

Stereoselective Synthesis of a Conformationally Defined Cyclohexyl Carnitine Analogue that Binds CPT-1 with High Affinity

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Abstract—Carnitine (**1**, 3-hydroxy-4-trimethylammonibutyrate) is important in mammalian tissue as a carrier of acyl groups. In order to explore the binding requirements of the carnitine acyltransferases for carnitine, we designed conformationally defined cyclohexyl carnitine analogues. These diastereomers contain the required *gauche* conformation between the trimethylammonium and hydroxy groups but vary the conformation between the hydroxy and carboxylic acid groups. Here we describe the synthesis and biological activity of the all-trans diastereomer (**2**), which was prepared by the ring opening of *trans*-methyl 2,3-epoxycyclohexanecarboxylate with NaN_3 . Racemic **2** was a competitive inhibitor of neonatal rat cardiac myocyte CPT-1 (K_i 0.5 mM for racemic **2**; K_m 0.2 mM for L-carnitine) and a noncompetitive inhibitor of neonatal rat cardiac myocyte CPT-2 (K_i 0.67 mM). These results suggest that **2** represents the bound conformation of carnitine for CPT-1. © 1999 Published by Elsevier Science Ltd. All rights reserved.

Introduction

Carnitine is widely distributed in nature, and research over the last three decades has provided a better understanding of the biochemical roles for carnitine.¹ These include mitochondrial long-chain fatty acid oxidation, buffering the mitochondrial acyl CoA/CoA couple, a scavenger system for acyl groups, and branched-chain amino acid metabolism. Most of carnitine's functions involve the transfer of an acyl residue from an acyl-CoA to the β -hydroxy group followed by translocation from one cellular compartment to another.^{2–4} In this process, R-carnitine serves as a substrate for a reversible reaction catalyzed by the carnitine acyltransferases, a class of enzymes which differ in their chain length specificity (see Fig. 1). Carnitine acetyltransferase (CAT) is important for the transfer of short chain acyl groups, particularly acetate, and for maintaining the CoASH/acetyl-S-CoA ratio.^{5,6} CAT is present in both

the mitochondria and peroxisomes. Carnitine octanoyltransferase (COT) is selective for transferring medium chain acyl groups and is found in peroxisomes and to a lesser extent in microsomes. It is believed that COT is responsible for shuttling medium chain acylcarnitines out of peroxisomes. Carnitine palmitoyltransferases 1 and 2 (CPT-1 and CPT-2) are responsible for the transfer of long chain acyl groups and are essential for the entry of fatty acids into mitochondria prior to β -oxidation.^{7–10} Recently, CPT-1 has been found to exist in two isoforms with different physical and kinetic properties, liver CPT-1 (L-CPT-1) and skeletal muscle CPT-1 (M-CPT-1). Rat heart mitochondria contain both isoforms of CPT-1, and the minor component is identical to L-CPT-1.

In order to probe potential differences among the carnitine binding sites on the different carnitine acyltransferases, we have designed rigid carnitine analogues **2–5**, which utilize a cyclohexyl framework. Because the bulky trimethylammonium group locks the cyclohexane ring in a single chair conformation, these carnitine analogues contain defined spatial relationships between the quaternary ammonium, hydroxyl, and carboxylate moieties. The syntheses and biological evaluations

Key words: Carnitine; carnitine acyltransferase; enzyme inhibition; conformationally defined analogue; bound conformation.

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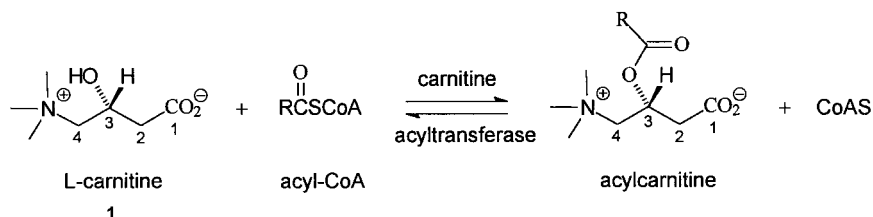


Figure 1. Reaction of carnitine with an Acyl-CoA.

(CAT and CPT-2) for **3–5** have been previously described.¹¹ Here we report the stereoselective synthesis of **2** and compare the effects of **2–5** on neonatal rat cardiac myocyte CPT-1 and CPT-2 and pigeon breast CAT.

