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## **ACYL-COENZYME A FORMATION OF SIMVASTATIN IN MOUSE LIVER PREPARATIONS**

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Running title: Acyl-CoA Formation of Simvastatin in Mice

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Abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; SVA, hydroxy acid form of simvastatin; LVA, hydroxy acid form of lovastatin; SVA-CoA, SVA-*S*-acyl coenzyme A; P1-CoA, *S*-acyl coenzyme A of P1 metabolite; P2-CoA, *S*-acyl coenzyme A of P2 metabolite; PA-CoA, palmitoyl-*S*-acyl coenzyme A; OA-CoA, octanoyl-*S*-acyl coenzyme A; HPLC, high-pressure liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; MS/MS, tandem mass spectrometry; Q-TOF, quadrupole time-of-flight.

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**Abstract:**

Formation of an acyl-CoA thioester has been proposed, but not directly demonstrated, to be a key step in mediating both lactonization and atypical  $\beta$ -oxidation of 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors. Here, we describe studies to characterize formation of acyl-CoA thioesters in vitro in mouse liver preparations, using the hydroxy acid form of simvastatin (SVA) as a model substrate. With an optimized chromatography method, three new products were detected in addition to the dehydration product (P1) and the lactone form of simvastatin, which have been characterized previously (Prueksaritanont et al., 2001). Based on HPLC analysis, UV spectroscopy, mass spectrometry and NMR spectral characterization, two metabolites were identified as acyl-CoA thioester conjugates of SVA and P1, respectively, whereas the third metabolite (M1) was confirmed to be the L- $\beta$ -hydroxy isomer of simvastatin. M1 was likely formed by stereospecific hydration, a previously reported reaction, and subsequent lactonization of P1-S-acyl CoA. Among all the mouse liver subcellular fractions, microsomes exhibited the highest capacity to catalyze the CoASH-dependent metabolism of SVA, while such activity was totally absent in cytosol. Together, these results provide direct experimental evidence that SVA (and conceivably other statins as well) is able to form an acyl-CoA thioester, possibly by microsomal long-chain acyl-CoA synthetase(s), leading to formation of two parallel metabolic pathways; one resulting in the two diastereomers of statin lactones (simvastatin and M1), and the other to the  $\beta$ -oxidation pathway of statin hydroxy acids.

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### Introduction

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) are a well-established class of drugs used widely in the treatment of hypercholesterolemia due to their tolerability, proven efficacy and convenient administration. Except for simvastatin and lovastatin, all current statins are administered as the pharmacologically active hydroxy acid form. Simvastatin and lovastatin are administered as inactive lactones, which, upon hydrolysis to their respective hydroxy acids (SVA and LVA), serve as potent competitive inhibitors of HMG-CoA reductase, the rate-limiting enzyme in de novo cholesterol biosynthesis (Duggan and Vickers, 1990).

All statins, with the exception of pravastatin (Hatananka, 2000) and recently approved rosuvastatin (Martin et al., 2000), are extensively metabolized before elimination (Reinoso et al., 2002). The common metabolic pathways of these statins include P450-mediated oxidative metabolism (Igel et al., 2001), acyl glucuronidation (Prueksaritanont et al., 2002a, 2002b) and  $\beta$ -oxidation (Reinoso et al., 2002). Like the acyl glucuronidation, the  $\beta$ -oxidation occurs at the dihydroxy heptanoic or heptenoic acid side chain, a structural feature common to all statin hydroxy acids (SVA structure as an example shown in Figure 1). The pentanoic and/or propanoic acid products corresponding to one and/or two cycles of  $\beta$ -oxidation, respectively, have been reported to occur exclusively in rodents following lovastatin, simvastatin and cerivastatin administration and predominantly so for other statins (Vickers et al., 1990; Halpin et al.,

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1993; Boberg et al., 1998; Reinoso et al., 2002). Acyl-CoA thioesters of these statin hydroxy acids have been proposed to be the key intermediates for the formation of these  $\beta$ -oxidation metabolites (Figure 1). Evidence that supports this proposal, however, is very limited. Recent studies in our laboratory have provided some evidence that formation of the  $\beta$ -oxidation products of SVA in vitro in mouse liver preparations was mediated by CoASH-dependent enzyme(s) (Prueksaritanont et al., 2001). Unfortunately, acyl-CoA thioesters of SVA and its  $\beta$ -oxidation intermediates were not detected in the study. Therefore, the present studies were undertaken, using an in vitro approach and SVA as a model substrate, to characterize the formation of these acyl-CoA thioester intermediates in mouse liver subcellular fractions, and to provide support for the mechanism of the  $\beta$ -oxidation of statins proposed by Halpin et al. (1993). Here, we demonstrate that acyl-CoA conjugates of SVA and its dehydration product (P1) were formed in vitro in mouse liver preparations and were primarily catalyzed by microsomal enzymes. Additionally, the findings of CoASH-dependent formation of simvastatin and its diastereomer M1 substantiate the mechanism proposed previously for the in vivo lactonization of statin hydroxy acids (Duggan and Vickers, 1990).

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### Methods

**Materials.** Simvastatin and SVA were synthesized at Merck Research Laboratories (Rahway, NJ). Triton X-100, CoASH, ATP, and palmitoyl-S-acyl coenzyme A (PA-CoA) were obtained from Sigma (St. Louis, MO). [1-<sup>14</sup>C]-Palmitic acid and [1-<sup>14</sup>C]-octanoic acid were obtained from Amersham Bioscience (Piscataway, NJ) and American Radiolabeled Chemicals, Inc. (St. Louis, MO), respectively. All other agents were of analytical or HPLC grade.

**Animals.** All animal studies were approved by the Merck Research Laboratories Institutional Animal Care and Use Committee. Ten male CD-1 mice (25-40 g) were obtained from Charles River Laboratories (Wilmington, MA). Following cervical dislocation, livers were quickly removed, weighed, and washed with 1.15% potassium chloride. The livers were homogenized in 4 volumes of ice cold medium containing 250 mM sucrose, 10 mM HEPES and 1 mM EDTA. The liver subcellular fractions were prepared by differential centrifugation as described previously with minor modifications (Bjorge and Baillie, 1991). Briefly, liver homogenates were centrifuged first at 600 g for 10 min to remove nuclei and cell debris. The supernatant (S0.6) was centrifuged at 3,300 g for a further 10 min to sediment the heavy mitochondria (MT-H). The resulting supernatant (S3) was then centrifuged at 27,000 g for 10 min to obtain the light mitochondria fraction (MT-L). The corresponding supernatant (S27) was finally centrifuged at 100,000 g for 1 hr to prepare the liver microsomes. The residue supernatant was taken as the cytosolic fraction. The purity of each of the fractions was

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assessed by the activity of their respective marker enzymes: catalase for peroxisomes (Baudhuin et al., 1964), cytochrome c oxidase for mitochondria (Sellinger et al., 1960), lactate dehydrogenase for cytosol (Volkl and Fahimi, 1985) and NADPH-cytochrome c reductase for microsomes (Beaufay et al., 1974).

**In Vitro Metabolism.** Incubations of SVA (100  $\mu$ M) with mouse liver subcellular fractions (1.5 mg/mL) were carried out in triplicate in 150 mM Tris-HCl buffer (pH 8.0) containing 0.05% Triton X-100, 1.2 mM CoASH, 10 mM MgCl<sub>2</sub>, and 5 mM ATP in a final volume of 0.2 mL. After a 3-min preincubation, reactions were initiated by addition of ATP and incubated for up to 60 min at 37°C. The reactions were terminated at appropriate time intervals by addition of 120  $\mu$ L of ice cold acetonitrile. After centrifugation at 10,000 g for 10 min, the supernatant was analyzed immediately by HPLC or LC-MS with an autosampler set at 5°C to minimize any degradation that might have occurred during the sample handling and analysis. Preliminary stability studies showed that SVA-*S*-acyl coenzyme A (SVA-CoA) and its subsequent metabolites were stable under the condition described above. Control experiments included incubation mixtures with one or more components missing.

