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Molecular Recognition in Carnitine Acyltransferases

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Abstract. We are designing and synthesizing rigid guests to probe the topography of the carnitine acyltransferases, regulatory enzymes in lipid metabolism. Our designs are based on structural studies of substrates and possible molecular mechanisms of enzymatic activity. Recent X-ray, ^{1}H NMR, and force-field computational studies on carnitine and acetylcarnitine, coupled with the known stereospecificity for activity in carnitine acyltransferases, have led us to propose a molecular mechanism for acyl transfer in these enzymes. The 'folded' conformation of an acylcarnitine is most populated and should be preferred for binding to these enzymes, because, in this conformation, the acyloxy is the most sterically accessible. There are four key recognition sites on the enzymes: I, carboxylate; II, trimethylammonium; III, coenzyme A; IV, acyl. Sites, I, II and III serve as the three loci required to create a chiral environment on the enzymes for carnitine. An addition-elimination reaction involving the formation of a tetrahedral intermediate is suggested as the mechanism for O-to-S acyl transfer. This proposed tetrahedral intermediate is chiral and the enzymes should prefer the *R* configuration at this center. Based on this proposal, conformationally rigid tetrahedral-intermediate analogues have been designed, synthesized and assayed. Morpholinium and 2-hydroxymorpholinium derivatives inhibit carnitine acetyltransferase and palmitoyltransferase. Because of rigidity at their two chiral centers, these inhibitors serve as probes of molecular topography of recognition sites, I, II, and IV.

Key words. Enzyme, inhibitor, force field calculations.

1. Molecular Recognition

Molecular recognition refers to all interactions between two molecules or two remote pieces of the same molecule. A time-dependent phenomenon, it includes both stable and unstable complexes. Host–guest chemistry, which covers stable complexes from ions–ionophores, substrates–enzymes, drugs–receptors, antigens–antibodies, is a major chemical research area with substantial practical applications in catalysis, separation science, medicine, and material science. Recognition by a host requires varying degrees of flexibility. An implicit assumption is that a perfectly constructed cavity will have ultraselectivity.

1.1. FLEXIBILITY OF HOST

The structural flexibility of a crown ether and its tendency to adopt a conformation appropriate to its environment have been recognized since the first structures appeared [1]. Ligand flexibility is necessary for complexation by cryptands [2]. Complexation of ions by these flexible ligands largely depends on neutralizing the charge on the metal, which requires an appropriate number of donors in an appropriate topography. Cram and Trueblood [3] have enunciated the principle that guests organize the hosts and that preorganization of the host improves binding. Gokel [4] has proposed the idea that cavity sizes of crowns are adjustable

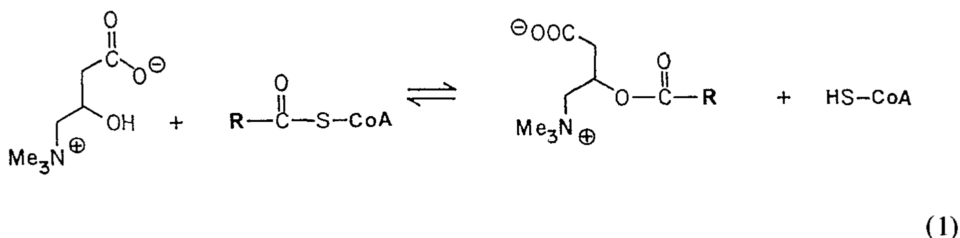
and has investigated the dynamics of binding by retaining a flexible component in his captivating lariat ethers. We have subsequently demonstrated [5] that the guest determines the cavity size of the host, depending on the number and identity of donors in the macrocycle. The carbon framework in these macrocycles primarily maintains the connectivity relationships among the donors rather than imposing a rigid conformational or steric bias on the system.

1.2. TOPOGRAPHICAL MAPPING OF FLEXIBLE HOSTS WITH RIGID GUESTS

When the guests are rigid ions, then the host simply must accommodate. In applying Cram and Trueblood's hypothesis [3] to the design of guests, we conclude that rigid guests can probe the structure of unknown hosts, an idea that has its origins in medicinal chemistry [6]. We are using this concept of a rigid guest's organizing a flexible host (induced-fit model [7]) to design enzyme inhibitors in order to map the topographies of catalytic centers in enzymes. Mapping topographies of the active sites of enzymes is a formidable task, which we are undertaking by preparing conformationally rigid analogs of reaction intermediates proposed for carnitine-acyltransferase catalyzed reactions. These analogs have groups anchored to a rigid molecular framework in a well-defined stereochemistry. Their inhibitory ability depends on their complementing the topography of the catalytic center.

2. Carnitine

Carnitine, the biological carrier-molecule of fatty acids destined for transport into and oxidation by mitochondria [8], is required for efficient metabolism of long-chain fatty acids [9–11]. Carnitine transports fatty acyl groups across mitochondrial membranes after accepting the acyl group from an acyl CoA in a reaction, Eq. 1, catalyzed by carnitine palmitoyltransferase (CPT).