Results and Discussion

Chemistry

We have been interested in evaluating differences in carnitine binding requirement among the different carnitine acyltransferases.^{11–17} Gandour et al. previously identified the relative populations for the three possible low energy conformations of carnitine about C2–C3 in solution using ¹H NMR techniques (1(A)–1(C) in Fig. 2).¹⁸ It was found that all three low-energy conformations about C2–C3 are significantly populated, although two of these were dominant (Fig. 2). About C3–C4, carnitine was reported to exist almost exclusively in a conformation, which places the hydroxy and bulky trimethylammonium group in a *gauche* relationship. In fact, this conformation about C3–C4 was suggested to be

the active conformation of carnitine for binding to the carnitine acyltransferases. However, the conformation of carnitine about C2–C3 involved in binding to the carnitine acyltransferases is not yet known. To address this question, our laboratory has designed a series of rigid carnitine analogues (**2–5** in Fig. 2) which utilize a cyclohexyl ring to control the stereochemistry. These carnitine analogues all maintain the *gauche* relationship between the hydroxy and trimethylammonium groups (C3–C4) while varying the stereochemistry between the hydroxy and carboxylic acid groups (C2–C3). Compounds **3–5** (Fig. 2) each mimic one of the three low energy conformations of carnitine about C2–C3, and their preparation and biological evaluations were previously described.¹¹ Although **3** and **5** were selective inhibitors of CAT over CPT-2 (CPT-1 was not evaluated), **3–5** were only low mM inhibitors of carnitine acyltransferases. We therefore pursued compound **2**. Compound **2**, like analogue **3**, mimics conformation A about C2–C3 but, unlike **3**, contains all of the functional groups in equatorial positions. The evaluation of **2** was desirable since, relative to **3**, it contains the cyclohexyl ring residues in a different region of space and

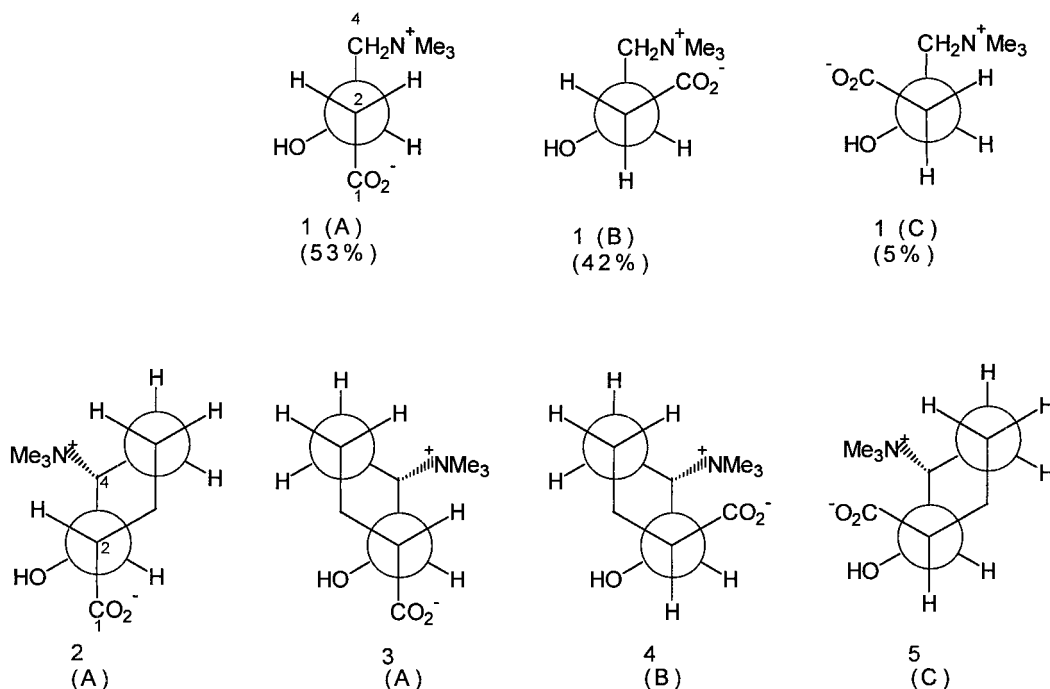


Figure 2. Solution conformations for carnitine (1(A)–1(C)) and their relative populations (%) as compared to the conformationally defined cyclohexyl carnitine analogues **2–5**. Compounds **2–5** all maintain the *gauche* arrangement about C3–C4 while varying the conformation about C2–C3. Note that compounds **2** and **3** both mimic conformation 1(A) of carnitine but contain functional groups in different axial versus equatorial arrangements.

may more readily present key functional groups to the carnitine binding site. Such information may prove useful for designing selective inhibitors of individual carnitine acyltransferases as therapeutic agents.

Since the synthetic approaches previously published for **3–5** could not provide usable quantities of compound **2**, an alternative approach to this compound was developed. Compound **2** was synthesized from 3-bromocyclohexene (**6**) as shown in Scheme 1. The conversion of **6** to **7** has been reported by Davies and Whitman¹⁹ using sodium cyanide in *N*-methyl-2-pyrrolidinone. We employed a variation of this method and found that bromide **6** could be cleanly converted to the nitrile **7** by stirring with KCN and 18-crown-6 in CH₂Cl₂ for several days. Compounds **8–10** were then prepared according to literature procedures.^{19,20} Ester **8** was obtained by treating the nitrile **7** with methanolic HCl, and **8** underwent stereoselective epoxidation with peracetic acid. The desired *trans*-epoxide **9** was obtained in a ratio of 3:2 over the *cis*-epoxide **10**, which was determined by comparing the integrations of the C2 protons by ¹H NMR. These diastereomers were then separated by flash chromatography.