Incubations with [1-<sup>14</sup>C]- palmitic acid (50  $\mu$ M) and [1-<sup>14</sup>C]- octanoic acid (50  $\mu$ M) were performed under the same conditions as described above for SVA, except that the mixtures contained 0.025 mg/mL of mouse liver subcellular fractions for palmitic acid and 0.125 mg/mL for octanoic acid and were incubated for 5 min. Under such incubation conditions, the formation of PA-CoA and octanoyl-*S*-acyl coenzyme A (OA-CoA) was linear with incubation time and protein concentration.

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For isolation and purification of metabolites of SVA, large scale incubations of SVA (500  $\mu$ M) were carried out with mouse liver microsomes (1.5 mg/mL), CoASH (1.2 mM) and ATP (5 mM) for 40 min in a final volume of 12 mL for the isolation of SVA-CoA and P1-S-acyl coenzyme A (P1-CoA) or for 3 hr in a final volume of 60 mL for M1. The incubation mixtures were terminated with half a volume of acetonitrile and loaded onto C<sub>18</sub> solid phase extraction cartridges (Oasis HLB SPE cartridge, Waters Corporation, Milford, MA), which were pre-equilibrated with water and acetonitrile. The acyl-CoA thioesters-containing fractions were eluted with 70% acetonitrile, while M1-containing fraction was eluted with 90% acetonitrile. The resulting solid phase eluates were evaporated to dryness, reconstituted in 5-10 mL of the mobile phase (20% acetonitrile in 10 mM ammonium acetate buffer, pH = 7) and isolated by preparative HPLC as described below. The purified metabolites were characterized by NMR spectroscopy.

**Analytical Methods.** SVA and its metabolites were analyzed using published HPLC methods with some modifications (Prueksaritanont et al., 1999). In brief, samples held in an autosampler (set at 5°C) were chromatographed on a C<sub>18</sub> Zorbax column (150 x 4.6 mm, 5  $\mu$ m; Agilent Technologies, Foster City, CA) with a linear gradient of acetonitrile and 10 mM ammonium acetate (pH 7). The eluates were monitored by UV absorption at 240 nm and 260 nm. Due to the unavailability of authentic standards for SVA-CoA and P1-CoA, the concentrations of these metabolites in the in vitro incubation mixtures were estimated using standard curves for PA-CoA at 260 nm, assuming similar extinction coefficients between SVA-CoA (or P1-CoA) and PA-CoA. Evidence that

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supports the assumption includes the observations that SVA, P1 and palmitic acid all had minimal UV absorption at 260 nm, whereas the corresponding acyl-CoA thioesters and free CoASH exhibited maximal UV absorption at 260 nm. In addition, PA-CoA was selected because of its similar HPLC retention time to SVA-CoA and P1-CoA under the HPLC conditions described above. Quantification of P1 and M1 in the in vitro incubations was accomplished using standard curves for SVA and simvastatin, respectively, assuming identical extraction recoveries and extinction coefficients at their maximal UV absorption at 240 nm.

Metabolite isolation was accomplished by semi-preparative HPLC (Waters, Milford, MA) coupled with a fraction collector (Foxy 200; ISCO, Inc., Lincoln, NE). The reconstituted reaction mixtures were separated on a Luna C<sub>18</sub> (2) column (250 x 10 mm, 5 μm; Phenomenex, Torrance, CA) with a 40-min gradient from 80% A (10% methanol in 10 mM ammonium acetate, pH 7) to 70% B (90% acetonitrile in water). Fractions corresponding to the peaks for the SVA metabolites were collected according to UV absorbance at 260 nm for SVA-CoA and P1-CoA and 240 nm for M1.

Analysis of PA-CoA and OA-CoA in mouse liver preparations was performed on a BDS Hypersil C<sub>8</sub> column (150 x 4.6 mm, 5 μm; Thermo Hypersil-Keystone, Bellefonte, PA) with a linear gradient of acetonitrile and 25 mM ammonium acetate (pH 7). The acyl-CoA thioesters were monitored by UV absorption at 260 nm and by an on-line IN/US β-radiation detector (IN/US Systems, Tampa, FL).

LC-MS analysis was conducted on a Finnigan MAT LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA), interfaced with a PerkinElmer HPLC system (PerkinElmer Life and Analytical Sciences, Boston, MA) equipped with two

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Series 200 micro pumps and an autosampler set at 5°C. LC-MS and LC-MS/MS experiments were carried out using electrospray ionization in the negative ion mode (for SVA, P1, SVA-CoA, and P1-CoA) or positive ion mode (for lactones: simvastatin and M1). The electrospray ionization voltage was set at 3.5 kV with the heated capillary temperature held at 175°C. A C<sub>18</sub> Zorbax column (150 x 4.6 mm, 5 µm; Agilent Technologies, Foster City, CA) was used for chromatographic separation and was eluted at 1.0 mL/min with a gradient of 10 mM ammonium acetate (pH 7) in water with 10% methanol (A) and 90% acetonitrile in water (B). The initial mobile phase consisted of 15% B, which remained unchanged for 5 min, and increased linearly to 70% B over 22 min, then to 90% over 1 min, and held for 5 min. One fifth of the flow was directed into the mass spectrometer, and the remainder was used for UV detection at 240 nm.

Accurate mass measurements were conducted on a Micromass Q-TOF (quadrupole time-of-flight) II mass spectrometer (Waters, Milford, MA) operated in the positive electrospray ionization mode with a LockSpray reference sprayer. The source and desolvation gas temperatures were set at 80°C and 180°C, respectively. The capillary voltage, cone voltage and collision energies were 2.5kV, 25 V and 15V, respectively. The instrument was calibrated by infusing aqueous 0.01% phosphoric acid solution through a syringe pump at 5 µL/min prior to the experiment. The same calibration solution was used as lock mass in the sample analysis. The chromatographic conditions used in Q-TOF experiments were the same as described in LCQ section.

NMR studies were performed at 10°C for CoASH and acyl-CoA thioesters, and at 25 °C for the SVA, simvastatin, and M1 samples. All NMR data were collected either on a 500 or 600 MHz spectrometer (Varian Inc., Palo Alto, CA) both equipped with a 5 mm

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HCN Cold Probe (Varian Inc.). The HPLC isolates or the authentic standard(s) were dissolved in ~160  $\mu\text{L}$  of  $\text{CD}_3\text{OD}$  (except for CoASH, where 10%  $\text{D}_2\text{O}$  v/v was added to promote solubility) and transferred to 3 mm tubes. 1D  $^1\text{H}$ , 2D  $^1\text{H}$ - $^1\text{H}$  correlation, 2D  $^1\text{H}$ - $^{13}\text{C}$  one-bond and multiple-bond correlation data sets were acquired for all samples and used to elucidate the structures.

