(+)-Hemipalmitoylcarnitinium Strongly Inhibits Carnitine Palmitoyltransferase-I in Intact Mitochondria

Richard D. Gandour,*,† On-tai Leung,†,‡ Anthony T. Greway,§ Rona R. Ramsay,⊥ Nóirín Nic a' Bháird,⊥ Frank R. Fronczek,† Bret M. Bellard,†,∥ and G. Kumaravel†

Department of Chemistry, Louisiana State University, Baton Rouge, Louisiana 70803-1804, Department of Pharmacology, Cardiovascular Research, Hoffmann-La Roche, Inc., 340 Kingsland Street, Nutley, New Jersey 07110-1199, Department of Veterans Affairs Medical Center, 4150 Clement Street, San Francisco, California 94121, and Department of Biochemistry/Biophysics, University of California, San Francisco, California 94143

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The reaction of the methyl ester of (*R*)-norcarnitine with 1-bromo-2-heptadecanone produces (+)-6-[(methoxycarbonyl)methyl]-2-pentadecyl-4,4-dimethylmorpholinium bromide, **3**, which hydrolyzes to (+)-6-(carboxylatomethyl)-2-pentadecyl-4,4-dimethylmorpholinium (hemipalmitoylcarnitinium, HPC) upon treatment with aqueous sodium hydroxide. Single-crystal X-ray analyses have confirmed the structures of (+)-HPC and **3**. (+)-HPC inhibits carnitine palmitoyltransferase (CPT-I) activity for the forward reaction (palmitoyl-CoA + carnitine →) in intact mitochondria from rat heart and rat liver. (+)-HPC competitively (versus carnitine) inhibits CPT-I activity in both rat heart and liver mitochondria with K_i = 2.8 ± 0.5 and 4.2 ± 0.7 μM, respectively. As one of the strongest specific inhibitors of CPT-I, HPC is a potential therapeutic agent in myocardial ischemia and Type II diabetes.

Introduction

Inhibitors of carnitine palmitoyltransferase (CPT) alleviate symptoms in two pathologies—myocardial ischemia[1] and Type II diabetes.[2] During the early stages of myocardial ischemia, deposits of long-chain acylcarnitines accumulate in the sarcolemmal membrane. These deposits may contribute to the irreversible necrosis of the affected myocardial tissue. CPT inhibitors would reduce the amount of infarcted tissue in the ischemic area by lowering the concentration of acylcarnitines. CPT inhibitors, e.g., the coenzyme A (CoA) esters of 2-tetradecyloxiranecarboxylate[3] (TDG-CoA) and ethyl-2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate[4] (etoxomir-CoA), do protect ischemic myocardium from reperfusion injury and increase mechanical function in hearts from diabetic animals. Etomoxir-CoA also improves heart function in rats with cardiac hypertrophy induced by pressure overload.[5]

CPT inhibitors also may prove helpful in Type II diabetes as oral hypoglycemic agents, which remain a target of medicinal chemists.[6] TDG-CoA[7] and etomoxir-CoA[8] show hypoglycemic effects in diabetic animals. Insulin deficiency leads to rapid mobilization of fatty acids from adipose tissue, which in turn increases ketone production in the liver. Malonyl-CoA (the acknowledged physiological inhibitor) regulates CPT,[9] but insulin deficiency leads to lower concentrations of malonyl-CoA. CPT limits the rate of ketogenesis, and thus, exogenous CPT inhibitors are anti-ketotics in Type II diabetes. Figure 1 shows a potential mechanism for the effect of CPT inhibitors on fatty acid oxidation.

Fatty acid oxidation in mitochondria requires two CPT activities. CPT-I, located on the outer membrane, catalyzes acyl transfer from acyl-CoA to carnitine. Acylcarnitine translocase (ATL) transports acylcarnitines across the inner mitochondrial membrane. CPT-II, located in this membrane, catalyzes the reverse reaction to give acyl-CoA, the feedstock of fatty acid oxidation. Although questions remain about the similarities of these two enzymes, all agree that they perform separate activities.[10] Malonyl-CoA regulates the activity of CPT-I, but not that of CPT-II. Because the purification of CPT-I is difficult, most workers use intact mitochondria for their assays. CPT-II is isolated by treating mitochondria with detergents.

