Comparison of (+)- and (–)-Hemipalmitoylcarnitinium as Inhibitors of Hepatic Mitochondrial Carnitine Palmitoyltransferases in Diabetic Rats

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Abstract: The syntheses of (*R*)- and (*S*)-norcarnitine ethyl esters are described starting with an optimized, chiral chemical reduction of ethyl 4-chloroacetoacetate followed by azide substitution, reduction, and dimethylation. The reaction of (*R*)- and (*S*)-norcarnitine ethyl esters with 1-bromoheptadecan-2-one gives (+)- and (–)-6-[(methoxycarbonyl)methyl]-2-pentadecyl-4,4-dimethylmorpholinium bromide, respectively, which hydrolyzes to (+)- and (–)-6-(carboxylatomethyl)-2-pentadecyl-4,4-dimethylmorpholinium (hemipalmitoylcarnitinium, **(+)-** and **(–)-HPC**), respectively, upon treatment with a hydroxide resin. **(+)-** and **(–)-HPC** are reversible active-site directed inhibitors of hepatic mitochondrial CPTs. Both stereoisomers inhibit CPT I and CPT II in control and streptozotocin diabetic rat to the same extent (I_{max} = 100%). Using intact mitochondria (CPT I), I_{50} values for **(–)-HPC** and **(+)-HPC** were 15.5 μM and 47.5 μM, respectively. The I_{50} values for CPT II were 6.7 μM and 38.5 μM for **(–)-HPC** and **(+)-HPC**, respectively. The mode of inhibition was uncompetitive for CPT I with respect to acyl-CoA. The apparent K_i for **(–)-HPC** is about 5 μM. These data suggest that **(–)-HPC** may be useful for further evaluation as an antidiabetic agent.

Key Words: carnitine palmitoyltransferase, antidiabetic agent, chiral synthesis, enzyme inhibition.

INTRODUCTION

Carnitine palmitoyltransferase (CPT; EC 2.3.1.21) is an important regulatory enzyme in the hepatic fatty acid oxidative (FAO) pathway. The regulatory properties of this enzyme are derived in part from the ability of malonyl-CoA, an intermediate in the fatty acid biosynthetic pathway, to inhibit this enzyme at concentrations that exist in the liver [1] and in part as a result of changes in the sensitivity of the enzyme to inhibition by malonyl-CoA (i.e., the K_i of CPT for malonyl-CoA) that occur during transitions in physiological and pathophysiological states [2–7]. The most significant change in CPT that occurs during fasting or with the onset of diabetes is a greater than 10-fold increase in the apparent K_i of CPT for malonyl-CoA, with an increase in the activity of the enzyme in both of these states [7,8].

Two CPT activities are expressed in the mitochondria, one associated with the inner aspect of the mitochondrial inner membrane (CPT-II) and the other with the mitochondrial outer membrane (CPT-I) [9]. Controlling the FAO pathway is reported to regulate blood-glucose levels and ameliorate some symptoms of type II (non-insulin-dependent) diabetes mellitus (NIDDM), a condition that accounts for about 90% of diabetic cases. For example, sulfonylureas that are used in treating type II diabetes can decrease FAO, in part, by inhibiting CPT-I [10]. Therefore, formulating an effective NIDDM drug that selectively targets liver mitochondrial CPTs compared to those in heart mitochondria is a rationale for possible treatment of this condition. However, Anderson [11] has struck a cautionary note with his account of the development of CPT-I inhibitors as drugs for NIDDM. Bebernitz and Schuster [12] have recently reviewed FAO and its utilization; they point out that liver CPT inhibitors can be part of a broader strategy that might include muscle CPT activators and CoA sequestering agents. Recently, Gianessi [13] has presented a more promising picture for selective CPT inhibitors in his comprehensive review, specially with regard to an aminocarnitine derivative, ST-1326.

(+)-Hemipalmitoylcarnitinium (**(+)-HPC**) potently inhibits the activity of CPT-I in intact heart and liver mitochondria, with K_i = 2.8 and 4.2 μM, respectively [14]. It more potently inhibits the activity of purified CPT-II with K_i = 0.16 μM [15]. We have confirmed that this compound is a potent active-site directed inhibitor of CPT-I of outer mitochondrial membranes [16]. The inhibitor **(+)-HPC** contains the natural configuration of L-carnitine and L-palmitoylcarnitine, which has the R absolute configuration. For CPT-I, **(+)-HPC** should be considered as a conformationally constrained substrate (L-palmitoylcarnitine) analog and not a transition-state or reaction-intermediate analog as previously suggested [14]. It can serve as prototype for our design of a bisubstrate reaction-intermediate analog inhibitor, which must include a CoA moiety to complete the analogy.

For racemic drugs, generally one enantiomer is more active. For carnitine acyltransferases, this generalization may not hold. For carnitine acetyltransferase (CAT), the (*R*)-carnitine and (*R*)-acetylcarnitine are substrates while (*S*)-

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carnitine and (*S*)-acetylcarnitine are equally potent competitive inhibitors [17,18]. This is to say that both stereoisomers bind CAT with similar affinities, e.g., K_m for (*R*)-carnitine equals K_i for (*S*)-carnitine. One can interpret this phenomenon as the carboxylato and trimethylammonio groups on both enantiomers occupying the same regions in CAT. The hydroxyl on the R enantiomer is positioned for acetyl transfer from CoA; the hydroxyl on the S enantiomer is not. Consequently, the R enantiomer behaves as a substrate whereas S enantiomer behaves as an inhibitor for the enzyme. The CPT-I appears to discriminate between the two enantiomers of carnitine. For CPT-I and CPT-II, (*S*)-palmitoylcarnitine inhibits weakly [19]; in the pathway for FAO, (*R*)-palmitoylcarnitine is a product for CPT-I and a substrate for CPT-II.