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### Results

**Metabolite Identification.** Figures 2A and 2B illustrate typical HPLC chromatograms derived from incubations of mouse liver subcellular fractions (S0.6 and microsomes) with SVA in the presence of CoASH and ATP. Five metabolites (designated as SVA-CoA, P1-CoA, P1, M1, and simvastatin) were detected by UV absorption at 240 nm. P1, M1 and simvastatin exhibited UV absorption spectra similar to SVA, whereas SVA-CoA and P1-CoA had UV absorption peak at 260 nm (characteristics for CoASH) in addition to the well-characterized UV peak for SVA at 240 nm. P1, which gave a deprotonated ion  $[M - H]^-$  at  $m/z$  417, has been identified previously by NMR as the dehydration product of SVA (Prueksaritanont et al., 2001). The product, which eluted last in the chromatogram, was identified as the simvastatin lactone based on the identical HPLC retention time and UV spectrum compared with the authentic standard. Except for a small amount of simvastatin (< 0.1% of initial concentration), the remaining four SVA metabolites were not detectable in incubations with mouse liver cytosol (Figure 2C) and control incubations that lacked liver protein, ATP, or CoASH. The results suggested that formation of SVA-CoA, P1-CoA, P1, and M1 was mediated by CoASH- and ATP-dependent enzyme(s), whereas simvastatin was formed by both enzymatic (by CoASH- and ATP-dependent enzymes) and chemical (to much less extent) processes under the conditions described in the methods.

The mass spectrum of SVA-CoA showed a prominent  $[M - H]^-$  ion at  $m/z$  1184.4, 749 (CoASH - H<sub>2</sub>O) mass units higher than the  $[M - H]^-$  ion of SVA. MS/MS fragmentation of  $m/z$  1184.4 showed peaks indicative of a CoASH moiety attached to SVA, including ions at  $m/z$  766.2 ( $[CoAS]^-$ ),  $m/z$  426.1 ([adenosine diphosphate]),  $m/z$

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408.2 ([adenosine diphosphate – H<sub>2</sub>O]<sup>-</sup>), *m/z* 1104.5 ([SVA-CoA – H<sub>2</sub>PO<sub>3</sub>]<sup>-</sup>), *m/z* 1086.5 ([SVA-CoA – dimethyl-oxobutyl]<sup>-</sup>), *m/z* 1049.4 ([SVA-CoA – adenine – 2H]<sup>-</sup>), and *m/z* 837.4 ([SVA-CoA – adenosine monophosphate – 2H]<sup>-</sup>) (Figure 3A). NMR studies with the isolated metabolite confirmed it to be an acyl-CoA conjugate of SVA with a thioester bond between SVA and CoASH. The combined NMR data clearly showed that the SVA and CoASH moieties were fully intact in the adduct spectrum. The methylene group, labeled **3**, in the SVA portion of the adduct appeared at  $\delta_{\text{H}}$  2.71, 2.77 ppm /  $\delta_{\text{C}}$  50.8 ppm (Table 1). The  $\beta$ -methylene group, labeled **a'**, of the mercaptoethylamine subunit in CoASH appeared at  $\delta_{\text{H}}$  3.00 ppm /  $\delta_{\text{C}}$  27.9 ppm (Table 1). A 2D <sup>1</sup>H-<sup>13</sup>C multi-bond correlation (a select expansion of the spectrum displayed in Figure 4) was observed from both methylene protons **3** and **a'** to the carbonyl carbon **2** (at 197.9 ppm) in the SVA heptanoic acid side chain. Therefore, the combined NMR data showed that the sulfur of the CoASH was conjugated to the acyl carbon **2** of the heptanoic acid side chain in SVA.

The identification of P1-CoA was based on UV, MS, MS/MS, and NMR spectra. P1-CoA gave rise to a deprotonated ion [M – H]<sup>-</sup> at *m/z* 1166.4, 749 (CoASH – H<sub>2</sub>O) mass units higher than the [M – H]<sup>-</sup> ion of P1 and 18 mass units less than that of SVA-CoA thioester (*m/z* 1184.4), suggesting possible dehydration of SVA-CoA. Consistently, the MS/MS spectrum of P1-CoA displayed a family of product ions, including ions *m/z* 1086.5 ([P1-CoA – H<sub>2</sub>PO<sub>3</sub>]<sup>-</sup>), *m/z* 1068.5 ([P1-CoA – dimethyl-oxobutyl]<sup>-</sup>), *m/z* 1031.4 ([P1-CoA – adenine – 2H]<sup>-</sup>), and *m/z* 819.4 ([P1-CoA – adenosine monophosphate – 2H]<sup>-</sup>) (Figure 3B), which were all 18 mass units less than the corresponding fragment ions of SVA-CoA. In addition, the product ions at *m/z* 426.1 and 408.2, corresponding to adenosine diphosphate and its dehydration ions, remained the same as those of SVA-

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CoA, indicating that the adenosine phosphate moiety was intact (Figure 3 B). NMR data supported that P1-CoA was an acyl-CoA conjugate of P1 with a thioester bond between P1 and CoASH. The combined NMR data conclusively indicated that the P1 and CoASH moieties were intact. The proton shifts of P1 moiety were similar to the previously published data on P1, the dehydration metabolite of SVA (Prueksaritanont et al., 2001). Alkene carbons **3** and **4** in the P1 moiety appeared at  $\delta_{\text{H}}$  6.22 ppm /  $\delta_{\text{C}}$  129.9 ppm and  $\delta_{\text{H}}$  6.90 ppm /  $\delta_{\text{C}}$  142.6 ppm, respectively (Table 1). The  $\beta$ -methylene group, labeled **a'**, of the mercaptoethylamine subunit in CoASH appeared at  $\delta_{\text{H}}$  3.04 ppm /  $\delta_{\text{C}}$  27.6 ppm (Table 1). A common 2 D  $^1\text{H}$ - $^{13}\text{C}$  multi-bond correlation was observed from the protons **3** and **4** of P1 moiety and methylene protons **a'** to the carbonyl carbon **2** at 190.0 ppm (not shown). Therefore, NMR data conclusively indicated the site of CoASH sulfur conjugation to be at the acyl carbon **2** of P1 moiety.

M1 was identified as L- $\beta$ -hydroxy isomer of simvastatin based on UV, accurate MS, MS/MS, and NMR spectra. Accurate mass measurements by a Q-TOF instrument revealed that the  $[\text{M} + \text{H}]^+$  of simvastatin standard and M1 were at  $m/z$  419.2788 and 419.2804, respectively, which were all within 2.2 ppm of the calculated mass of  $[\text{simvastatin} + \text{H}]^+$ . In the high resolution MS/MS experiment the residual parent ion signal was used as the internal lock mass. MS/MS spectra of simvastatin and M1 exhibited almost identical fragmentation patterns except for the relative intensities of fragment ions (not shown). The observed and calculated  $m/z$  values of the most abundant fragment ions of simvastatin and M1 are shown in Table 2, which confirmed the fragmentation pathway proposed in Figure 5. Therefore, high resolution MS data strongly suggested that simvastatin and M1 were positional isomers. The NMR data on M1 was

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consistent with a chiral center inversion at carbon **4** that is part of the six-membered lactone ring.  $^1\text{H}$  chemical shifts of simvastatin standard and M1 are shown in Table 1. Comparing the two 1D  $^1\text{H}$  NMR spectra, the spectral changes were observed for protons in the six-membered lactone ring. The CHOH methine protons, labeled **4** and **6**, appeared at 4.24 and 4.61 ppm, respectively, in simvastatin (Table 1). In contrast, H4 and H6 were overlapped and appeared at 4.19 ppm in the M1 spectrum (Table 1). The methine carbons at **4** and **6** were resolved in the 2D  $^1\text{H}$ - $^{13}\text{C}$  one-bond correlation spectrum of M1 at 62.8 ppm and 77.8 ppm, respectively (not shown).