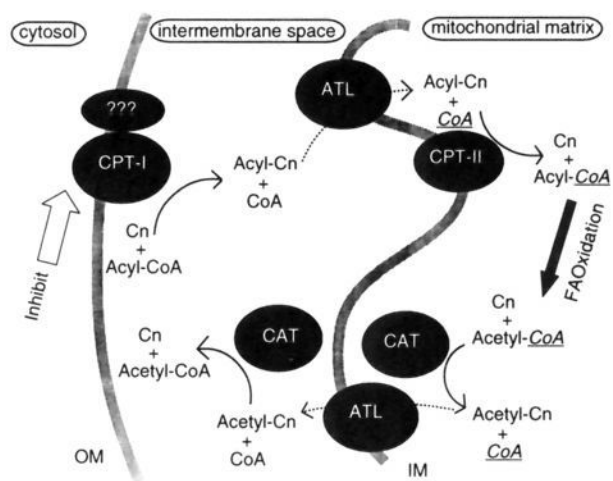

Figure 1. Schematic representation of the inhibition of carnitine-dependent import of acyl groups into mitochondria. Cn, carnitine; CoA, cytosolic coenzyme A; *CoA*, mitochondrial coenzyme A; FA, fatty acid; CPT-I, outer CPT; ???, regulatory subunit or not?; CPT-II, inner CPT; ATL, translocase; OM, outer membrane; IM, inner membrane.

(±)-Hemipalmitoylcarnitinium (HPC), **1**, inhibits rat liver mitochondrial CPT-II.[11] HPC belongs to a class of carnitine acyltransferase inhibitors that we call hemiacylcarnitiniums.[12] These conformationally constrained analogues resemble the tetrahedral intermediate **2** that we have proposed for carnitine acetyltransferase catalysis and that we presume occurs in CPT catalysis. Substituent

† Louisiana State University.
‡ Present address: University of Toronto, Toronto, ON, Canada.
§ Hoffmann-La Roche.
⊥ University of California.
∥ Present address: Baylor School of Medicine, Houston, TX.

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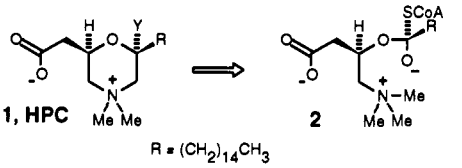
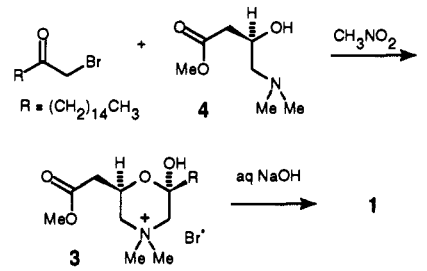


Y is a possible location for coenzyme A. Hemiacylcarnitiniums (Y = OH) are a prototype for our design of a bisubstrate reaction-intermediate analogue inhibitor. The carboxymethyl and the alkyl chain are cis diequatorial in the most stable conformation of this six-membered ring.

Since our report of inhibition of CPT-II, we have made improvements in the synthesis and purification of HPC that yielded sufficient quantities to enable us to further evaluate its therapeutic potential. Toward this end, we have prepared (+)-HPC with the *R* configuration at the carbon that mimics the chiral carbon in (*R*)-carnitine. The improvements in synthesis, the X-ray structural analysis of HPC, and the evaluations of inhibition of CPT-I in intact mitochondria from both rat heart and liver prompt us to describe these latest results.

Chemistry

Our new synthesis of HPC improves upon our earlier method.[11] The reaction of 1-bromoheptadecan-2-one and (*R*)-norcarnitine methyl ester, **4**, which was prepared by



refluxing (*R*)-norcarnitine[13] and trimethyl orthoformate,[14] proceeds more cleanly at 50 °C in nitromethane than in refluxing tetrahydrofuran.[15] We isolate the crystalline product **3** in 78% yield. The highly favored ring closure only gives an axial hydroxyl. We only detect the hemiketal form in NMR and IR spectra. One equivalent of sodium hydroxide hydrolyzes the methyl ester to give the zwitterion. Single crystal X-ray analyses have confirmed both structures.

Figures 2 and 3 present the structures of HPC and the methyl ester **3**. HPC crystallizes as the monohydrate and with two independent molecules in the unit cell. The most notable difference between the two molecules is the gauche versus anti conformation of the methyl group at the end of the alkyl chain. Compound **3** crystallizes with the long-chain alkyl fully extended. The structures of **3** and HPC differ with respect to the conformation of the carboxymethyl side chain on the morpholinium rings. In **3**, the carboxyl carbon is gauche to the oxygen of the morpholinium, but in HPC (both molecules), it is anti. Conformational studies on this torsion angle in carnitine and acetylcarnitine[16] and crystal structural studies of hemiacylcarnitiniums[12] reveal little preference for gauche or anti. The structural parameters for the morpholinium ring in **3** and HPC resemble those of other hemiacylcarnitiniums.[12]

Biological Evaluation

We have evaluated (+)-HPC as an inhibitor of CPT-I activity for the forward reaction (palmitoyl-CoA + carnitine →) in intact mitochondria from both rat heart and liver. Double-reciprocal plots (Figures 4 and 5) of (+)-HPC demonstrate simple competitive inhibition (versus carnitine) of CPT-I activity in both rat heart and liver mitochondria. (+)-HPC also inhibits CPT-I activity in rat liver mitochondria under the same assay conditions that were used for TDG-CoA and etomoxir-CoA.[17,18] Table I lists the inhibition constants, which are 100-fold better than the K_m of (*R*)-carnitine.