Recent work has shown that CPT-I discriminates among enantiomers of certain active-site directed inhibitors. The acylamidomorpholinium analogs, which are closely related to **(+)-HPC**, show distinct stereoselectivity in inhibiting activities of rat-liver mitochondrial CPT-I and CPT-II. The (2*R*,6*S*)-tetradecanamidomethyl analog activates CPT-I but inhibits CPT-II [20]. For sulfonamidic and sulfamidic aminocarnitine derivatives as inhibitors of the activities of hepatic and heart mitochondrial CPT-I, the R enantiomers are significantly more active than the S enantiomers [21].

A preliminary study dealing with the inhibitory effects of **(–)-HPC** and **(+)-HPC** isomers has been reported previously [22]. A more detailed report including the synthesis of both isomers is presented herein.

RESULTS AND DISCUSSION

Chemistry

Synthesis of HPC

Fig. (**1**) describes our latest synthesis of **(+)-** and **(–)-HPC**. The key step is the condensation of bromoketone **4** with norcarnitine ester **3**. The enantiomers of **3** are readily prepared by chiral reduction of ethyl 4-chloroacetoacetate followed by azide substitution of chloride and reduction of the azide to amine with concurrent dimethylation. As considerable improvements have been made with the objective of scaling up the synthesis for further testing, descriptions of the optimization for each step are provided below.

Step a. Enantiomers, (R)- and (S)-1

Chiral-ruthenium-catalyzed hydrogenation of ethyl 4-chloroacetoacetate [23] served as the starting point for the synthesis. The active catalyst was generated *in situ* by mixing $[RuCl_2(R\text{-BINAP})]_x$ and triethylamine in alcohol. With the H_2 pressure at 60 psi and the temperature at 65 °C, several amines and alcohols were tested with the goal of maximizing the %ee. *tert*-Butyl alcohol gave the best results (>97% ee), producing a 2% higher ee than did ethanol. Other primary and secondary alcohols gave transesterification. Other tertiary alcohols gave similar results to *tert*-butyl alcohol. Diisopropylethylamine gave similar results as triethylamine; other tertiary amines, benzyldimethylamine and *N*-methylmorpholine, gave lower % ee's.

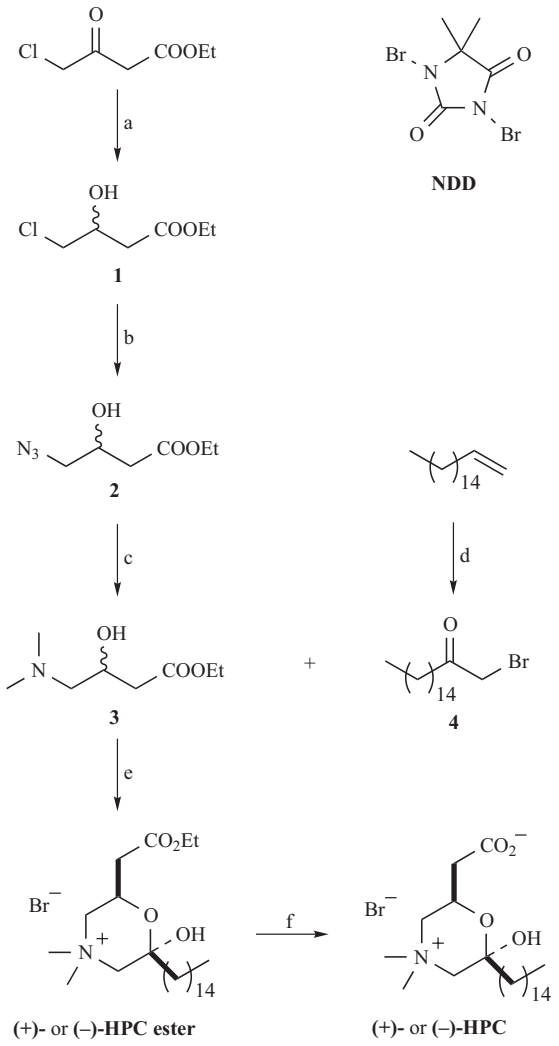

Fig. (1). Synthesis of (+)- or (–)-HPC. Reagents and conditions: (a) $[RuCl_2(S\text{-BINAP})]_x$ or $[RuCl_2(R\text{-BINAP})]_x$, NEt_3, *t*-BuOH, H_2, 60 psi, 68 °C, 48 h; (b) NaN_3, DMF, 105–110 °C, 3.5 h; (c) H_2CO in H_2O, 10% Pd/C, HCOOH, H_2, 70 psi, 60 h; (d) NDD, H_2O:Me_2CO (1:50), 2.3% $FeCl_3 \cdot 6H_2O$, 3h, rt; then, Jones reagent, SiO_2, 24 h, rt; (e) MeCN, 60–65 °C, 24 h; (f) hydroxide resin, 95% EtOH, rt, 18 h.

The %ee's were analyzed by a modification of an HPLC method [24] as the *O*-benzoyl derivatives of **1**. Determining the %ee by this method gave reliable results as we discovered that the specific rotations of **(*R*)-** and **(*S*)-1** in chloroform are very sensitive to concentration whereas in methanol they are not. (Table **1**). Our estimate for $[\alpha]_D^{23}$ is lower than $[\alpha]_D^{20}$ reported earlier [25].

Steps b and c. Enantiomers, (R)- and (S)-3

In hot DMF, azide readily substituted for chloride to produce in good yield the azidohydrin **2** that still contained 2% solvent. Without further purification, **2** was reduced and dimethylated in a hydrogenator to give in excellent yield **3** that still contained 2% DMF. Our experience suggested that this material was suitable for further reactions.

Table 1. Dependence of [α] on Solvent and Concentration

c; $CHCl_3$	$[\alpha]_D^{23}$ °	c; MeOH	$[\alpha]_D^{23}$ °#
0.51	+23.2	1.07	+12.3
1.17	+22.4	1.42	+12.1
2.04	+21.6	2.14	+12.2
3.02	+21.7	3.03	+12.2
4.20	+21.0	4.34	+12.2
5.07	+21.0	5.92	+12.1
6.38	+20.6	7.2	+12.3

$[\alpha]_D^{23}$ = +12.5°, 100% ee.