**Metabolism of SVA in mouse liver subcellular fractions.** Time-dependent studies with mouse liver S0.6 showed that formation of SVA-CoA and P1-CoA increased rapidly during the first 10-20 min incubations and then decreased steadily thereafter, whereas formation of P1, M1, and simvastatin appeared to increase gradually with incubation time (Figure 6A). In addition to mouse liver S0.6, CoASH-dependent metabolism of SVA was also observed in incubations with other mouse liver subcellular fractions, including mitochondria (MT-H and MT-L) and microsomes (Figure 7C). The identities and purities of these subcellular fractions were confirmed by marker enzyme analysis (Table 3). Among all the mouse subcellular fractions, microsomes exhibited the highest capability to catalyze CoASH-dependent metabolism of SVA on a per mg protein basis, whereas cytosol was totally incapable of catalyzing the reaction (Figures 2C and 7C). As illustrated in Figures 2B and 6B, incubations of SVA with mouse liver microsomes produced significantly higher levels of SVA-CoA and P1-CoA thioesters (approximately 5 to 7 fold higher after 20 min incubations) than that of mouse liver S0.6. Consistently, the lactonization products, simvastatin and M1, were also found to be

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approximately 5 to 7 fold higher after 1-hr incubations with mouse liver microsomes than the corresponding incubations with S0.6. These results provided additional support for the acyl-CoA thioester-dependent mechanism proposed previously for the lactonization of the hydroxy acid forms of statins observed *in vivo* (Duggan and Vickers, 1990). Interestingly, P1 levels in microsomes were not significantly more than in S0.6.

Due to the apparent subsequent metabolism of SVA-CoA in mouse liver preparations, the sum of the five metabolites (SVA-CoA, P1-CoA, P1, M1, and simvastatin) was used to compare the rates of CoASH-dependent metabolism of SVA in mouse liver subcellular fractions. Preliminary studies showed that CoASH-dependent metabolism of SVA was linear with time up to 30 min and with mouse liver S0.6 up to 3 mg/mL. As shown in Figure 7C and Table 3, the rate of CoASH-dependent metabolism of SVA in mouse liver subcellular fractions appeared to correlate well with that of acyl-CoA formation of palmitic acid (a substrate for long-chain acyl-CoA synthetase) (Figure 7 A) and the activity of cytochrome c reductase, a marker enzyme for microsomes, but not with the rate of acyl-CoA formation of octanoic acid (a substrate for medium-chain acyl-CoA synthetase) (Figure 7B) and the activities of marker enzymes for other subcellular fractions. These observations clearly indicated that acyl-CoA formation of SVA is catalyzed preferentially by microsomal enzyme(s).

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### Discussion

The present studies provide direct experimental evidence for the formation of acyl-CoA thioester of SVA *in vitro* in mouse liver preparations. Employing the HPLC methods described above, acyl-CoA thioester intermediates of SVA were completely resolved from other peaks present following incubations with mouse liver preparations. Under such HPLC conditions optimized for acyl-CoA thioesters, we were able to isolate and fully characterize these acyl-CoA thioester intermediates by LC-MS/MS and NMR analysis. The optimized HPLC method also provides a possible explanation for the reason that the CoA thioester conjugates of SVA were not observed in our previous studies with mouse liver preparations (Prueksaritanont et al., 2001). Preliminary studies showed that SVA-CoA and P1-CoA were sensitive to pH of mobile phase and did not chromatograph well under acidic conditions, such as 0.1% formic acid, the conditions used in our previous studies (Prueksaritanont et al., 2001). Similar observations were also made for other xenobiotic acyl-CoA thioesters, such as 2-phenylpropionyl-*S*-acyl CoA (Li et al., 2002), 2,4-dichlorophenoxyacetyl-*S*-acyl CoA (Li et al., 2003) and naproxen-*S*-acyl CoA (Olsen et al., 2002). Recently, Sidenius et al. (2004) reported several LC chromatographic methods developed for analysis of acyl-CoA thioesters of eight structurally diverse carboxylic acids, including ibuprofen, clofibric acid, indomethacin, fenbufen, tolmetin, salicylic acid, 2-phenoxypropionic and 4-chloro-2-methylphenoxyacetic acid. Notably, the pH of mobile phases used for the eight acyl-CoA thioesters were all between 6 and 7.

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As pointed out by Halpin et al. (1993),  $\beta$ -oxidation of fatty acids requires that hydroxylated intermediates be in the L-configuration, while in the case of statins, both the 4- and 6-hydroxyl groups on the side chain possess the D-configuration required for pharmacological activity (Figure 1). An epimerization to L-configuration has been proposed to be an essential step for D- $\beta$ -hydroxyacyl CoA thioester to enter the  $\beta$ -oxidation cycles (Halpin et al., 1993) (Figure 1). Recent in vitro studies with SVA provided evidence supporting that epimerization of D- $\beta$ -hydroxy statin acyl CoA proceeded via the dehydration at the  $\alpha$  and  $\beta$  carbons with respect to the carboxy carbon to give a trans-enoyl-CoA, followed by stereospecific hydration of the unsaturated acyl-CoA to give rise to the L- $\beta$ -hydroxy isomer (Prueksaritanont et al., 2001) (Figure 1). However, the corresponding dehydration (P1) and stereospecific rehydration (P2: L,D-dihydroxy isomer of SVA) intermediates were detected only in their free acid forms (Prueksaritanont et al., 2001). Under the current HPLC conditions, we were able to detect the acyl-CoA thioester of the dehydration product, namely P1-CoA, in addition to its free acid form P1. However, the peak corresponding to acyl-CoA thioester of P2 (P2-S-acyl coenzyme A, P2-CoA) was not observed in the present study. The reason for this remains unclear. It is possible that formation of P2-CoA is low under current incubation conditions and could not be detected by HPLC analysis.

In addition to mediating the  $\beta$ -oxidation of statins, the bioactivated acyl-CoA thioesters have been hypothesized to be a key intermediate for the formation of statin lactones (Duggan and Vickers, 1990). The hypothesis has been substantiated by recent findings of CoASH-dependent formation of simvastatin lactone in vitro in mouse liver preparations (Prueksaritanont et al., 2001). Similar observations on simvastatin formation

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were also made in the present study. In addition to simvastatin, we found a new lactone metabolite of SVA, M1 (L- $\beta$ -hydroxy isomer of simvastatin), possibly formed by lactonization of P2-CoA, and its structure has been identified definitively (Figure 1). Our finding that the extent of formation of simvastatin and M1 increased when the corresponding acyl-CoA formation (SVA-CoA and P1-CoA) increased (Figure 6) provides additional evidence that the lactonization of the statin hydroxy acids (SVA and P2) could be mediated by acyl-CoA thioesters via an intramolecular acylation. Considering the well-documented interconversion between SVA and simvastatin, it is anticipated that interconversion between P2 and M1 might also exist (Figure 1). Both P2 and M1 are pharmacologically inactive, and the relevance of the *in vitro* formation of M1 to *in vivo* situation is currently unclear. The formation of M1 has not been reported *in vivo* following administration of SVA or simvastatin to humans and animals. This might be partly due to the chromatographic conditions used in the previous *in vivo* studies, which were not optimized for the detection of M1. It is also possible that formation of M1 is relatively low *in vivo* if the subsequent  $\beta$ -oxidation of P2-CoA is more favorable than the corresponding lactonization process (Figure 1).