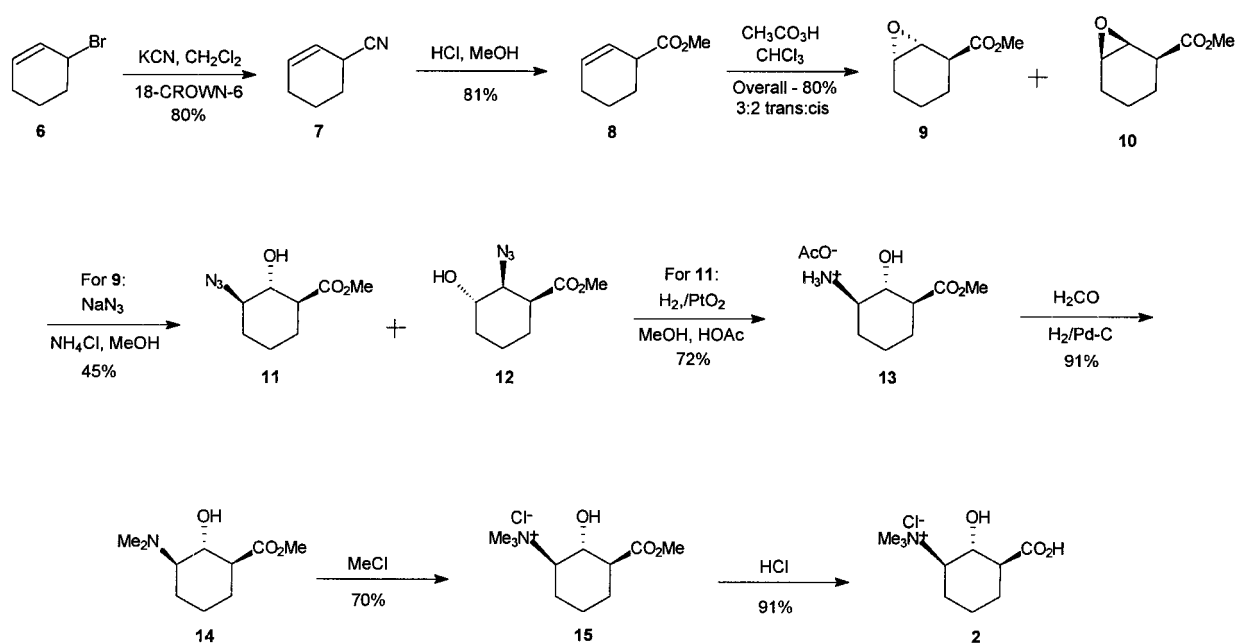
Attempts were made to improve the stereoselectivity of epoxidation of compound **8**. It was thought that a bulkier ester might increase the selectivity of epoxidation by providing steric hindrance to the 'cis' face of the ring. The methyl ester of **8** was changed to a *t*-butyl ester and epoxidized with mCPBA and peracetic acid at –40, 0, and 25 °C. In all cases, the selectivity of the epoxidation was not improved.

Following a procedure reported by Swift and Swern for the conversion of 1,2-epoxycyclohexane to *trans*-2-azidocyclohexanol,²¹ epoxide **9** underwent ring opening with NaN₃ to give a 5:4 mixture of **11** and **12**, which was

separated by HPLC to give pure **11**. Azide **11** was reduced with H₂ and Pd/C to yield the acetate salt **13**.²² Reductive alkylation of **13** under Eschweiler–Clarke conditions²³ provided dimethylamine **14**. Quaternization with chloromethane followed by hydrolysis of the ester provided the final product **2**.

The relative configuration of **2** was determined from coupling constants between H2, H3, and H4 using ¹H NMR. Compound **2** is locked in a single chair conformation since the bulky trimethylammonium group can only occupy the equatorial position. This places H4 axial, which is used as the reference proton in determining the relative configuration. The residual splittings between H2, H3, and H4 were determined by single frequency off-resonance decoupling experiments (recorded at 400 MHz).