Step d. Optimization of 1-Bromoalkan-2-one Preparation

In our previous work [26], we slightly modified the procedure of Zav'yalov *et al*. [27]—a. NBS, Me_2CO:H_2O (25:1), 6.3 mol % $FeCl_3 \cdot 6H_2O$; b. silica gel, Jones reagent—by decreasing the amount of water (Me_2CO:H_2O, 50:1) to accommodate the low aqueous solubility of heptadec-1-ene; yields ranged from 30–55% of recrystallized material. In later work [28], we used THF:water (1:1) as a solvent instead because of a higher regioselectivity in the bromohydroxylation step [29]. We achieved higher yields (70%) of material, which was isolated by flash column chromatography and appeared clean enough by ^{1}H NMR for further reactions. This latter method proved difficult to scale-up to multi-gram batches because chromatography was required. Furthermore, this procedure used three-fold more oxidizing agent than needed in acetone:water. Returning the original method, the brominating agent was changed from NBS to *N*,*N*'-dibromo-5,5-dimethylhydantoin (NDD) [30], which is less expensive than NBS and provides two bromine atoms per molecule. *In-situ* Jones oxidation in the presence of silica gel required vigorous stirring to complete the oxidation. The minor isomer in the bromohydroxylation part oxidized to an acid, which absorb to the solids. Compound **4** was readily isolated and purified by recrystallization.

Biology

Inhibition of the Activity of CPT-I by (–)- and (+)-HPC

Both **(–)-** and **(+)-HPC** are excellent inhibitors of the activity of CPT-I in intact hepatic mitochondria; **(–)-HPC** is a much more potent inhibitor compared to **(+)-HPC** (Fig. (**2**)). Both enantiomers inhibit the activity of inner membrane CPT-II better than they inhibit the activity of CPT-I (data not shown). From similar data to that shown in Fig. (**2**), the measured values of I_{50} for **(–)-** and **(+)-HPC** are 15.5 μM and 47.5 μM, respectively for CPT-I and 6.7 μM and 38.5 μM, respectively for CPT-II. These data clearly show that **(–)-HPC** is a more potent inhibitor than **(+)-HPC**. These agents are better inhibitors of CPT-II than CPT-I as we expected from our studies a decade ago [14,15]. The values of I_{50} are considerably larger than the previously reported K_i's. This increase can be attributed to different reaction conditions, which in general can give varying I_{50}'s but constant K_i values.

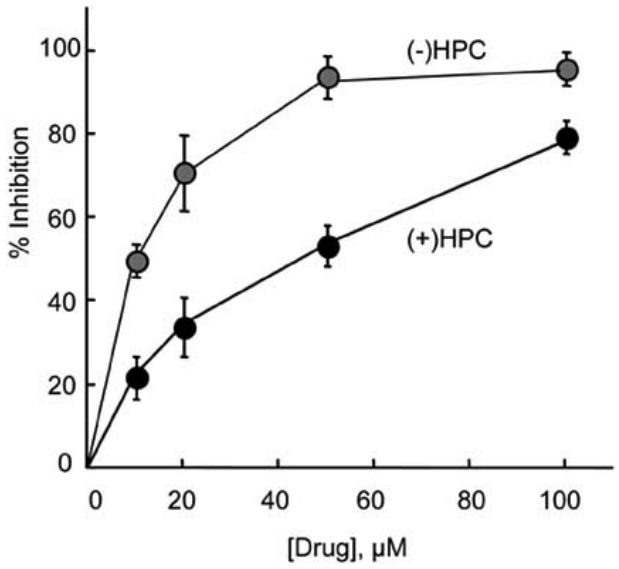

Fig. (2). Inhibition of CPT-I by (–)- and (+)-HPC. Intact rat liver mitochondria were assayed for the outer carnitine palmitoyltransferase activity as described under Materials and Methods for Biology with 40-μM myristoyl-CoA and 0.5-mM carnitine. Results are means ± SEM for three different preparations of mitochondria. Specific activity of the enzyme in the absence of any inhibitor was 8.5 ± 0.5 nmol/min/mg protein.

Binding of Inhibitors at the Active Site

These agents are designed [31] as substrate analogues and are expected to bind at the palmitoylcarnitine binding site of CPT [14,15]. The palmitoyl group should occupy the same binding site as when it is attached to CoA. When intact mitochondria are treated with the protease Nagarse, the inhibitory properties of malonyl-CoA are significantly reduced and the activity of CPT-I is virtually unchanged [9,32,33]. Under similar conditions of protease treatment that reduce malonyl-CoA inhibition, the inhibitory effects of other known inhibitors of CPT-I are not affected [33,34].

These studies clearly suggest that the enzyme has more than one binding domain—one for malonyl-CoA and the other(s) for other inhibitors.

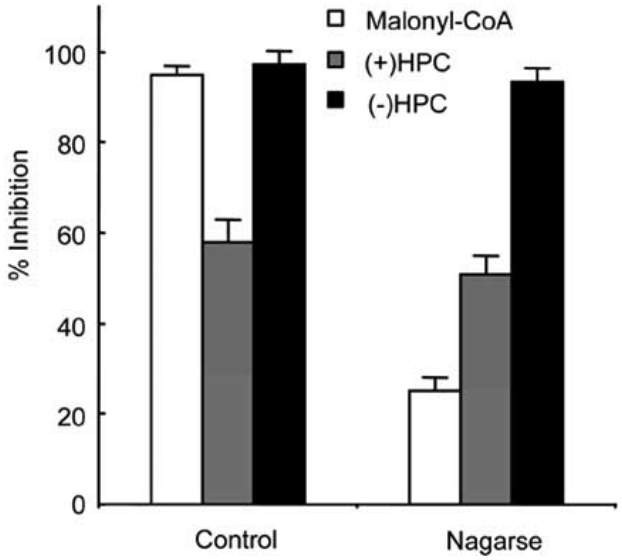

Fig. (3). Inhibition of CPT-I in Control and Nagarse-treated Mitochondria. Intact mitochondria were assayed for the activity of outer CPT in the presence and absence of malonyl-CoA, **(+)-HPC** and **(–)-HPC** as described under Materials and Methods for Biology with 40-μM myristoyl-CoA and 0.5-mM carnitine in control and Nagarse-treated mitochondria. Results are means ± SEM for three different preparations of mitochondria. Specific activity of the enzyme in the absence of any inhibitor was 8.5 ± 0.5 nmol/min/mg protein.