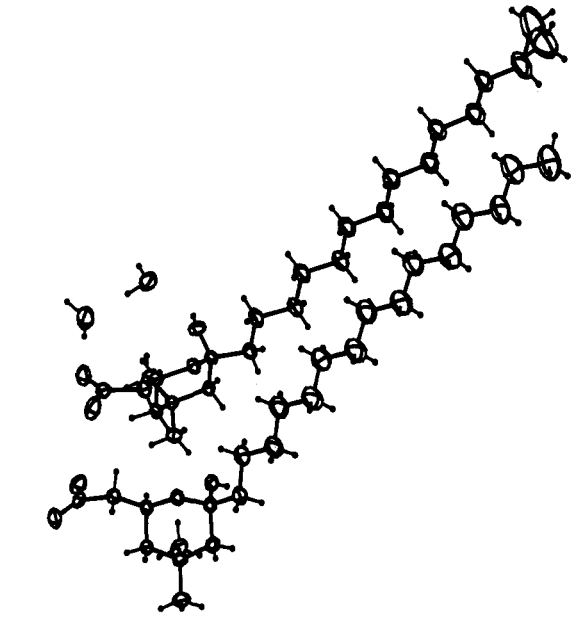


Figure 2. ORTEP drawing of the crystal structure of HPC.

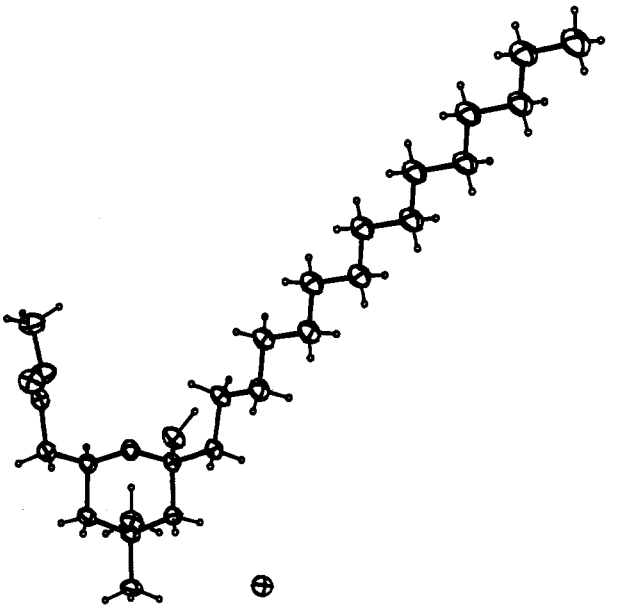


Figure 3. ORTEP drawing of the crystal structure of 3.

Discussion

Inhibition by HPC. HPC competitively inhibits CPT-I activity, which means that HPC prevents carnitine from binding at the catalytic center of CPT-I when palmitoyl-CoA is saturating the enzyme. This suggests that the catalytic center can accommodate both the long chain on palmitoyl-CoA and on HPC. With both rat heart and liver mitochondria, the inhibition patterns and constants of HPC on CPT-I activities are similar.

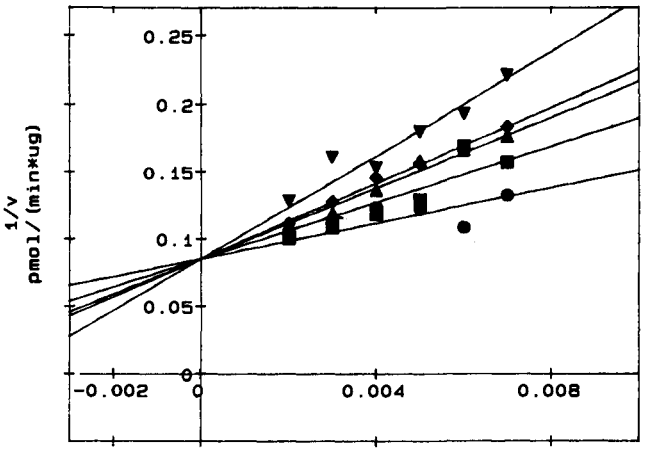


Figure 4. Double-reciprocal plot of inhibition of CPT-I with HPC versus carnitine in rat heart mitochondria. [HPC] = 0.0 (●), 0.7 (■), 2.0 (▲), 3.0 (♦), and 5.0 (▼) μM.