In agreement with previous data (Krisans et al., 1980 and Knights and Drogemuller, 2000), our study showed that long chain acyl-CoA synthetase (measured by PA-CoA synthetase) was primarily localized in microsomes and to less extent in mitochondria (MT-H and MT-L) (Figure 7A), while medium chain acyl-CoA synthetase (measured by OA-CoA synthetase) was mainly located in mitochondria (MT-H and MT-L) (Figure 7B), which further confirmed the purity of subcellular fractions obtained. Studies with these subcellular fractions revealed that CoASH-dependent SVA

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metabolism occurred preferentially in microsomes and to much less extent in mitochondria (MT-H and MT-L) with no activity observed in cytosol (Figure 7C). This distribution of activity correlated well with that of long chain acyl-CoA synthetase, but not with that of medium chain acyl-CoA synthetase, suggesting the potential involvement of long chain acyl-CoA synthetase in the acyl-CoA formation of SVA. This observation was further supported by our preliminary studies which showed that CoASH-dependent metabolism of SVA was effectively inhibited by palmitic acid and triacsin C (10  $\mu$ M), a potent and selective inhibitor for long-chain acyl-CoA synthetase (Vessey et al., 2004). In contrast, the enzymes potentially involved in the hydrolysis of SVA-CoA and P1-CoA were still poorly understood. It appeared that other subcellular fractions (e.g. mitochondria and cytosol), in addition to microsomes, might contribute significantly to the hydrolysis of P1-CoA, based on the fact that P1 levels were not significantly increased when the corresponding P1-CoA was increased by 5-7 fold in liver microsomes as compared to liver S0.6 (Figure 6). This observation is consistent with the previous reports on the wide subcellular distribution of acyl-CoA hydrolases (Urrea and Bronfman, 1996 and Garras et al., 1997).

It is noteworthy that formation of statin acyl-CoA thioesters is not expected to be unique for SVA. Other statin hydroxy acids, including cerivastatin and atorvastatin, which all share the common D,D-dihydroxy acid side chain undergo  $\beta$ -oxidation, and therefore, are anticipated to be able to form the acyl-CoA thioesters. In fact, our preliminary studies with cerivastatin and atorvastatin showed that acyl-CoA thioesters of these statin acids were readily detectable *in vitro* in mouse liver preparations. Additionally, consistent with the *in vivo* observations on the  $\beta$ -oxidation of statins

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(Reinoso et al., 2002), species differences in the formation of acyl-CoA thioesters were also observed in our preliminary studies, which showed that acyl-CoA thioesters of statin hydroxy acids, including SVA, cerivastatin and atorvastatin, were detectable only with liver preparations from mice and rats, but not from dogs and humans. However, such marked species differences were not observed *in vivo* for the formation of statin lactones. Significant lactone formation was reported to occur *in vivo* following administration of SVA or cerivastatin to dogs and of cerivastatin and atorvastatin to humans (Vickers et al., 1990; Boberg et al., 1998; Kantola et al., 1998, 1999), in addition to rodents, suggesting that more than one metabolic pathway might contribute to the formation of statin lactones. In fact, recent study showed that acyl glucuronides also mediated the lactone formation (Prueksaritanont et al., 2002a). Quantitatively, there appears to be differences in the relative contribution of these two metabolic pathways to the lactone formation of different statins in different species. In rodents, acyl-CoA pathway is likely to contribute significantly to the formation of statin lactones based on the fact that  $\beta$ -oxidation of statins has been shown to be a major pathway in these species (Reinoso et al., 2002). Conversely, in dogs, acyl glucuronidation appears to play a more important role in the lactonization of statins than acyl-CoA formation; statins have been shown to undergo significant acyl glucuronidation and minimal  $\beta$ -oxidation *in vivo* in dogs (Vickers et al., 1990; Boberg et al., 1998; Black et al., 1999; Prueksaritanont et al., 2002a). Unfortunately, such conclusion could not be readily drawn in humans, due to limited *in vivo* data. Nevertheless, based on the findings on acyl glucuronidation in human liver preparations *in vitro* and in animals *in vitro* and *in vivo* (Prueksaritanont et al., 2002a), it

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is anticipated that acyl glucuronidation plays a role in the lactonization of statin acids in vivo in humans.

In conclusion, this was the first study to demonstrate the formation of acyl-CoA thioesters of SVA and subsequent metabolites in vitro in mouse liver preparations. Our results support the hypothesis that  $\beta$ -oxidation occurs as a consequence of acyl-CoA thioester formation, possibly by microsomal long-chain acyl-CoA synthetase(s), and subsequent epimerization. Additionally, the present studies also provide evidence that formation of these thioesters contributes, at least in part, to interconversion between SVA and its diastereomer and between these hydroxy acids and their corresponding lactones. Similar observations are also anticipated with other hydroxy acid forms of statins (and their diastereomers) and the corresponding lactones.

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Footnotes

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### Figure Legends

Figure 1. Proposed pathway for CoASH-dependent metabolism of SVA.

Figure 2. Representative HPLC-UV profiles of SVA metabolites in incubations with mouse liver S0.6 (A), microsomes (B) and cytosol (C).

Incubations were carried out at 37°C for 20 min using mouse liver subcellular fractions (1.5 mg/mL) and SVA (100 µM) with 1.2 mM CoASH and 5 mM ATP.

Figure 3. MS/MS spectra of SVA-CoA (A) and P1-CoA (B) and tentative assignments of the fragment ions.

Figure 4. Select expansion of a 2D <sup>1</sup>H-<sup>13</sup>C multi-bond correlation NMR spectrum of SVA-CoA. Labels are shown in Figure 1.

Figure 5. Proposed fragmentation pathway for simvastatin and M1. The accurate mass measurement data are summarized in Table 2.

Figure 6. Time-dependent formation of SVA metabolites following incubations with mouse liver S0.6 (A) and microsomes (B).

Incubations were carried out in triplicate at 37°C using mouse liver subcellular fractions (1.5 mg/mL) and SVA (100 µM) with 1.2 mM CoASH and 5 mM ATP. Values represent means ± SD from triplicate incubations.

Figure 7. Mouse liver subcellular distribution of enzyme activity responsible for the formation of PA-CoA (A), OA-CoA (B) and CoA-dependent metabolism of SVA (C).

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Incubations of SVA (100  $\mu$ M) with mouse liver subcellular fractions (1.5 mg/mL) were carried out in triplicate at 37°C for 20 min in 150 mM Tris-HCl buffer (pH 8.0) containing 1.2 mM CoASH and 5 mM ATP. Incubations with [1-<sup>14</sup>C]- palmitic acid (50  $\mu$ M) and [1-<sup>14</sup>C]- octanoic acid (50  $\mu$ M) were performed under the same conditions as described above for SVA, except that the mixtures contained 0.025 mg/mL of mouse liver subcellular fractions for palmitic acid and 0.125 mg/mL for octanoic acid and were incubated for 5 min. Values represent means  $\pm$  SD from triplicate incubations.

Table 1.  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts of SVA, simvastatin and CoASH-dependent SVA metabolites.

All chemical shifts are referenced to  $\text{CD}_2\text{HOD}$  peak set at  $\delta_{\text{H}}$  3.30 /  $\delta_{\text{C}}$  47.9 ppm. The decalin ring and the dimethyl butanoic acid moieties are unchanged in all structures and their chemical shifts in all structures were practically identical to previously published data on SVA (Prueksaritanont et al., 2001).