(1)

After being transported across the inner mitochondrial membrane, acyl carnitine donates the acyl group to an endogenous CoA molecule in the mitochondrial matrix. After fatty-acid oxidation, the acetyl CoA transfers the acetyl group to carnitine in a reaction catalyzed by carnitine acetyltransferase (CAT). Acetylcarnitine is transported out of the mitochondrial matrix and donates the acetyl group to exogenous CoA.

3. Reaction-Intermediate Analogs

Wolfenden [12] has pioneered the development of transition-state analog inhibitors of enzymes. The idea is that enzymes bind transition structures or reaction intermediates more tightly than reactants or products (Figure 1). Molecules that resemble the structures of transition states or reaction intermediates but are unreactive will bind strongly to the enzyme. Our goal is to design a conformationally rigid, reaction-intermediate analog inhibitor in order to map the topographies of the catalytic centers of CAT and CPT.

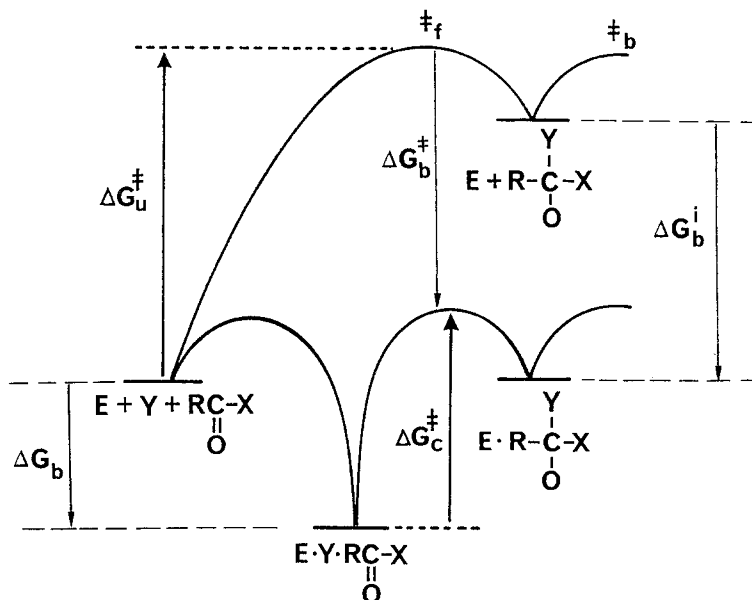
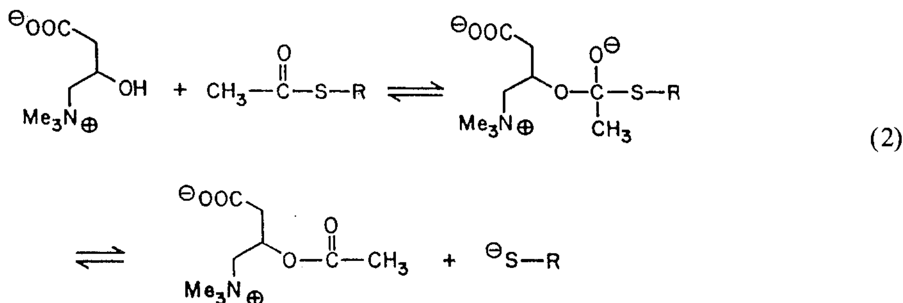


Fig. 1. Free-energy diagram illustrating the energetic advantage of binding transition structures and reaction intermediates. For a complete discussion of the ideas from which this diagram is derived, see the excellent review by Schowen [13].

We have proposed [14] a mechanism (Eq. 2) for acetyl transfer in CAT involving a tetrahedral intermediate, which contains both carnitine and coenzyme A. We assume that a similar mechanism operates in CPT.



(2)

The catalytic centers of both CPT and CAT contain a carnitine recognition site and a CoA recognition site, as well as an alkyl recognition site that is juxtaposed to the other two sites (Figure 2). The carnitine recognition site has two recognition points, one for carboxylate, the other for trimethylammonium. Together with the CoA site, these two recognition points create the stereoselectivity observed for the reaction. The alkyl group is probably not detached from its recognition site during the transfer, hence creating an additional chirality for the active site (i.e., the tetrahedral intermediate). This chirality is recognized during the transfer and must be mimicked by a reaction-intermediate analog.

To design a rigid analog, we must know which of the nine possible conformations of carnitine is (are) preferred on the surface of the enzyme. This question is still unanswered but we have addressed the question of the preferred conformation of carnitine and acetylcarnitine in other states of matter. We have used single crystal X-ray for the solid state, NMR for the solution state, and *ab-initio*-enhanced MM2 for the computational state.