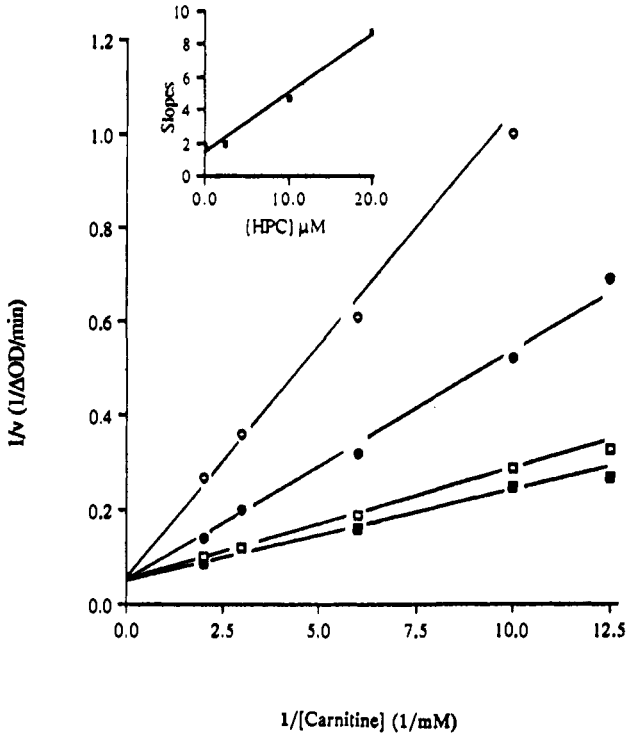
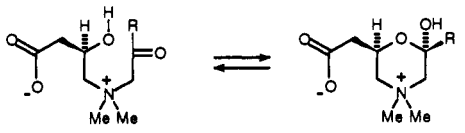


Figure 5. Double-reciprocal plot of inhibition of CPT-I with HPC versus carnitine in rat liver mitochondria. [HPC] = 0.0 (■), 2.5 (□), 10 (●) and 20 (○) μM. (Inset) Plots of slopes versus [HPC].

Table I. Inhibition Constants of (+)-HPC for CPT-I Activity in Intact Mitochondria from Various Sources

source of mitochondria	substrate	K_m (μM)	inhibition constant, K_i (μM)
rat heart	(*R*)-carnitine	500	2.8 ± 0.5
rat liver	(*R*)-carnitine	350 ± 30 (mean ± SD, *n* = 2)	4.2 ± 0.7 (mean ± SD, *n* = 2)

HPC presumably inhibits as a cyclic structure, but the evidence is circumstantial. A small fraction of the



compound in solution might be in the open form, but none is seen by NMR. The NMR spectrum of HPC further suggests that the six-membered ring strongly favors the conformation seen in the crystal. Axial and equatorial signals are resolved. We cannot completely exclude selective binding of the open form, which might react with CoASH or a thiol of a cysteine in the catalytic center to form a hemithioketal.

Comparison with Other Inhibitors. Most effective CPT-I inhibitors have a long-chain alkyl group attached to either CoA or to a carnitine analogue. Malonyl-CoA is a reversible inhibitor, but does not show competitive inhibition. In contrast, TDG-CoA, etomoxir-CoA, and 2-bromopalmitoyl-CoA are irreversible inhibitors of CPT-I in intact mitochondria[17,18] and, presumably, form a covalent bond to some functional group in the catalytic center. Carnitine analogues, racemic palmitoyl- and decanoylaminocarnitine, inhibit fatty acid oxidation in mice,[19] and (*R*)-palmitoyl- and (*R*)-myristoylaminocarnitine inhibit fatty acid oxidation in intact rat liver mitochondria.[20] Presumably, the site of inhibition is CPT-I, but (*R*)-aminocarnitine more strongly inhibits CPT-II than CPT-I.[21] HPC inhibits better than (*R*)-aminocarnitine, as well as malonyl-CoA, but significantly less than etomoxir-CoA.

HPC reversibly and competitively inhibits both CPT-I and CPT-II with equal potency. (±)-HPC inhibits CPT-II with an apparent K_i of 5.1 ± 0.7 μM.[11] In contrast, etomoxir-CoA, TDG-CoA, and 2-bromopalmitoyl-CoA irreversibly inhibit CPT-I,[17] but reversibly inhibit CPT-II much less effectively than HPC. Malonyl-CoA inhibits CPT-I, but not CPT-II.[9] That HPC *competitively* inhibits both CPT activities argues favorably for interactions at the catalytic centers of both proteins. Although not surprising, the data demonstrate that HPC is as effective an inhibitor in intact mitochondria as it is in a purified protein. This specificity will make HPC useful for analyzing CPT activity, exploring topography of various CPT enzymes, and characterizing new CPTs.