Under the same experimental conditions, **(+)-HPC** is an active site directed inhibitor of CPT-I [16]. Fig. (**3**) shows that pretreatment of intact liver mitochondria with Nagarse significantly reduces inhibition by malonyl-CoA; however, the inhibitory effects of **(+)-HPC** and **(–)-HPC** are not affected. These data strongly suggest that like **(+)-HPC** [16], **(–)-HPC** is also an active-site directed inhibitor of CPT-I and is a much better inhibitor compared to **(+)-HPC**.

Therefore, these agents are excellent inhibitors of CPT-I and CPT-II of rat liver mitochondria and that they act at the active site of the enzyme. But what is their mode of inhibition?

Mode of Inhibition

Fig. (**4**) compares the activity of the rat liver mitochondrial outer membrane CPT in the presence and absence of **(–)-HPC** as a function of myristoyl-CoA concentration. Both V_{max} of CPT and the K_m for myristoyl-CoA were decreased by **(–)-HPC**, suggesting that the type of inhibition is uncompetitive.

This mode of inhibition may be a result of irreversible binding or slow dissociation of the enzyme-inhibitor complex. Therefore, to probe this further we have done a dilution and a wash-out experiment to verify the uncompetitive inhibition. The results of such an experiment are shown in Fig. (**5**). When intact mitochondria were

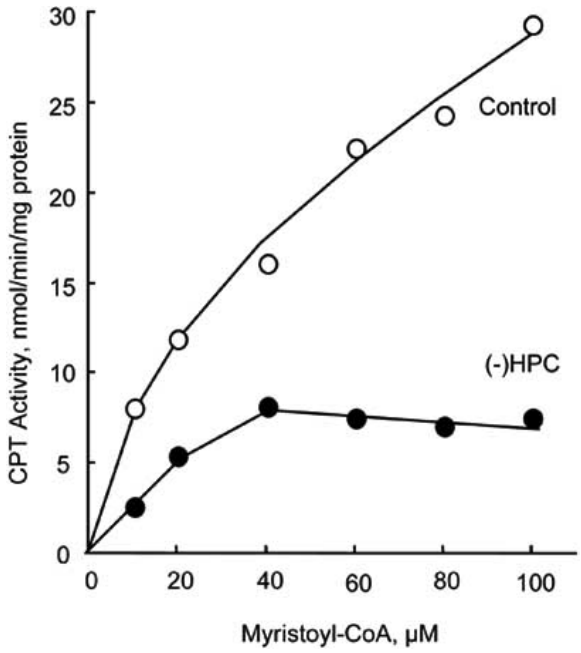

Fig. (4). Inhibitory Effect of (–)-HPC on Isolated Mitochondrial Outer Membrane CPT. Inhibition by 50-μM **(–)-HPC** was compared with activity in absence of **(–)-HPC**. Mitochondrial outer membrane CPT was assayed at a constant myristoyl-CoA to BSA ratio (3:1) and 0.5-mM carnitine. Results represent three different preparations of mitochondrial outer membranes.

incubated with 50-μM **(–)-HPC** for 10 min and an aliquot of this incubation mixture was assayed for activity of CPT, the original enzyme activity is recovered. Upon resuspension of the incubated mitochondrial mixture, the activity of CPT also returns to that of the control. These experiments suggest that the inhibition seen by **(–)-HPC** is essentially reversed.

The Dixon plots [35], Fig. (**6A**), corresponding to the inhibition of the enzyme by **(–)-HPC** indicate that the inhibition is uncompetitive with respect to myristoyl–CoA as substrate. Because Dixon plots could not be used to determine the K_i values for the inhibitor, Cornish-Bowden [36] plots are constructed (Fig. (**6B**)), from which the apparent K_i for **(–)-HPC** is about 5 μM. This apparent K_i indicates that **(–)-HPC** is an extremely good inhibitor of the activity of CPT-I; in fact, it is as good as malonyl-CoA, the physiological inhibitor. One should note that it is possible to have an effective inhibitor of the activity of CPT-I that acts at a site other than the malonyl-CoA binding domain and that the CoA moiety is not an absolute necessity for an effective inhibitor. Fig. (**6**) also shows that at higher concentrations of **(–)-HPC**, the lines curve upwards suggesting that the enzyme contains more binding domains than one or **(–)-HPC** may bind to BSA or **(–)-HPC** forms micelles in the medium.

Inhibition of CPT Activity in Diabetic Mice

In order to ascertain whether these agents inhibit the activity of CPT in the diabetic animal, we produced highly ketotic animals by injecting them with streptozotocin at a

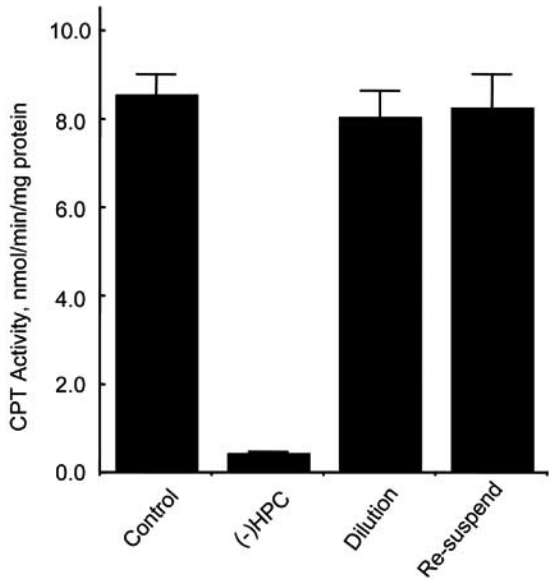

Fig. (5). Reversibility of CPT-I inhibition by (–)-HPC. Intact mitochondria were assayed for CPT activity as described under Materials and Methods for Biology with 40-μM myristoyl-CoA and 0.5-mM carnitine. Results are means ± SEM for three different preparations of mitochondria.