The chemical shifts for H2 (2.52), H3 (3.38), and H4 (4.08) were initially assigned by comparison to diastereomers **3–5**. The ¹H NMR spectrum indicated that H2, H3, and H4 were all axial based upon the following observations. The presence of a pseudo triplet (overlapping doublet of doublets) for H3 with large coupling constants (10.0 and 10.1 Hz) indicated two axial–axial interactions. These assignments were confirmed by decoupling experiments. Irradiation of H2 resulted in the collapse of H3 into a doublet (residual *J*_{3,4} = 10.1 Hz). A similar result was obtained by irradiating H4 causing collapse of H3 into a doublet (residual *J*_{2,3} = 10.0 Hz). This confirmed that H3 has axial–axial couplings to both H2 and H4. The irradiation of H3 collapsed both H2 and H4 to doublets of doublets. Each doublet of doublets contained large splittings (*J*_{2,7a} = 12.1 Hz, *J*_{4,5a} = 12.0 Hz), as well as small splittings (*J*_{2,7e} = 3.7 Hz, *J*_{4,5e} = 3.8 Hz). This shows that H2 and H4 both have axial–axial coupling, as well as axial–equatorial coupling, confirming that H2, H3, and H4 are all axial.



Scheme 1.

Enzyme kinetics assays

Racemic **2** was evaluated as an inhibitor of purified pigeon breast carnitine acetyltransferase (CAT) in the forward direction (acylcarnitine formation), as well as mitochondrial carnitine palmitoyltransferases 1 and 2 (CPT-1 and CPT-2) in cultured neonatal rat cardiac myocytes (in the direction of acylcarnitine formation) (Figs. 3 and 4). The effects of **3–5** on CPT-2 and CAT were previously reported, and for comparison purposes in the present study these were also assayed with CPT-1 (Figs. 5 and 6). As reported earlier,¹¹ racemic **3** and **5** are modest inhibitors of CAT but do not inhibit CPT-2. Studies performed here with CPT-1 (Table 1) reveal that **5** is a weak competitive inhibitor, while compound **3** is specific for the inhibition of CAT. In contrast to **3–5**, racemic **2** did not inhibit CAT. However, **2** was an effective noncompetitive inhibitor of CPT-2 ($K_i = 0.67$ mM). The only competitive inhibition for **2** was observed with CPT-1, and the K_i for racemic **2** (0.5 mM) was similar to the K_m observed for L-carnitine (0.2 mM). This is the most potent inhibition observed for **2–5** and suggests that the carnitine conformation in **2** may represent the bound conformation of carnitine with CPT-1. These results also suggest that properly designed carnitine analogues may be useful as selective small molecule inhibitors of carnitine acyltransferases.

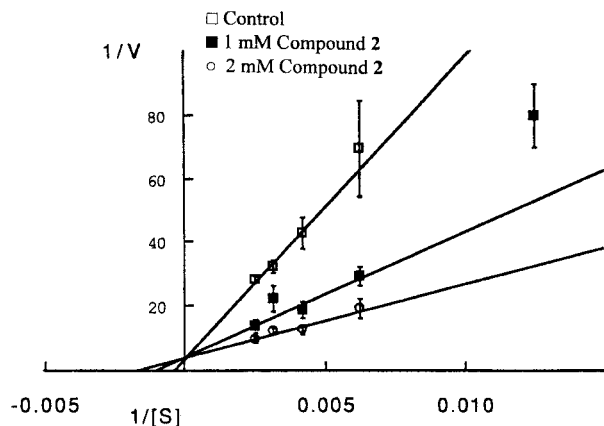


Figure 3. Lineweaver–Burke plot of compound **2** and CPT-1, where $S = \text{L-carnitine}$ (concentration = M).

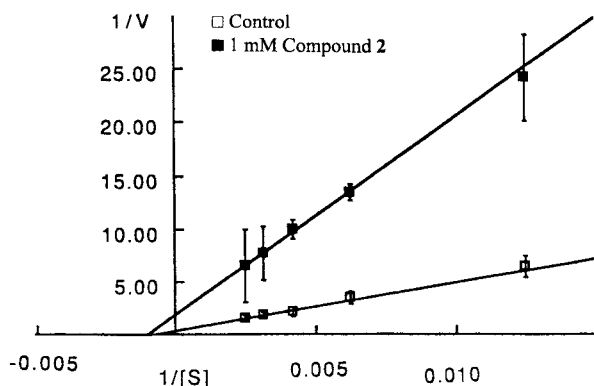


Figure 4. Lineweaver–Burke plot of compound **2** and CPT-2, where $S = \text{L-carnitine}$ (concentration = M).

Conclusions

While all of the rigid cyclohexyl carnitine analogues contain the same hydroxy to trimethylammonium (O to N) distances, the hydroxy to carboxy distances vary considerably. The results from evaluation of these compounds as inhibitors of carnitine acyltransferases (Table 1) permit formulation of the following structural hypotheses. (1) The poor binding of cyclohexyl carnitine analogues to CAT and CPT-2 may result from interference by the cyclohexyl ring residues. (2) The biologically active conformation of carnitine for CPT-1 is contained in both rigid analogues **2** and **3**, but **3** contains the cyclohexyl ring residues in a region of space that interferes with binding. (3) The carnitine binding site of CPT-1 is distinctly different from that for CAT and CPT-2.