Label <sup>a</sup>	SVA & CoASH <sup>b</sup>		SVA-CoA		P1-CoA		simvastatin		M1	
	$^1\text{H}$	$^{13}\text{C}$	$^1\text{H}$	$^{13}\text{C}$	$^1\text{H}$	$^{13}\text{C}$	$^1\text{H}$	$^{13}\text{C}$	$^1\text{H}$	$^{13}\text{C}$
2	—	178.5	—	197.9	—	190.0	—	172.0	—	172.3
3	2.28, 2.39	43.9	2.71, 2.77	50.8	6.22	129.9	2.52, 2.71	37.7	2.35, 2.85	38.7
4	4.08	67.8	4.21	66.8	6.90	142.6	4.24	61.9	4.19	62.8
5	1.55	43.8	1.60	43.4	2.35	40.0	1.74, 1.91	35.2	1.49, 2.24	37.5
6	3.69	70.3	3.63	69.9	3.57	71.0	4.61	76.7	4.19	77.8
7	1.27, 1.54	34.4	1.17, 1.54	32.9	1.17, 1.55	34.1	1.35, 1.82	32.7	1.36, 1.85	32.6
8	1.40	24.5	1.34	24.4	1.37	24.7	1.39, 1.49	23.9	1.41, 1.49	24.6
a'	2.58	23.1	3.00	27.9	3.04	27.6				
b'	3.31	42.5	3.31	38.6	3.32	38.8				
c'	—	172.8	—	172.7	—	172.7				
d'	2.45	35.3	2.41	35.2	2.43	35.3				
e'	3.46	35.1	3.45	35.1	3.46	35.1				
f'	—	174.3	—	174.4	—	174.4				
g'	4.05	73.9	4.06	73.5	4.08	73.5				
h'	—	38.8	—	38.7	—	38.7				
h'-Me	0.82, 1.02	18.3, 20.8	0.83, 1.05	18.1, 21.0	0.82, 1.05	18.1, 20.9				
i'	3.59, 3.96	71.8	3.56, 3.98	71.8	3.57, 3.99	71.7				
R1	6.13	87.4	6.12	87.1	6.14	87.1				
R2, R4	4.76, 4.92	74.1, 74.6	4.79, 4.86	74.1, 74.4	4.79, 4.92	74.1, 74.5				
R3	4.52	83.3	4.50	83.4	4.50	83.4				
R5	4.28	64.9	4.24	65.1	4.23, 4.30	64.9				
A2	8.22	150.3	8.20	152.4	8.20	152.4				
A6	8.62	140.6	8.57	139.6	8.59	139.7				

<sup>a</sup> Labels are shown in Figure 1.

<sup>b</sup> Chemical shifts of a' to i', R1 to R5 and A2 & A6 in this column correspond to the CoASH standard in 90%  $\text{CD}_3\text{OD}/10\% \text{D}_2\text{O}$ .

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Table 2. Observed and calculated  $m/z$  values of the most abundant fragment ions in the MS/MS spectra of simvastatin standard (A) and M1 (B).

The calculated values and formulas are based on the fragmentation assigned in Figure 5. The MS/MS spectra were obtained using a Micromass Q-TOF mass spectrometer (see text for details).

### A. simvastatin standard

Elemental Composition	Observed $m/z$	Calculated $m/z$	Deviation (mDa)	Deviation (ppm)
$C_{13}H_{17}^+$	173.1328	173.1325	-0.3	-1.7
$C_{15}H_{19}^+$	199.1486	199.1481	-0.5	-2.5
$C_{17}H_{21}^+$	225.1638	225.1638	0.0	0.0
$C_{17}H_{23}O^+$	243.1744	243.1743	-0.1	-0.4
$C_{19}H_{23}O^+$	267.1762	267.1743	-1.9	-7.1
$C_{19}H_{25}O_2^+$	285.1856	285.1849	-0.7	-2.5
$C_{19}H_{27}O_3^+$	303.1964	303.1955	-0.9	-3.0

### B. M1

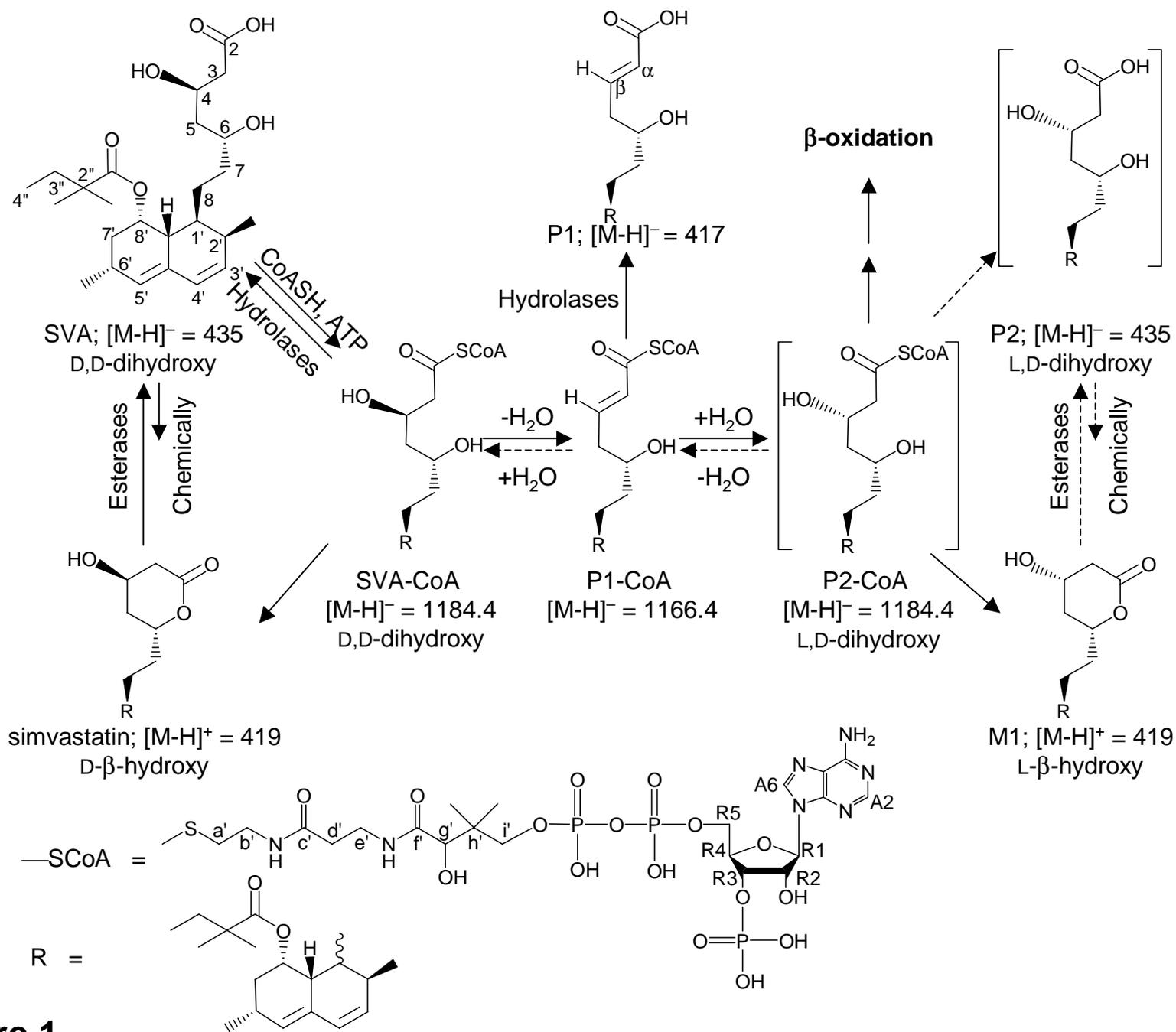
Elemental Composition	Observed $m/z$	Calculated $m/z$	Deviation (mDa)	Deviation (ppm)
$C_{13}H_{17}^+$	173.1326	173.1325	-0.1	-0.6
$C_{15}H_{19}^+$	199.1490	199.1481	-0.9	-4.5
$C_{17}H_{21}^+$	225.1655	225.1638	-1.7	-7.6
$C_{17}H_{23}O^+$	243.1744	243.1743	-0.1	-0.4
$C_{19}H_{23}O^+$	267.1742	267.1743	0.1	0.4
$C_{19}H_{25}O_2^+$	285.1855	285.1849	-0.6	2.1
$C_{19}H_{27}O_3^+$	303.1945	303.1955	1.0	3.3

Table 3. Marker enzyme activities in mouse liver subcellular fractions.