concentration of 150 mg/kg body weight through the tail vein. Fig. (**7**) shows that the activity of CPT-I, as expected, is significantly higher in the diabetic animals and that the inhibitory effects of malony-CoA are much lower. However, in contrast to malonyl-CoA, the inhibitory effects of **(–)-HPC** are not reduced in the diabetic animal. Similar qualitative results are seen with **(+)-HPC** (data not shown). The success of these compounds in inhibiting the activity of CPT-I in a diabetic animal provides an opportunity for evaluating these inhibitors as possible therapeutic agents. The concentrations needed to significantly inhibit CPT-I activity by these agents is in the μM range, which is necessary for a clinically useful drug. Other agents such as glyburide and tolbutamide that are used in treatment of type II diabetes, inhibit both CPT-I and CPT-II [8]. However, their inhibitory effects although substantial, is lower in the diabetic animal [8].

SUMMARY

The present work has shown that both **(–)-HPC** and **(+)-HPC** are excellent inhibitors of the activities of CPT-I and CPT-II in rat liver mitochondria. They are both reversible, active-site directed inhibitors, however, **(–)-HPC** is a much more potent inhibitor. **(–)-HPC**, which has the unnatural configuration, inhibits by an uncompetitive mechanism as compared to **(+)-HPC**, which has the natural configuration and inhibits by a competitive mechanism. Uncompetitive inhibition implies that **(–)-HPC** binds to the enzyme-myristoyl-CoA complex; while **(+)-HPC** binds to the free enzyme.

This improvement in activity with the unnatural enantiomer contrasts previous studies of other inhibitors [21]. The most significant structural difference between HPC and these other inhibitors is the conformationally constrained ring in **HPC**. Perhaps, these constraints give rise to the improvement in inhibition for the unnatural enantiomer.

With an apparent K_i of about 5 μM for inhibition of the activity of CPT-I, **(–)-HPC** inhibits as well as the physiological inhibitor malonyl-CoA. The efficacy of these agents to inhibit CPT-I so effectively in a diabetic animal suggests that they are worthy candidates for further evaluation as antidiabetic agents.

EXPERIMENTAL

General Methods for Chemistry

^{1}H NMR spectra were recorded at 400 and 500 MHz. Unless noted otherwise, all NMR spectra were recorded in

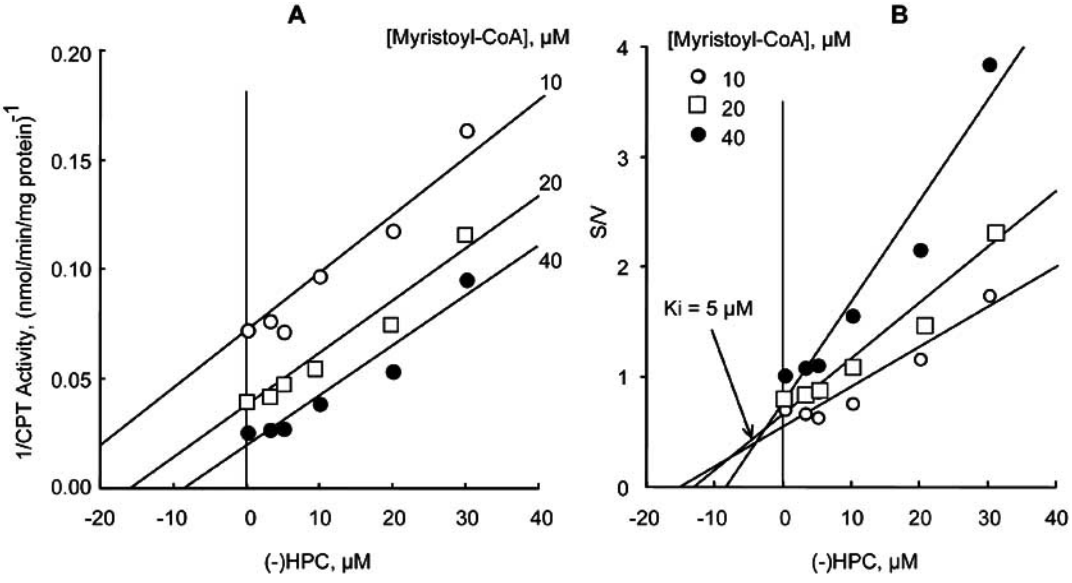

Fig. (6). Dixon (A) and Cornish–Bowden (B) Plots for Inhibition by (–)-HPC. Isolated mitochondrial outer membranes were assayed as described under Materials and Methods for Biology with 10-μM (○), 20-μM (□), and 40-μM (●) myristoyl-CoA and 0.5-mM carnitine.