Experimental

General

Melting points were determined on an Electrothermal melting point apparatus and are uncorrected. The ^1H NMR spectra were recorded on a Bruker DRX-400 (400 MHz) or a Bruker ARX-300 (300 MHz) spectrometer. The ^{13}C NMR spectra were recorded on a Bruker

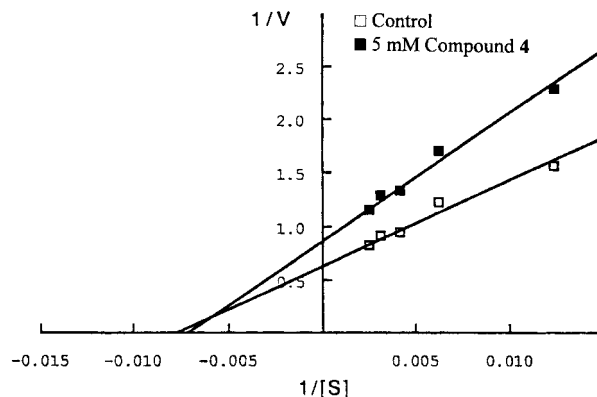


Figure 5. Lineweaver–Burke plot of compound **4** and CPT-1, where $S = \text{L-carnitine}$ (concentration = M).

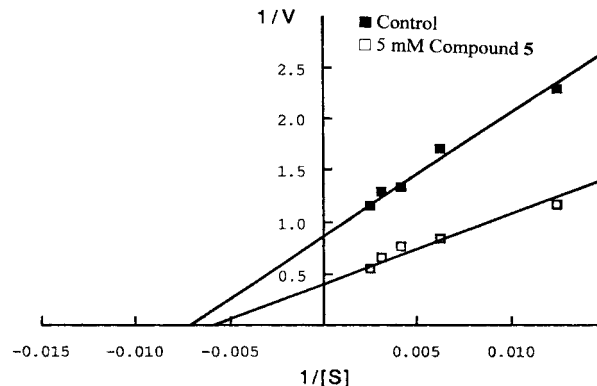


Figure 6. Lineweaver–Burke plot of compound **5** and CPT-1, where $S = \text{L-carnitine}$ (concentration = M).

Table 1. Kinetic constants for conformationally defined carnitine analogues with selected carnitine acyltransferases^a

Compound	K_i , mM, CPT-1 ^b (Type Inhib)	K_i , mM, CPT-2 ^b (Type Inhib)	K_i , mM, CAT ^c (Type Inhib)
(<i>R,S</i>)-2	0.5 (Competitive)	0.67 (Noncompetitive)	No Effect
(<i>R,S</i>)-3	No Effect	No Effect ^d	4.1 (Competitive) ^d
(<i>R,S</i>)-4	9.5 (Noncompetitive)	No Effect ^d	3.7 (Competitive) ^d
(<i>R,S</i>)-5	9.3 (Competitive)	5.3 (Competitive) ^d	2.9 (Competitive)

^a All assays were in the forward direction (acylcarnitine formation) and used varying concentration of L-carnitine (K_m 0.2 mM for CPT-1 and 0.2 mM for CPT-2).

^b Assayed in cultured neonatal rat cardiac myocytes.

^c Purified pigeon breast CAT was utilized.

^d Taken from ref 11.

DRX-400 (100 MHz) or a Bruker ARX-300 (75 MHz). Chemical shifts (ppm) are referenced to internal TMS for ¹H and ¹³C NMR spectra, when recorded in CDCl₃ solvent, and samples recorded in D₂O were referenced to either H₂O (¹H NMR) or dioxane (¹³C NMR). IR spectra were obtained on a Bruker Vector 22 FT-IR or a Perkin–Elmer 1310 IR. Mass spectra were recorded by electrospray on a Perkin–Elmer Sciex API-III spectrometer. Flash chromatographic separations were performed on Baker silica gel, 40 μm, and TLC was performed on Whatman brand silica gel plates (250 μm layer, 5×10 cm). HPLC separations utilized a Rainin Dynamax silica column on a Rainin Rabbit HPLC system. All liquid reagents were distilled prior to use. Methanol was distilled over magnesium methoxide and methylene chloride was distilled over phosphorus pentoxide. Acetone was dried by distilling over potassium permanganate and then over anhydrous calcium sulfate. All other solvents were reagent grade, obtained from Fisher Scientific or Aldrich Chemical Company, and used without further purification. Elemental analyses were performed at Atlantic Microlab of Atlanta, Georgia.