Marker enzymes	Specific activity of S0.6 <sup>a</sup>	Specific activity relative to S0.6					
		S3	MT-H	S27	MT-L	cytosol	microsomes
Catalase	167 ± 13	0.83 ± 0.01	1.26 ± 0.08	0.52 ± 0.04	2.79 ± 0.10	0.68 ± 0.04	0.41 ± 0.01
Cytochrome c oxidase	217 ± 3	0.72 ± 0.04	3.21 ± 0.23	0.29 ± 0.07	2.89 ± 0.52	0.02 ± 0.03	0.93 ± 0.16
Lactate dehydrogenase	11.1 ± 1.0	0.99 ± 0.11	0.18 ± 0.11	1.08 ± 0.08	0.06 ± 0.003	1.63 ± 0.18	0.08 ± 0.004
Cytochrome c reductase	9.7 ± 0.6	0.94 ± 0.10	0.03 ± 0.01	1.26 ± 0.05	0.10 ± 0.02	0.24 ± 0.01	3.56 ± 0.19

<sup>a</sup>Units for catalase and lactate dehydrogenase are  $\mu\text{mol}/\text{min}/\text{mg}$  protein; units for cytochrome c oxidase and cytochrome c reductase are  $\text{nmol}/\text{min}/\text{mg}$  protein.

Values represent means  $\pm$  SD from triplicate incubations.