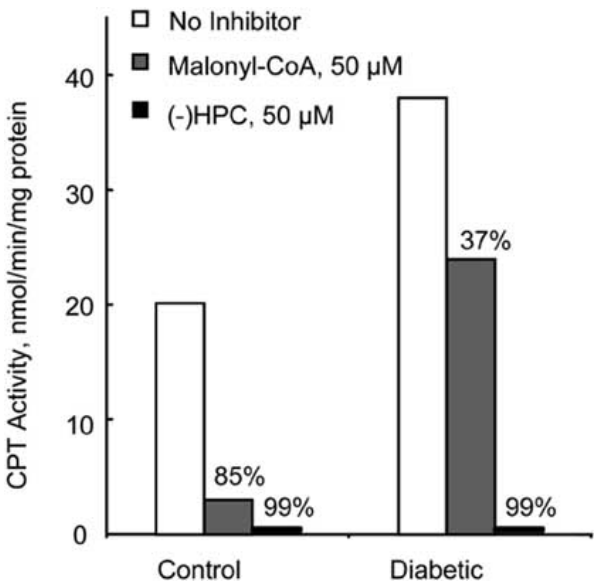

Fig. (7). Comparative Effects of Malonyl-CoA and (–)-HPC on CPT-I in Normal and Diabetic Rats. Isolated mitochondrial outer membranes from normal and diabetic animals were assayed for CPT-I as described under Materials and Methods for Biology with 40-µM myristoyl-CoA and 0.5-mM carnitine. Percent inhibition due to each inhibitor is shown above the bar graphs.

$CDCl_3$. Proton chemical shifts are expressed in ppm downfield from internal TMS standard. Coupling constant, expressed as J_{app}'s in Hz, were observed separation between the lines. FAB-MS samples were prepared by suspending in glycerol. Unless otherwise noted, materials were obtained from commercial sources and used without further purification. Pyridine was distilled from potassium hydroxide and stored under an atmosphere of nitrogen in a freezer at –20 °C. Benzoyl chloride was distilled from calcium hydride and stored under an atmosphere of nitrogen in a freezer at –20 °C in the absence of light. Benzene was stored over 4-Å molecular sieves. The catalysts, $[RuCl_2(S\text{-BINAP})]_x$ and $[RuCl_2(R\text{-BINAP})]_x$, were stored under argon in a dessicator in the absence of light. Solutions were dried over $MgSO_4$ and concentrated by rotary evaporation, unless specified otherwise.

Ethyl (*R*)-4-chloro-3-hydroxybutanoate, (*R*)-1

Ethyl 4-chloroacetoacetate (5.00 g, 30.4 mmol) was placed in a 250-mL hydrogenator vessel, to which was added *t*-BuOH (15.2 mL) and Et_3N (14 drops, ≈ 82 mg, 0.81 mmol). Catalyst, $[RuCl_2(S\text{-BINAP})]_x$ (24.2 mg, 30.5 µmol), was added last and the mixture was then shaken in a Parr hydrogenator at 65 °C and 60 psi H_2 pressure for 48 h. The mixture was removed from the hydrogenator, allowed to cool, diluted with EtOAc (20 mL), filtered, and concentrated to yield 4.90 g (97%) of yellow oil. Bulb-to-bulb distillation at 80 °C produced 3.5 g (69%) of almost colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25 (3H, t), 2.61 (2H, ddd), 3.26 (1H, broad s), 3.59 (2H, ddd), 4.18 (2H, q), 4.22 (1H, dddd). (lit. [37] 300 MHz) $[\alpha]^{23}_D$ +12.2 (*c* 1.11, MeOH).

Ethyl (*S*)-4-chloro-3-hydroxybutanoate, (*S*)-1

Prepared in a similar manner as **(*R*)-1** with $[RuCl_2(R\text{-BINAP})]_x$ as the catalyst. $[\alpha]^{23}_D$ -12.3 (*c* 1.08, MeOH).

Analysis of Enantiomeric Excess with (*R*)- and (*S*)-1-(chloromethyl)-3-ethoxy-3-oxopropyl benzoate, (*R*)- and (*S*)-1(OBz)

A solution of ethyl (*R*)-4-chloro-3-hydroxybutanoate (100 mg, 0.600 mmol) was mixed with PhH (0.3 mL) and pyridine (0.3 mL) in a 5-mL conical reaction vial. Benzoyl chloride (400 mg, 2.85 mmol) was added and the mixture was stirred at ambient temperature for 30 min. Ethanolamine (180 mg, 2.95 mmol) was added dropwise and the solution was stirred an additional 15 min. The mixture was concentrated and redissolved in chloroform (1 mL) for preparative TLC. Preparative TLC of the mixture (1:1 hexane/EtOAc) was performed and the band at R_f = 0.68 was removed. The compound was extracted from the silica gel with EtOAc, which was then filtered and concentrated to yield 110 mg (70%) of colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.2 (3H, t), 2.9 (2H, d), 3.85 (2H, m), 4.15 (2H, q), 5.63 (1H, m), 7.43 (2H, t), 7.58 (1H, t), 8.03 (2H, d).

HPLC analyses were carried out on variable wavelength detector set at 228 nm with a Chiralcel OD chiral column fitted with a cooling jacket and chilled to 10 °C. The solvent system was hexane:*i*-PrOH, 99:1, at a flow rate of 1 mL/min. Enantiomeric excess of 97% was determined: **(*S*)-1(OBz)** and **(*R*)-1(OBz)**: t_R 11.2 and 12.5 min, respectively; Rs 1.5.

(*R*)-Ethyl 4-azido-3-hydroxybutanoate, (*R*)-2

Compound **(*R*)-1**, (38.13 g, 228.9 mmol) was combined with DMF (286 mL) and NaN_3 (29.78 g, 458.0 mmol) in a 500-mL round-bottom flask. The mixture was stirred at 105–110 ºC for 3.5 h with a condenser and drying tube. The flask was cooled to rt and the DMF distilled off at 70 ºC under vacuum. After distillation, equal volumes of H_2O and $CHCl_3$ (225 mL) were added to the flask. The organic phase was removed and the aqueous was extracted with $CHCl_3$ (2x200 mL). The combined organic phases were washed with H_2O (3x100 mL). The mixture was dried, concentrated, and distilled under high vacuum in a bulb-to-bulb apparatus, first at 60 ºC to remove any remaining DMF, then at 85 ºC to separate the desired compound from impurities. A colorless oil (29.96 g, 75%) was recovered. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.26 (3H, t), 2.55 (2H, dd), 3.33 (2H, dd overlapped with 1H, s), 4.16 (2H, q), 4.18 (1H, dddd) plus 2% DMF 2.86 (3H, s), 2.94 (3H, s), 7.95 (1H, s). The product was used without further purification.