Summary and Questions. As one of the strongest specific inhibitors of CPT-I, HPC is a potential therapeutic agent in myocardial ischemia and Type II diabetes. Questions remain as to the behavior of HPC in mitochondria. Does HPC interfere with transport across the mitochondrial membrane? Does HPC gain access to CPT-II in intact mitochondria? For therapeutic effects, the crucial question is how much HPC crosses the cell membrane via diffusion and via transport by the carnitine receptor? Studies are in progress to answer these and other questions regarding the therapeutic potential of HPC. Nevertheless, even if HPC does not have therapeutic value, it can help clarify the physiological role of CPT in fatty acid oxidation.

Experimental Section

General. Hexane was distilled from CaH_2; Et_2O, from Na. Nitromethane and MeOH were distilled and stored over 4-Å sieves. *i*-PrOH was distilled from CaO and stored over 5-Å sieves. Reagent-grade acetone, CH_2Cl_2, $CHCl_3$, CH_3CN, palmitoyl chloride, and trimethyl orthoformate, and absolute EtOH were used as received. Sodium (*R*)-norcarnitine was prepared by the demethylation[13] of (*R*)-carnitine (Sigma Tau) and recrystallized from hot *i*-PrOH. FAB MS samples were suspended in glycerol. Oneida Research Services of Whitesboro, NY, did the elemental analyses. J_{app} refers to peak separations in ^1H NMR spectra. The optical rotations were recorded in a 10 × 100 mm cell. Concentration of solutions were done by rotary evaporation.

1-Diazo-2-heptadecanone. To a solution of CH_2N_2, prepared by distilling from a mixture of Diazald (10.5 g) and KOH (6 g)

in Et_2O/EtOH (aq),[22] in Et_2O (75 mL) at 0 °C was added a solution of palmitoyl chloride (2.5 g, 9.09 mmol) in Et_2O (50 mL) in portions over 15 min with stirring. After addition, the solution was continuously stirred at 0 °C for another 15 min. Concentration gave a yellow solid, which was recrystallized from CH_2Cl_2/EtOH (ca. 2:1 v/v) at 20 °C as pale yellow plates (1.70 g). Concentration of the filtrate gave more crystals for a total weight of 2.0 g (79% yield): mp 61–62 °C; ^{1}H NMR (100 MHz, $CDCl_3$, TMS) δ 0.88 (3 H, t, J_{app} = 6.0 Hz, CH_3), 0.90–1.70 (26 H, m, $CH_3(CH_2)_{13}CH_2$), 2.33 (2 H, t, J_{app} = 7.2 Hz, CH_2CO), 5.20 (2 H, s, $COCH_2Br$); IR (film) ν_{max} 2122, 2100, 1621 cm^{-1}.

1-Bromo-2-heptadecanone. To a yellow solution of 1-diazo-2-heptadecanone (2.70 g, 9.64 mmol) in CH_2Cl_2 (50 mL) at 0 °C was added a solution of Et_2O saturated with HBr at 0 °C slowly until no air bubbles appeared. The resulting pale yellow solution was stirred for 5 min at 0 °C and then concentrated. The residue was recrystallized from hexane as plates (2.86 g). Concentration of the mother liquid gave more crystals for a total weight of 2.96 g (92% yield): mp 64.5–65.5 °C (lit.[23] mp 64–65 °C); ^{1}H NMR (100 MHz, $CDCl_3$, TMS) δ 0.75–1.75 (26 H, m, $CH_3(CH_2)_{13}CH_2$-CO), 2.65 (2 H, t, J_{app} = 7.1 Hz, CH_2CO), 3.89 ($COCH_2Br$); IR (film) ν_{max} 1719 (lit.[23] ν_{max} 1725 cm^{-1}); MS m/z (EI) 334 (M (with Br^{81})$^+$, 0.6), 332 (M (with Br^{79})$^+$, 0.6), 239 (M – CH_2Br^+, 54), 43 (100).