**Figure 1**

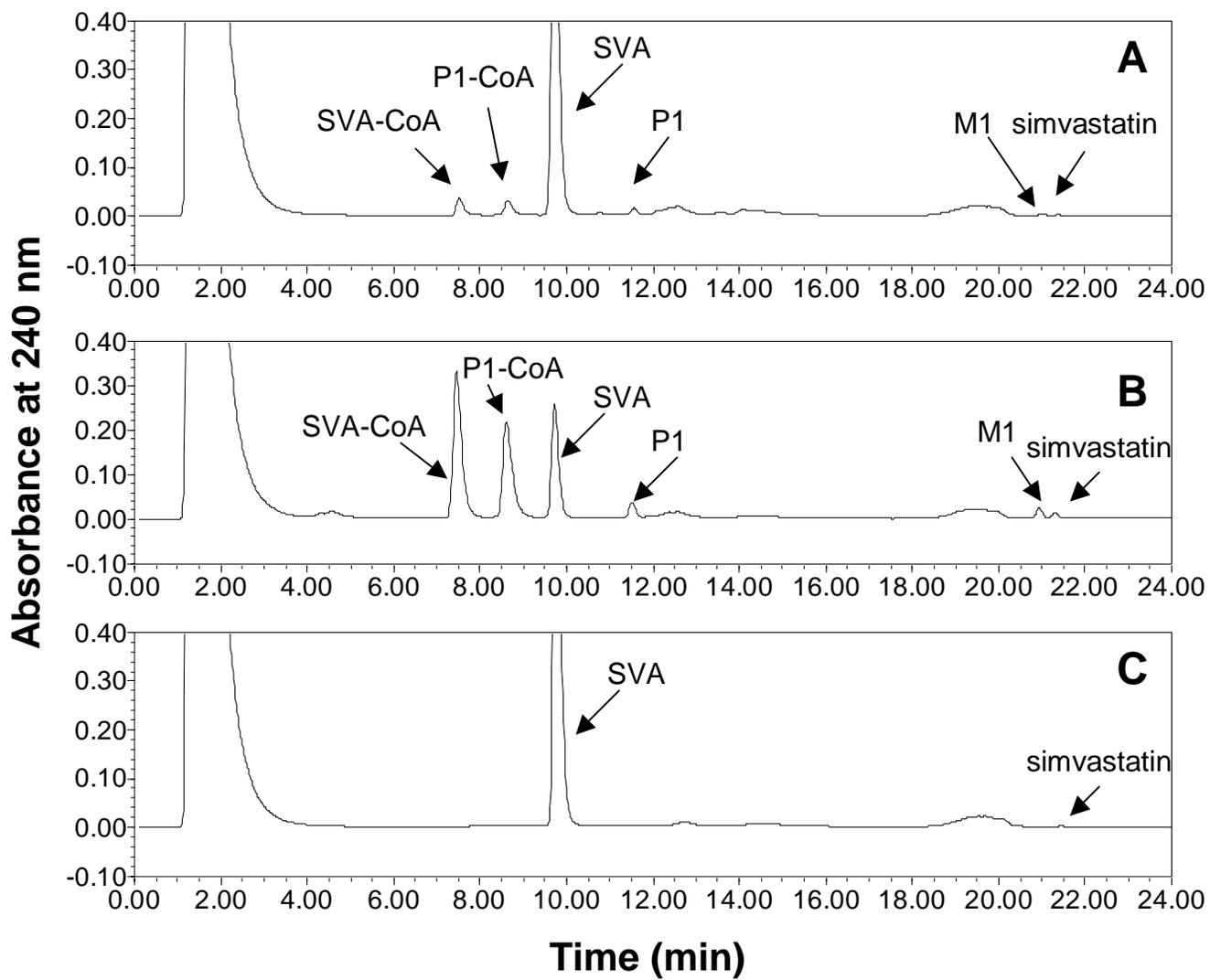


Figure 2

Figure 3

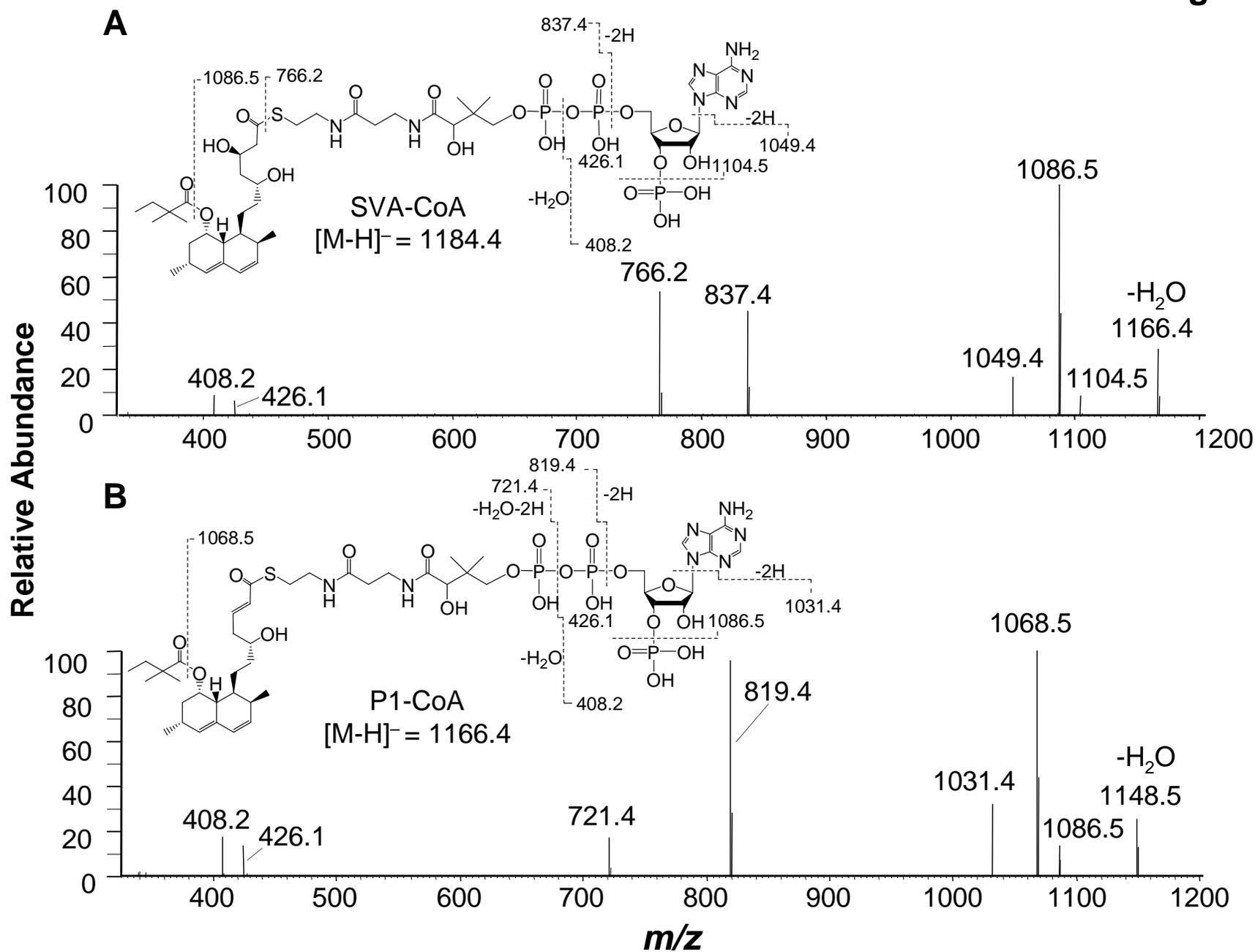
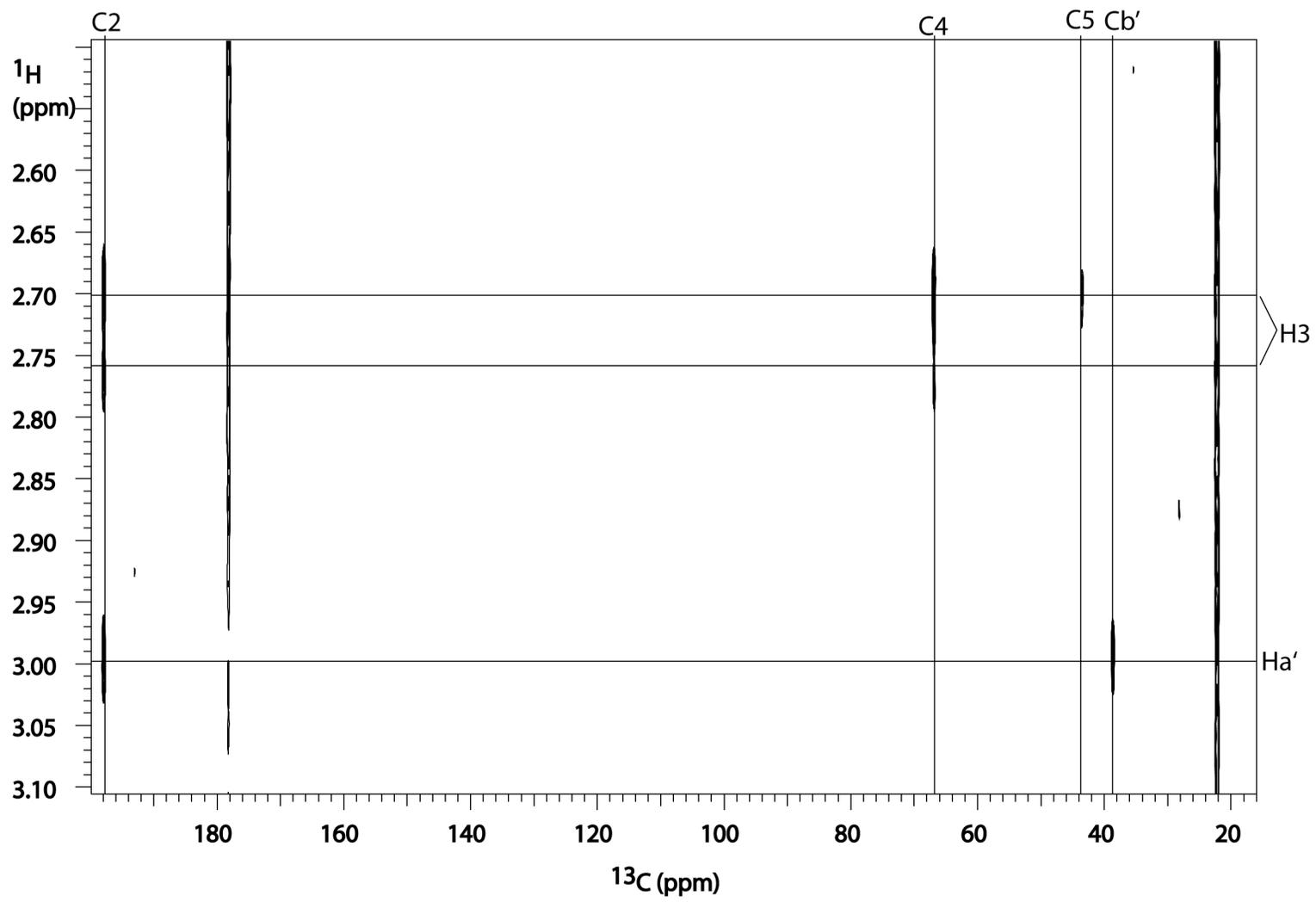


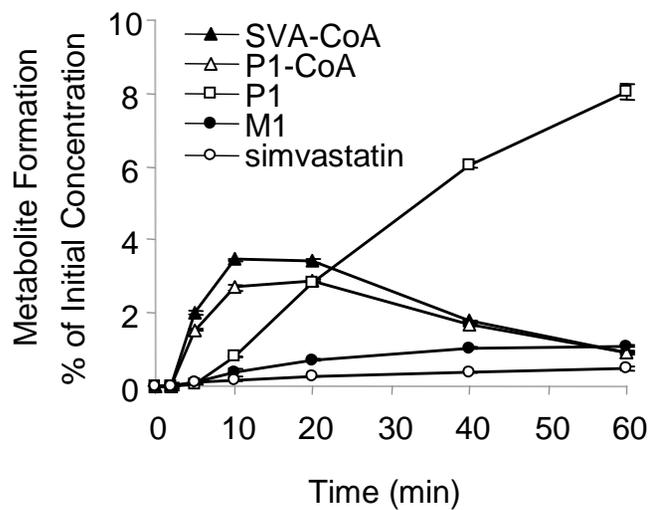
Figure 4



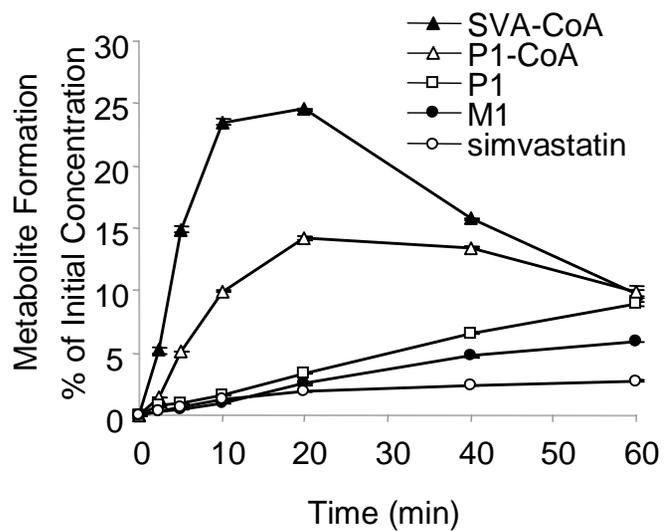


**Figure 6**

**A.**



**B.**



**Figure 7**