(*S*)-Ethyl 4-azido-3-hydroxybutanoate, (*S*)-2

Prepared in a similar manner as **(*R*)-2** with **(*S*)-1** as the starting material. The product was used without further purification.

Ethyl (*R*)-4-dimethylamino-3-hydroxybutanoate, (*R*)-3

Compound **(*R*)-2** (14.99 g, 86.55 mmol) was combined with 37 % H_2CO in H_2O (14.84 g, 182.9 mmol) and 10% Pd/C (0.7510 g, 5 wt.% of **(*R*)-2**) in a 500-mL hydrogenator vessel. The mixtures vessel were cooled in an ice bath for 5 min before HCOOH (19.93 g, 433.2 mmol) and H_2O (10

mL) was added to the vessels. The mixture was cooled an additional 5 min and then placed in the hydrogenator at 70 psi H_2 pressure for 60 h. The vessel was both flushed and refilled with H_2 after 1 h and after running overnight. The mixture was filtered through a Buchner funnel through 2 pieces of filter paper; the filter cake was washed with H_2O (3x10 mL). Solid K_2CO_3 was added to adjust the filtrate to pH 10; a greenish grey oil layer formed. The mixture was extracted with $CHCl_3$ (3x50 mL). The organic layer was dried, and concentrated to produce a light yellow oil (15.00 g, 96%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.22 (3H, t), 2.22 (6H, s), 2.25 (2H, ddd), 2.37 (2H, ddd), 3.6 (1H, broad s), 4.05 (1H, dddd), 4.12 (2H, q) plus 2% DMF at 2.82 (3H, s), 2.91 (3H, s), 7.95 (1H, s). The product was used without further purification.

Ethyl (*S*)-4-dimethylamino-3-hydroxybutanoate, (*S*)-3

Prepared in a similar manner as **(*R*)-3** with **(*S*)-2** as the starting material. The product was used without further purification.

1-Bromoheptadecan-2-one, 4

A mixture of heptadec-1-ene (25 mL, 83 mmol), $FeCl_3 \cdot 6H_2O$ (561.3 mg, 2.077 mmol), H_2O (3 mL), and acetone (150 mL) was prepared in 500-mL round-bottom flask. The flask containing the pale yellow solution was covered with aluminum foil and placed in an ice-water bath. *N*,*N*'-Dibromo-5,5-dimethylhydantoin (14.033 g, 49.078 mmol) was added slowly by spatula during 10 min to the stirred solution. The orange solution was stirred for 3 h at rt. After cooling the stirred solution in an ice bath, silica gel (4.54 g) was added, followed by dropwise addition of CrO_3 (9.8 g)/conc H_2SO_4 (6.5 mL)/H_2O (19.5 mL). The reaction was then stirred vigorously for about 24 h at rt. The reaction was quenched with water (80 mL) and extracted with $CHCl_3$ (3x200 mL). The extract was washed with satd $NaHCO_3$ (80 mL) and brine (80 mL), dried over $MgSO_4$, and filtered through Celite®. Concentration by rotary evaporation of the pale green solution gave a greenish solid, which was dried under high vacuum for 6 h. Recrystallization in hexane:EtOH (50:2, 80 mL) gave a white solid, which was further purified by dissolving in $CHCl_3$ (75 mL), filtering through a pad of silica gel (20 g), and concentrating to give a white solid (13.67 g, 41.00 mmol, 49%). Concentrating the filtrate by two-thirds and then filtering yielded a colorless solid. The solid was dissolved in hexane:EtOH (50:2, 8 mL) and cooled in the refrigerator to form crystals, which were treated in a similar manner as the first crop to give a white solid (3.40 g, 6.97 mmol, 8%). To ensure the highest purity for the next step in the synthesis, the extensive purification procedure was conducted until the crucial feature in the ^{1}H NMR had the correct integration for all the signals, the most critical being the broad signal for the twelve overlapping methylene groups. mp 66.3–66.7 °C (lit. [14] 64.5–65.5 °C) FTIR (diamond ATR, neat) 2913 (s), 2847 (m), 1718 (m), 1470 (m), 1390 (m), 718 (m) cm^{-1}. (lit. [14,28] IR, film) ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, 3H, J_{app} = 7.0), 1.30–1.24 (b, 24H), 1.61 (m, 2H), 2.64 (t, 2H, J_{app} = 7.4), 3.88 (s, 2H). (lit. [14] 100 MHz, lit. [28] MHz) ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.3, 22.9, 24.1, 29.3, 29.5, 29.6, 29.7, 29.8, 29.9, 32.2, 34.5, 40.1, 202.5.

(6*R*)-HPC Ester Bromide

Compound **4** (63.34 g, 0.190 mol) was added to an MeCN solution (190 mL) of **(*R*)-3** (33.29 g, 0.190 mol) in a 500-mL round-bottom flask. After stirring under N_2 at rt for 1 h, the flask was placed in a oil bath at 60–65 °C for about 24 h. The reaction mixture was concentrated to a sticky yellow liquid. After drying under vacuum for 2 d, 95.66 g (99%) was obtained as lemon powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.85 (t, 3H), 1.1–1.45 (m, 27H), 1.65–1.8 (m, 2H), 2.65 (ddd, 2H), 3.22 (d, 1H), 3.35 (t, 1H), 3.51 (s, 3H), 3.75 (s, 3H), 4.01 (t, 2H), 4.16 (q, 2H), 4.61 (s, 1H), 4.75–4.85 (m, 1H). The product was used without further purification.

(6*S*)-HPC Ester Bromide

Prepared in a similar manner as **(6*R*)-HPC ester bromide** with **(S)-3** as the starting material. The product was used without further purification.

