.C. De Klerk, S.K. Wadman, *cis*-4-Decenoic acid in plasma: a characteristic metabolite in medium-chain acyl-CoA dehydrogenase deficiency, Clin. Chem. 34 (1988) 548–551.
[6] M. Kimura, H.R. Yoon, P. Wasant, Y. Takahashi, S. Yamaguchi, A sensitive and simplified method to analyze free fatty acids in mitochondrial beta oxidation disorders using gas chromatography/mass spectrometry and dried blood spots, Clin. Chim. Acta 316 (2002) 117–121.
[7] W. Le, A.S. Abbas, H. Sprecher, J. Vockley, H. Schulz, Long-chain acyl-CoA dehydrogenase is a key enzyme in the mitochondrial β-oxidation of unsaturated fatty acids, Biochim. Biophys. Acta 1485 (2000) 121–128.
[8] R.J. Wanders, S. Denis, J.P. Ruiter, L.I. Jlst, G. Dacremont, 2,6-Dimethylheptanoyl-CoA is a specific substrate for long-chain acyl-CoA dehydrogenase (LCAD): evidence for a major role of LCAD in branched-chain fatty acid oxidation, Biochim. Biophys. Acta 1393 (1998) 35–40.
[9] E.J. Simon, D. Shemin, The preparation of *S*-succinyl coenzyme A, J. Am. Chem. Soc. 75 (1953) 2520.
[10] P. Goldman, P.R. Vagelos, The specificity of triglyceride synthesis from diglygerides in chicken adipose tissue, J. Biol. Chem. 236 (1961) 2620–2623.
[11] S. Werner, *S*-Palmitoyl coenzyme A, Biochem. Prep. 7 (1960) 80–83.
[12] A. Al-Arif, M. Blecher, Synthesis of fatty acyl CoA and other thiol esters using *N*-hydroxysuccinimide esters of fatty acids, J. Lipid Res. 10 (1969) 344–345.
[13] M. Blecher, Synthesis of long chain fatty acyl-CoA thioesters using *N*-hydroxysuccinimide esters, Methods Enzymol. 72 (1981) 404–408.
[14] D. Mangroo, G.E. Gerber, Synthesis of acyl-CoA thioesters, Biochem. Cell Biol. 68 (1990) 308–312.
[15] V.B. Rajgarhia, N.D. Priestly, W.R. Strohl, Efficient synthesis of radiolabeled propionyl-coenzyme A and acetyl-coenzyme A, Anal. Biochem. 224 (1995) 159–162.
[16] M.S. Chauhan, K. Dakshinamurti, Synthesis of *S*-erucyl coenzyme A, Prep. Biochem. 10 (1980) 37–41.
[17] P.E. Minkler, V.E. Anderson, N.C. Maiti, J. Kerner, C.L. Hoppel, Isolation and identification of two isomeric forms of malonyl-coenzyme A in commercial malonyl-coenzyme A, Anal. Biochem. 328 (2004) 203–209.
[18] G.L. Ellman, Tissue sulfhydryl groups, Arch. Biochem. Biophys. 82 (1959) 70–77.
[19] S.J. Jin, K.Y. Tserng, Metabolic origins of urinary unsaturated dicarboxylic acids, Biochemistry 29 (1990) 8540–8547.
[20] A. Poulos, E. Barone, Partial synthesis of [$^{1-14}$C]Phytanic acid, Lipids 15 (1980) 19–21.
[21] I. Richter, H.K. Mangold, Synthesis of muscalure and homologous hydrocarbons, Chem. Phys. Lipids 11 (1973) 210–214.
[22] K.Y. Tserng, S.J. Jin, D.S. Kerr, C.L. Hoppel, Abnormal urinary excretion of unsaturated dicarboxylic acids in patients with medium-chain acyl-CoA dehydrogenase deficiency, J. Lipid Res. 31 (1990) 763–771.
[23] T. Wieland, L. Rueff, Synthese von β-oxybutyryl-und *S*-acetacetyl-coenzyme A, Angew. Chem. 65 (1953) 186–187.
[24] P.E. Minkler, J. Kerner, S.T. Ingalls, C.L. Hoppel, Novel isolation procedure for short-, medium-, and long-chain acyl-coenzyme A esters from tissue, Anal. Biochem. 376 (2008) 275–276.
[25] C.L. Hoppel, J.P. DiMarco, B. Tandler, Riboflavin and rat hepatic cell structure and function: Mitochondrial oxidative metabolism in deficiency states, J. Biol. Chem. 254 (1979) 4164–4170.
[26] T. Saito, K. Hayamizu, M. Yanagisawa, O. Yamamoto, Spectral Database for Organic Compounds (SDBS), National Institute of Advanced Industrial Science and Technology (AIST), Japan.
[27] J.A. Zirrolli, P. Wheelan, R.C. Murphy, Analysis of long-chain acyl coenzyme A thioesters by negative ion fast-atom bombardment mass spectrometry and tandem mass spectrometry, J. Am. Soc. Mass Spectrom. 5 (1994) 416–424.
[28] T. Mauriala, K.H. Herzig, M. Heinonen, J. Idziak, S. Auriola, Determination of long-chain fatty acid acyl-coenzyme A compounds using liquid chromatography–electrospray ionization tandem mass spectrometry, J. Chromatogr. B 808 (2004) 263–268.
[29] V. Dommes, W.H. Kunau, Purification and properties of acyl coenzyme A dehydrogenases from bovine liver: Formation of 2-*trans*, 4-*cis*-decadienoyl coenzyme A, J. Biol. Chem. 259 (1984) 1789–1797.