Cyclohex-2-enecarbonitrile (7). Dry potassium cyanide (34.5 g, 530 mmol) and 18-crown-6 (600 mg, 2.20 mmol) were added to a solution of 3-bromocyclohexene (**6**, 30.0 g, 186 mmol) in dry CH₂Cl₂ (90 mL). The reaction mixture was stirred at 25°C for 4 days. The unreacted KCN was filtered and washed with CH₂Cl₂. The solvent was removed from the filtrate on a rotary evaporator to give **7** (19.4 g, 97.2%) as an oil: bp 83–84°C/20 mm Hg (lit.¹³ bp 89°C/23 mm Hg).

Methyl cyclohex-2-enecarboxylate (8). Anhydrous hydrogen chloride was bubbled into a refluxing mixture of **7** (8.60 g, 80.3 mmol) in dry methanol (100 mL, 79.1 g, 2.47 moles) for 60 min. The reaction mixture was heated at reflux for an additional 60 min and stirred at room temperature for 22 h. The reaction mixture was poured over ice (300 g) and stirred until the ice had melted. The mixture was extracted with ether (4×50 mL). The combined ether layers were washed with 5% NaHCO₃ (3×50 mL) and dried (Na₂SO₄), and the solvent was removed in vacuo to give **8** as an oil (R_f 0.45, 30% ethyl acetate/hexanes). The ¹H NMR spectrum was identical to that reported by Davies and Whitham.¹⁹ This product was carried on without further purification. ¹H NMR (CDCl₃) δ 1.4–1.6 (m, 1H), 1.7–1.9 (m, 3H),

1.9–2.0 (br s, 2H), 2.9–3.0 (m, 1H), 3.7 (s, 3H), 5.6–5.7 and 5.75–5.8 (m, 2H).

Methyl trans-2,3-epoxycyclohexanecarboxylate (9) and methyl cis-2,3-epoxycyclohexanecarboxylate (10). To a solution of **8** (16.6 g, 118 mmol) in CHCl₃ (250 mL) was added 32% peracetic acid (90.0 mL, 28.8 g, 427 mmol) and anhydrous sodium acetate (16.7 g, 203 mmol). The reaction mixture was stirred at 25°C for 16 h. The mixture was extracted with CHCl₃ (4×50 mL), the combined organic extracts were washed with 5% NaHCO₃ (4×100 mL), and the organic layer was dried (Na₂SO₄). The solvent was removed in vacuo to give a mixture of the *cis* and *trans* epoxyesters (17.1 g, 92.9%). Integration of the C2 proton resonances in the ¹H NMR spectrum gave the ratio of *trans* (C2 resonance at 3.4 ppm) to *cis* (C2 resonance at 3.45 ppm) as 3:2. Part of the mixture (5.0 g) was chromatographed (33% ether/hexanes) on a flash silica column (5×25 cm) to first provide **9** as an oil (2.3 g, R_f 0.47) followed by a mixture of **9** and **10** (0.70 g). Further elution gave pure **10** as an oil (1.5 g, R_f 0.31). The ¹H NMR spectra for **9** and **10** were identical to those reported by Davies and Whitman.²⁰

For **9**: ¹H NMR (CDCl₃) δ 1.3–1.5 (m, 3H), 1.7–1.9 (m, 2H), 2.0–2.1 (m, 1H), 2.9 (dd, 1H), 3.2 (m, 1H), 3.4 (d, 1H), 3.7 (s, 3H).

For **10**: ¹H NMR (CDCl₃) δ 1.20–1.94 (m, 6H), 2.81–2.89 (m, 1H), 3.19–3.25 (m, 1H), 3.45 (t, 1H), 3.78 (s, 3H).

Methyl 3-azido-2-hydroxycyclohexanecarboxylate (11) and methyl 2-azido-3-hydroxycyclohexanecarboxylate (12). Compound **9** (3.50 g, 22.7 mmol) was added to a flame dried round bottom flask containing dry methanol (75 mL) under a nitrogen atmosphere. Dry NaN₃ (2.58 g, 39.7 mmol) and NH₄Cl (1.83 g, 34.2 mmol) were added to the reaction mixture. This mixture was heated to reflux for 14 h. The reaction mixture was cooled to room temperature and the solvent was removed, leaving an oil. The oil was taken up in water (75 mL) and extracted with ethyl acetate (3×50 mL). The combined organic extracts were washed with saturated NaCl (2×50 mL) and dried (Na₂SO₄). Integration of the methyl ester resonances in the ¹H NMR spectrum revealed a 1:1.1 ratio of **11**:**12**. The mixture was filtered through a bed of silica gel on a sintered glass funnel and concentrated in vacuo to yield a mixture of **11** and **12** (3.65 g, 81.8%). This mixture was separated on a