(+)-HPC

Dissolved **(6*R*)–HPC** ester bromide (95.66 g, 0.1881 mol) in 95% EtOH (500 mL), and stirred for about 30 min. To the solution was added Amberlite IRA 402 (OH) anionic exchange resin (175.32 g, 1.3 eq); the mixture was stirred ***gently*** at rt overnight. The liquid was decanted, resin was washed with 95 % EtOH. The solvent was concentrated to give a sticky yellow liquid, which was dried under vacuum to afford light yellow powder (75.16 g). The mixture of this yellow powder and ether (~ 500 mL) was refluxed for 30 min to give a light yellow suspension. After cooling to rt, the suspension was filtered to give an off-white powder. This procedure was repeated three times. After stirring with EtOH (300 mL) and activated carbon (Norit SA3, ~100 mesh) at rt overnight, the solid was removed by filtration. The filtrate was concentrated and dried under high vacuum to afford white powder (59.24 g). Recrystallization of the white powder by vapor diffusion of ether into ether/EtOH solution gave fine white crystals, which were dried under high vacuum to give 51.01 g (68%) of fine, white crystals. mp 153–155 °C (dec) ^{1}H NMR (400 MHz, D_2O) δ 0.65 (t, 3H) 0.9–1.35 (m, 26), 1.4–1.5 (m, 2H), 2.1–2.3 (ddd, 2H), 2.85–3.05 (m, 2H), 3.13 (s, 3H), 3.27 (s, 3H), 3.42 (d, 2H), 4.5–4.6 (m, 1H). Anal. calcd. for $C_{23}H_{45}N_1O_4 \cdot H_2O$ C, 66.13; H, 11.35; N, 3.36 Found, C, 66.18; H, 11.32; N, 3.34. $[\alpha]^{23}_D$ +14.4 (*c* 0.2, MeOH).

(–)-HPC

Prepared in a similar manner as **(+)-HPC** with **(6*S*)-HPC ester bromide** as the starting material. Anal. calcd. for $C_{23}H_{45}N_1O_4 \cdot H_2O$ C, 66.13; H, 11.35; N, 3.36 Found, C, 66.17; H, 11.33; N, 3.36. $[\alpha]^{23}_D$ –14.5 (*c* 0.2, MeOH).

METHODS AND MATERIALS FOR BIOLOGY

Materials

Myristoyl-CoA, imidazole, L-carnitine hydrochloride, EDTA, essentially fatty-acid-free bovine serum albumin, streptozotocin , malonyl-CoA, and Nagarse (subtilisin BPN', P 4789) were purchased from Sigma (St. Louis, MO, U.S.A.). L-[methyl-^{3}H]Carnitine hydrochloride was obtained from Amersham Corp. (Arlington Heights, IL, U.S.A.).

Animals

Male, Sprague-Dawley rats weighing 180–240 g were obtained from Harlan Industries (Indianapolis, IN) and were fed on Purina Rat Chow (Ralston Purina Co., Richmond, IN) and water *ad libitum*. On the day of the experiment, rats were killed by decapitation and their livers were removed rapidly for preparation of mitochondria. Ketotic diabetic rats used in some experiments were produced by injecting rats with 150 mg of streptozotocin/kg of body weight into the tail vein [38] and were used 48 h after the injection. This high dose of streptozotocin produced rats that were highly ketotic (urine ketone bodies ≥80 mg/mL and urine glucose levels ≥2000 mg/mL using Multistix.

Isolation and Protease Treatment of Mitochondria

Intact liver mitochondria were isolated by the method of Johnson and Lardy [39] with a medium containing 210 mM mannitol, 70-mM sucrose, 0.1-mM EDTA, and 10-mM Tris-HCl (pH 7.4). Mitochondrial outer membranes were isolated by the method of Parsons *et al*. [40], and their purity was assessed as described previously [41]. Protease treatment of mitochondria was carried out as described previously [32,33]. Briefly, this method consisted of incubating the mitochondria (5 mg/mL), and outer membranes (1 mg/mL) with Nagarse (5 μg/mL) at 37 °C for 10 min after which the proteolytic activity was stopped by addition of 200 μl of 20% (w/v) BSA/mL of incubation volume plus 40 mL of ice-cold isolation medium. After centrifugation (5600 *xg* for 10 min), the mitochondria were resuspended (4 mg/mL) in isolation medium and used as indicated. Protein determination of the mitochondrial outer membranes was by the Lowry method [42]; protein determination of the mitochondria was by the biuret method [43].

Carnitine Palmitoyltransferase Assay

The activity of CPT was measured according to the method of Bremer [4] as modified and reported previously [7]. Each assay contained, in a total of 1 mL: 82-mM sucrose, 70-mM KCl, 70-mM imidazole, 1 μg of antimycin A, and 2 mg of bovine serum albumin. For assaying the outer CPT each assay also contained 0.5-mM L-carnitine (0.4 mCi of L-[methyl-^{3}H]carnitine) and 40-μM myristoyl-CoA; for assaying the inner CPT each assay contained 2-mM carnitine and 100-μM myristoyl-CoA.

Intact mitochondria were preincubated for 5 min with the inhibitors at the concentrations indicated before assaying for the activity of CPT. For assaying the inner membrane CPT, intact mitochondria were subjected to a freeze-thaw cycle; the assay was done in the presence of 0.04% Triton X-100. In some experiments, the intact mitochondria were preincubated as indicated but then an aliquot was taken and assayed for the activity of CPT (dilution experiments), the rest was diluted 70-fold and after centrifugation and resuspension, was assayed for the activity of CPT (wash-out experiments).

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ABBREVIATIONS

CPT	=	carnitine palmitoyltransferase
FAO	=	fatty acid oxidation
CoA	=	coenzyme A
NIDDM	=	noninsulin-dependent diabetes mellitus
CAT	=	carnitine acetyltransferase
HPC	=	Hemipalmitoylcarnitinium
BINAP	=	2,2'-bis(diphenylphosphino)-1,1'-binaphthalene
DMF	=	*N*,*N*-dimethylformamide
NBS	=	*N*-bromosuccinimide
NDD	=	*N*,*N'*-dibromo-5,5-dimethylhydantoin

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