Methyl (*R*)-4-(Dimethylamino)-3-hydroxybutanoate, 4. At room temperature, HCl (about 10 mL/min) was bubbled for 0.5–1.0 h into a solution of sodium (*R*)-norcarnitine (19.86 g, 0.117 mol, 1 equiv) in MeOH (350 mL) and trimethyl orthoformate (30 mL, 30.93 g, 0.291 mol, 2.5 equiv). Then, the solution was refluxed for 16–20 h. Filtration of the resulting precipitate and concentration of the filtrate gave an oily residue, which was dissolved in H_2O (30 mL). The pH was adjusted to 10.5 with 1 M NaOH, and the aqueous solution was extracted with $CHCl_3$ (3 × 30 mL). The combined organic layers were washed with brine (5–10 mL), dried (Na_2SO_4), and concentrated to give an orange oil (15.05 g). High-vacuum distillation gave a colorless oil (10.91 g, 58% yield): bp 73–76 °C (0.3 Torr); ^{1}H NMR (400 MHz, D_2O, TSP) δ 2.26 (6 H, s, $N(CH_3)_2$), 2.30–2.70 (4 H, m, $CH_2CHOHCH_2$), 3.72 (3 H, s, CO_2CH_3), 4.15–4.25 (1 H, m, $CH_2CHOHCH_2$); IR (film) ν_{max} 3441 and 1737 cm^{-1}; ^{13}C NMR (100 MHz, $CHCl_3$, TMS) δ 39.01 ($CH_2CO_2CH_3$), 42.54 and 44.47 ($N(CH_3)_2$), 51.45 (CO_2CH_3), 61.68 ($(CH_3)_2NCH_2$), 62.09 (CHOH), 170.48 (CO_2CH_3); MS m/z (EI) 161 (M^+, 3), 88 ($Me_2NCH_2CHOH^+$, 11), 58 ($Me_2NCH_2^+$, 100), $[\alpha]^{25}_D$ –5.50° (c = 1.026 mg/mL, H_2O). Anal. ($C_7H_{15}NO_3$) C, H, N.

(+)-6-[(Methoxycarbonyl)methyl]-2-pentadecyl-4,4-dimethylmorpholinium Bromide, 3. A suspension of the 1-bromo-2-heptadecanone (2.50 g, 7.51 mmol) and 4 (1.829 g, 7.51 mmol) in CH_3NO_2 (30 mL) was stirred as 50–55 °C for 3 h, after which time, the suspension dissolved. The resulting orange solution was cooled to room temperature and concentrated to give an orange oil, which was redissolved in hot CH_2Cl_2 (30 mL), treated with charcoal, and filtered through Celite. The filtrate was concentrated and the residue was solidified from EtOAc to give white solids (1.66 g). Concentration of the mother liquid gave more solids for a total weight of 3.37 g (78% yield); mp 114–116 °C. Recrystallization from CH_2Cl_2 gave a crystal for X-ray analysis: ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 0.88 (3 H, t, J_{app} = 6.8 Hz, $(CH_2)_{13}CH_3$), 0.90–1.50 (26 H, m, $(CH_2)_{13}CH_3$), 1.6–1.85 (2 H, m, $C(OH)CH_2(CH_2)_{13}CH_3$), 2.68, 2.69 (2 H, dd, J_{app} = 6.1 Hz, $CH_2CO_2CH_3$), 3.22 (1 H, d, J_{app} = 12.9 Hz, $C(OH)$-$CH_2(ax)N^+(CH_3)_2$), 3.34 (1 H, dd, J_{app} = 12.9 Hz, 12.9 Hz, $CHCH_2$-$(ax)N^+(CH_3)_2$), 3.51 (3 H, s, NCH_3 (eq)), 3.72 (3 H, s, N^+CH_3 (ax)), 3.77 (3 H, s, CO_2CH_3), 4.02 (2 H, d, J_{app} = 12.9 Hz, CH_2-$(eq)N^+(CH_3)_2CH_2(eq)$), 4.50 (1 H, s, OH), 4.80–4.85 (1 H, m, $OCHCH_2N$); ^{13}C NMR (100 MHz, $CDCl_3$, TMS) δ 13.95 (CH_2CH_3), 22.33, 22.52, 29.20, 29.46, 29.57, 31.77 ($(CH_2)_{13}CH_3$), 36.71 (CH_2-CO_2Me), 40.40 ($C(OH)CH_2(CH_2)_{13}CH_3$), 51.87 (CO_2CH_3), 52.06 (NCH_3 (ax)), 58.68 (NCH_3 (eq)), 60.88 ($OCHCH_2N$), 63.45 ($OCHCH_2N$), 64.56 (OCH_2OHCH_2N), 95.38 ($OC(OH)CH_2N$), 170.16 (CO_2CH_3); IR (film) ν_{max} 3428, 1735, 1148 cm^{-1}; FAB MS m/z, 414 (M^+, 100), 43 (M – 317, 8), $[\alpha]^{25}_D$ +19.0° (c = 1 mg/mL, MeOH). Anal. ($C_{24}H_{48}BrNO_4$) C, H, N.