Rainin Dynamax silica HPLC column (21.4×250 mm, 60 Å, 20% ethyl acetate/hexane, 489 mg/injection as a 54% ethyl acetate stock solution) using a flow rate of 5 mL/min to give **11** (1.47 g, 32.9%; t_R = 57.6 min). IR (neat) 3700–3000 (OH), 2100 (N₃), 1710 (C=O) cm⁻¹; ¹H NMR (CDCl₃) δ 1.27–1.45 (m, 3H), 1.80–1.85 (m, 1H), 1.95–2.10 (m, 2H), 2.36–2.42 (m, 1H), 3.15 (d, 1H), 3.25–3.32 (m, 1H), 3.65–3.76 (dd, 1H), 3.73 (s, 3H); ¹³C NMR (CDCl₃) δ 174.24, 74.28, 65.13, 51.99, 49.58, 29.80, 27.64, 23.31; MS (ES) m/z 200 (M+H)⁺. Anal. calcd for C₈H₁₃N₃O₃: C, 48.26; H, 6.60; N, 21.10. Found: C, 48.01; H, 6.56; N, 20.86.

For **12** (1.38 g, 30.9%; t_R = 74.2 min): IR (neat) 3700–3000 (OH), 2100 (N₃), 1720 (C=O) cm⁻¹; ¹H NMR (CDCl₃) δ 1.45–1.90 (m, 6H), 2.26 (s, 1H), 2.95–3.05 (m, 1H), 3.72 (s, 3H), 3.83 (t, 1H), 4.13–4.19 (br s, 1H); ¹³C NMR (CDCl₃) δ 173.4, 68.35, 64.38, 51.80, 42.09, 29.31, 23.94, 19.01; MS (ES) m/z 200 (M+H)⁺. Anal. calcd for C₈H₁₃N₃O₃: C, 48.26; H, 6.60; N, 21.10. Found: C, 48.05; H, 6.56; N, 20.85.

Methyl 3-ammonio-2-hydroxycyclohexanecarboxylate, acetate (13). Compound **11** (1.00 g, 5.02 mmol), methanol (25.0 mL), acetic acid (10.7 mL), and 10% Pd/C (0.41 g) were combined in a hydrogenation pressure bottle. The reaction mixture was shaken on a Parr shaker (50 psi H₂) for 1 h. The catalyst was filtered and washed with hot deionized water. The solvent was removed from the filtrate and dried under vacuum to yield **13** (1.07 g, 91.5%) as a white solid: mp 156–158°C (ethyl acetate); IR (KBr) 3500–2250 (OH), 1724 (C=O) cm⁻¹; ¹H NMR (D₂O) δ 1.34–1.46 (m, 3H), 1.74–2.22 (m, 2H), 1.81 (s, 3H), 2.43–2.49 (m, 1H), 2.98–3.14 (m, 1H), 3.62–3.68 (dd, 1H), 3.65 (s, 1H); ¹³C NMR (D₂O) δ 183.75, 178.93, 74.31, 57.79, 55.44, 53.12, 31.22, 30.56, 25.80, 25.21; MS (ES) m/z 174 (M+H)⁺. Anal. calcd for C₁₀H₁₉NO₅: C, 51.52; H, 8.23; N, 6.01. Found: C, 51.49; H, 8.18; N, 5.89.

Methyl 3-(*N,N*-dimethylamino)-2-hydroxycyclohexanecarboxylate (14). Compound **13** (580 mg, 2.49 mmol) was dissolved in deionized water (22.0 mL) and added to a hydrogenation pressure bottle. Formaldehyde (37%, 1.70 mL, 629 mg, 22.7 mmol) and 10% Pd/C (300 mg) were added to the reaction mixture, which was then shaken on a Parr shaker (50 psi H₂) for 18 h. The catalyst was filtered and washed with hot deionized water. The filtrate was allowed to cool to room temperature, saturated with potassium carbonate, and extracted with CHCl₃ (3×50 mL). The combined organic layers were dried (Na₂SO₄) and concentrated in vacuo to give **14** as a white solid (455 mg, 91.0%); mp 50–52°C (ether/hexanes). IR (KBr) 3550–3100 (OH), 1736 (C=O) cm⁻¹; ¹H NMR (CDCl₃) δ 1.11–1.53 (m, 3H), 1.72–1.88 (m, 3H), 2.21–2.42 (m, 2H), 2.26 (s, 6H), 3.58 (dd, 1H), 3.73 (s, 3H); ¹³C NMR (CDCl₃) δ 176.86, 72.09, 70.17, 53.77, 52.25, 42.22, 30.06, 26.20, 22.00; MS (ES) m/z 202 (M+H)⁺. Anal. calcd for C₁₀H₁₉NO₃: C, 59.68; H, 9.52; N, 6.96. Found: C, 59.51; H, 9.41; N, 7.04.