(+)-6-(Carboxylatomethyl)-2-pentadecyl-4,4-dimethylmorpholinium, 1. A solution of 3 (1.5 g, 3.036 mmol, 1 equiv) in 0.1 M NaOH (30.36 mL, 3.036 mmol, 1 equiv) was stirred at room temperature for 14 h. Concentration gave an orange oil, which was eluted in 20 portions through a reversed-phase HPLC column[24] with MeOH–H_2O (9:1 v/v) containing 0.2 M $Et_3NH^+AcO^-$ buffer. The retention time of the desired product was 7.9 min. Concentration gave a yellow oil, which upon trituration with CH_3CN gave a white solid. Recrystallization from MeOH–CH_3CN (~1:9 v/v) gave solid (0.59 g). Concentration of the filtrate gave more solid (0.1 g) for a total weight of 0.69 g (57% yield): mp 153–155 °C dec; RPTLC[25] R_f = 0.20–0.40 (MeOH–H_2O, 9:1 v/v); ^{1}H NMR (400 MHz, D_2O, TSP) δ 0.74 (3 H, t, J_{app} = 6 Hz, $(CH_2)_{14}CH_3$), 0.7–1.5 (28 H, m, $C(OH)(CH_2)_{14}CH_3$), 2.26, 2.37 (2 H, dd, J_{app} = 6.7, 14.5 Hz, $CH_2CO_2^-$), 2.97 (2 H, dd, J_{app} = 12.1, 11.9 Hz, $CH_2(ax)NCH_2(ax)$), 3.11 (3 H, s, $N^+CH_3(eq)$), 3.37 (3 H, s, $N^+(CH_3)_2(ax)$), 3.53 (2 H, d, J_{app} = 11.9 Hz, CH_2-$(eq)NCH_2(eq)$), 4.60–4.67 (1 H, m, $OCHCH_2N$); ^{13}C NMR (100 MHz, D_2O, TSP) δ 16.72 ($(CH_2)_{13}CH_3$), 25.05, 25.54, 32.50, 33.07, 34.93 ($(CH_2)_{13}CH_3$), 43.56 ($C(OH)CH_2(CH_2)_{13}$), 54.37 ($N^+CH_3(ax)$), 61.11 ($N^+CH_3(eq)$), 65.08 ($OCHCH_2CO_2^-$), 97.88 ($OC(OH)CH_2N^+$-$(CH_3)_2$), 79.21 ($CH_2CO_2^-$); IR (KBr) ν_{max} 3418, 1586, 1397, 1070 cm^{-1}; FAB MS m/z 400 (M + 1, 25), 84 (M – 316, 15), 58 (M – 342, 100); $[\alpha]^{25}_D$ +14.4° (c = 0.169/10 mL, MeOH). Anal. ($C_{23}H_{45}$-$NO_4 \cdot H_2O$) C, H, N.

Single Crystal X-ray Analyses. Table II contains the crystallographic data for HPC·H_2O and 3. X-ray data were collected on an Enraf-Nonius CAD4 diffractometer equipped with CuKα radiation (λ = 1.54184 Å) and a graphite monochromator, employing $\omega - 2\theta$ scans. Data reduction included corrections for background, Lorentz, polarization, and absorption by psi scans. Structures were solved by direct methods using programs MULTAN[26] for HPC·H_2O and SHELXS86[27] for 3. Refinement was carried out by full-matrix least squares based on F with weights $w = \sigma^{-2}(F_o)$, treating nonhydrogen atoms anisotropically. Hydrogen atoms on OH and H_2O were placed from difference maps and refined isotropically. Hydrogens on carbon were included as fixed contributions in calculated positions.

The absolute configuration of 3 was determined by refinement of the inversion-related structure, which yielded R = 0.030, R_w = 0.042, and GOF = 2.331.

Preparation of Rat Heart Mitochondria. Male Sprague–Dawley rats were anesthetized with $CHCl_3$ and their hearts and livers rapidly excised. Mitochondria were prepared essentially as described for those from rat liver[28] using a mannitol–sucrose–MOPS (220:70:5 mM, pH 7.4) homogenizing buffer. All manipulations of the tissue and mitochondria were done between 0 and 4 °C. The organs were rinsed with cold homogenizing