Methyl 2-hydroxy-3-(trimethylammonio)cyclohexanecarboxylate, chloride (15). A solution of **14** (250 mg, 1.24 mmol) in anhydrous acetone (5.0 mL) was added to

a pear-shaped pressure bottle and cooled to -78°C. Chloromethane was bubbled through the solution for 10 min. The bottle was capped and warmed to 30°C for 5 days. The solid was filtered and washed with anhydrous acetone (5.0 mL) to give **15** (183 mg, 58.7%); mp 217–218°C (ethyl acetate/hexanes). IR (KBr) 3250–3100 (OH), 1736 (C=O) cm⁻¹; ¹H NMR (D₂O) δ 1.30–1.55 (m, 3H), 1.79–1.90 (m, 2H), 2.20–2.28 (m, 1H), 2.52–2.62 (m, 1H, J_{2-7e} = 3.48 Hz, J_{2-7a} = 11.05 Hz), 3.11 (s, 9H), 3.34–3.45 (m, 1H, J_{4-5e} = 3.79 Hz, J_{4-5a} = 11.94 Hz), 3.64 (s, 3H, J_{2-3} = J_{3-4} = 10.18 Hz), 4.08 (dd, 1H); ¹³C NMR (D₂O with 0.01% dioxane) δ 175.76, 75.94, 70.77, 53.22, 52.65, 52.29, 27.51, 25.26, 23.09; MS (ES) m/z = 216 (M+H)⁺. Anal. calcd for C₉H₂₂ClNO₃: C, 52.56; H, 8.84; N, 5.57; Cl, 13.93. Found: C, 52.36; H, 8.66; N, 5.46; Cl, 13.74.

2-Hydroxy-3-(trimethylammonio)cyclohexanecarboxylic acid, chloride (2). A solution of **15** (100 mg, 398 mmol) in 17% HCl (4.0 mL) was heated to reflux for 1.5 h. The reaction mixture was cooled to rt, concentrated to dryness in vacuo, and dried overnight under high vacuum to yield **2** as a white solid (90.9 mg, 96.0%); mp 207–208°C (methanol/ether); IR (KBr) 3650–3100 (OH), 1718 (C=O) cm⁻¹; ¹H NMR (D₂O) δ 1.41–1.54 (m, 3H), 1.89–1.95 (m, 2H), 2.17–2.29 (m, 1H), 2.53–2.60 (m, 1H, J_{2-7a} = 12.11 Hz, J_{2-7e} = 3.70 Hz), 3.17 (s, 9H), 3.40–3.46 (m, 1H, J_{4-5a} = 11.95 Hz, J_{4-5e} = 3.77 Hz), 4.11 (dd, 1H, J_{3-4} = J_{2-3} = 10.11 Hz); ¹³C NMR (D₂O with 0.01% dioxane) δ 177.09, 75.83, 70.55, 53.10, 52.25, 27.61, 25.17, 23.09; MS (ES) m/z = 202 (M+H). Anal. calcd for C₁₀H₂₀ClNO₃: C, 50.52; H, 8.50; Cl, 14.91; N, 5.89. Found: C, 50.36; H, 8.41; Cl, 14.75; N, 5.68.

Cultured neonatal rat cardiac myocyte CPT-1 and CPT-2 assays. The details of these procedures have been previously published.¹⁷ Briefly, neonatal cardiac myocytes were isolated and cultured in 35 mm, 12-well plates (2×10⁵ cells/well) as described by McMillin et al.²⁴ For the CPT-1 assay, cell membranes were permeabilized with 10 mM digitonin (10 min at 37°C). After removal of the permeabilization medium, 0.5 mL of CPT assay medium buffered with 10 mM HEPES, pH 7.0, and containing 1% BSA, was added to each well. Palmitoyl-CoA was added to give a final concentration of 30 mM. The assay was initiated by adding increasing concentrations of [¹⁴C]carnitine (specific activity = 2000–3000 dpm/nmol) to a series of cell cultures (final concentrations varied from 0.08 to 0.40 mM). The rates of [¹⁴C]palmitoylcarnitine formation were linear for 40 min. CPT-2 assays were carried out separately in a similar manner, except the mitochondrial membranes of the cultured cells were permeabilized with 0.16% Triton X-100 to inactivate CPT-1 activity and to provide latent CPT-2 activity. The permeabilized medium was removed from each well and added to a solution (0.5 mL) containing 10 mM HEPES (pH 7.0) and 1% BSA. The final concentration of palmitoyl-CoA was 75 mM. Increasing concentrations of L-[¹⁴C]carnitine (specific activity = 2000–3000 dpm/nmol) were then added to a series of cell cultures (0.08–0.40 mM final concentration) to initiate the reaction. The initial rates, which were linear, were used to determine CPT-2 activity. The

K_i values of **2** for CPT-1 or CPT-2 were determined by adding inhibitor to a final concentration of 1 mM in each well. After gentle shaking at 37°C for 20 min, the reaction was quenched with butanol-saturated 0.73 M HCl. The amount of radioactive product in the butanol extract was determined by scintillation counting.

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