Table II. Crystallographic Data

	HPC·H_2O	3
formula	$C_{23}H_{47}NO_5$	$C_{24}H_{48}NO_4Br$
FW	417.6	494.6
cryst system	monoclinic	monoclinic
space group	$P2_1$	$P2_1$
a (Å)	11.504 (2)	5.6549 (4)
b (Å)	8.760 (4)	7.5877 (6)
c (Å)	25.586 (3)	31.459 (3)
β (deg)	97.89 (1)	94.628 (8)
V (Å^3)	2554 (2)	1345.4 (3)
Z	4	2
D (g cm^{-3})	1.086	1.221
T (°C)	21	24
μ (cm^{-1})	5.63	22.85
transmission coeff. (%)	90.7–99.9	80.0–96.6
cryst size, mm	0.04 × 0.25 × 0.57	0.10 × 0.15 × 0.45
θ limits	2–75	2–75
unique data	5597	2811
observed data	4005	2733
variables	547	274
R (obs data)	0.050	0.027
R_w	0.056	0.038
R (all data)	0.082	0.028
max residual (e Å^{-3})	0.27	0.31
min residual	–0.05	–0.44
color	colorless lath	colorless needle
extinction	9.6 (6) × 10^{-7}	2.13 (11) × 10^{-6}
GOF	2.161	2.108

buffer and diced with scissors. Homogenization of the tissue was performed with four passes of a tight-fitting Teflon pestle in a Potter–Elvejham tissue disrupter. The disrupted tissue was centrifuged at 700*g* in a HG-4L rotor (Sorvall centrifuge) for 10 min to sediment cellular debris. The supernatant was centrifuged at 9000*g* at 4 °C for 12 min to pellet the mitochondria. The mitochondria were resuspended with a hand homogenizer and the low-speed contrifugation repeated to pellet red blood cells and other macroscopic debris. The mitochondria were repelleted and washed twice by resuspension and centrifugation at the higher speed. Mitochondria at a final protein concentration of 30 mg/mL were kept at 0–4 °C.

Rat Heart Enzyme Assay. Activity of CPT-I was assayed in the direction of [^{3}H]-(*R*)-palmitoylcarnitine formation.[9] Substrates, assay buffer (20 mM Tris-HCl, 100 mM KCl, 500 mM sucrose, pH 7.2), and [^{3}H]-(*R*)-carnitine (~0.5 μCi) were deposited in 12 × 75 mm test tubes and equilibrated to 30 °C. The reactions, in a total volume of 200 μL, were initiated by adding mitochondria (~30–50 μg). The concentration of carnitine was varied from 100 to 1000 μM and that of palmitoyl-CoA was 50 μM. The inhibitor was added in a concentration range to bracket the K_i; preincubation with the enzyme was not necessary to achieve inhibition. After 3–6 min, the reactions were quenched by the adding 1 mL of H_2O-saturated BuOH. The test tubes were vortexed for 15 s and centrifuged at 300*g* in a GLC-2B (Sorvall) centrifuge to separate the layers. Seven hundred fifty microliters of the upper (BuOH) layer were removed and added to 250 μL of BuOH-saturated H_2O in mini Eppendorf tubes. The solutions were vortexed and centrifuged to separate the layers. Five hundred microliters of the upper layer were removed and added to 10 mL of scintillation cocktail (Aquasol-2, NEN). Incorporation of tritium into the organic phase as [^{3}H]-(*R*)-palmitoylcarnitine was determined. Both the time of incubation of the enzyme with the substrates and the concentration of mitochondria used in the assay were frequently verified to be in the linear range of activity. Inhibition and kinetic constants were determined from eq 1 and analyzed with a BASIC translation[29] of Cleland's COMP program.[30]

$$v = \frac{V[S]}{K_m\left(1 + \frac{[I]}{K_i}\right) + [S]} \quad (1)$$

Rat Liver Enzyme Assay. Mitochondria were prepared by differential centrifugation and gradient purification as described.[31] CPT-I activity in intact mitochondria was assayed essentially as described.[31] The standard assay contained the following in a final volume of 100 μL: 10 mM Tris HCl, pH 7.4, 220 mM sucrose, 40 mM KCl, 1 mM EGTA, 1.3 mg/mL fatty acid-free BSA, 100 μM palmitoyl-CoA, [^{3}H]-(*R*)-carnitine (10 Ci/mol), and 10 μg mitochondrial protein. HPC was added as appropriate. Assays were performed in 1.5-mL centrifuge tubes at 30 °C. The reaction was started by adding mitochondrial protein and stopped by adding 150 μL ice-cold 1 M HCl. The product, [^{3}H]-(*R*)-palmitoylcarnitine, was extracted into water-saturated BuOH for scintillation counting.[32]

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Supplementary Material Available: X-ray crystallographic data (atomic coordinates, anisotropic displacement coefficients, H-atom coordinates, bond lengths, and bond angles) for HPC·H_2O and **3** (15 pages). Ordering information is given on any current masthead.

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