4. Structural Studies

4.1. SINGLE CRYSTAL X-RAY ANALYSIS

We have determined [14] the crystal structures of the zwitterions of carnitine and acetylcarnitine, which are similar to those of hydrochloride salts. Because the zwitterion is the physiologically active form, we need to know if there are any

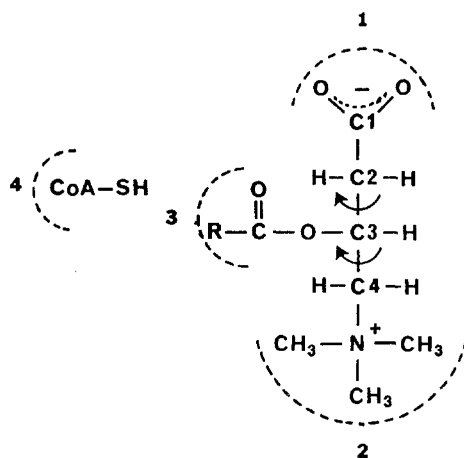


Fig. 2. Coenzyme A and acylcarnitine illustrating the possible recognition sites. The carnitine site has two recognition points: (1) carboxylate and (2) trimethylammonium. The acyl recognition site (3) is juxtaposed to the carnitine and coenzyme A site (4). Carnitine has flexibility about the C2—C3 and C3—C4 bonds. The preferred conformations about these bonds must be known in order to design rigid inhibitors.

changes in conformation arising from ionization of the carboxyl. In the solid state, the conformations do not change on ionization. Carnitine has a different conformation to acetylcarnitine. The conformation about C3—C4 is similar in both but the conformation about C2—C3 changes from *anti*(a) to g^-. Murray, Reed, and Roche [15] have labeled the conformation of carnitine as 'extended' and acetylcarnitine as 'folded' (Figure 3).

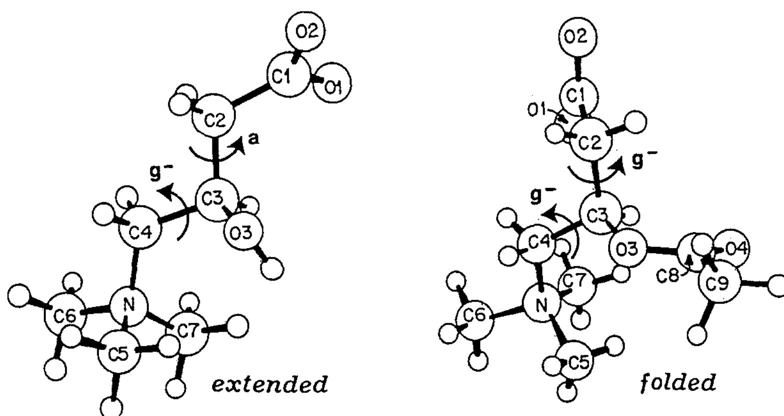
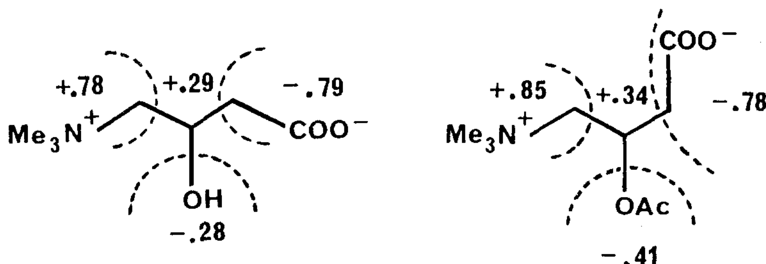


Fig. 3. Crystal structures of the zwitterions of carnitine (left) and acetylcarnitine (right) from ref. [14]. Carnitine is in the 'extended' conformation (a, g^-) and acetylcarnitine is in the 'folded' conformation (g^-, g^-).

4.2. COMPUTATIONAL STUDIES

We have computed the relative energies of the conformations of carnitine and acetylcarnitine [16]. Atomic point charges from a single-point *ab initio* (3–21G basis set) calculations of the zwitterions using the crystal-structure geometries form the electrostatic force field. The total charges on the three polar fragments of carnitine and acetylcarnitine are shown below.



The MM2 results show that electrostatic energy accounts for enhancement of the proportion of folded conformer. Attraction between carboxylato and quaternary

ammonium groups increases for both carnitine and acetylcarnitine. The greater enhancement for acetylcarnitine than carnitine results from relief of both electrostatic and steric repulsion between carboxylato and acetoxyl groups.

Because we wanted to determine the relative energetics of conformations in the physiological state, we explored the effect of dielectric. As we are dealing with charged structures the effects are quite dramatic. The dielectric has only small effects on populations down to a value of about $\varepsilon = 40$, below which electrostatics primarily determine the energy of the zwitterions.

Figure 4 shows computational results for a dielectric of water ($\varepsilon = 80$). As in the X-ray study, 'folded' was favored for acetylcarnitine and 'extended' for carnitine. Previous semi-empirical studies [15] on carnitine's conformation suggested a strong electrostatic attraction between the carboxylate and trimethylammonium. The difference between those calculations and ours is that the semi-empirical methods calculate the energy of an isolated molecule and thus resemble the gas phase. Because the dielectric constant is part of the force field in our MM2 calculations, we can simulate the solution state.

4.3. ^{1}H NMR

Our computational studies were done in concert with an ^{1}H NMR study of the conformations in deuterium oxide. The problem of how to determine the conformation of flexible molecules in solution has challenged researchers for decades and will continue to do so. The problem is even more difficult for flexible molecules that are either quite polar or charged, especially if these molecules interact with the solvent. The techniques of NMR spectroscopy and computational chemistry,

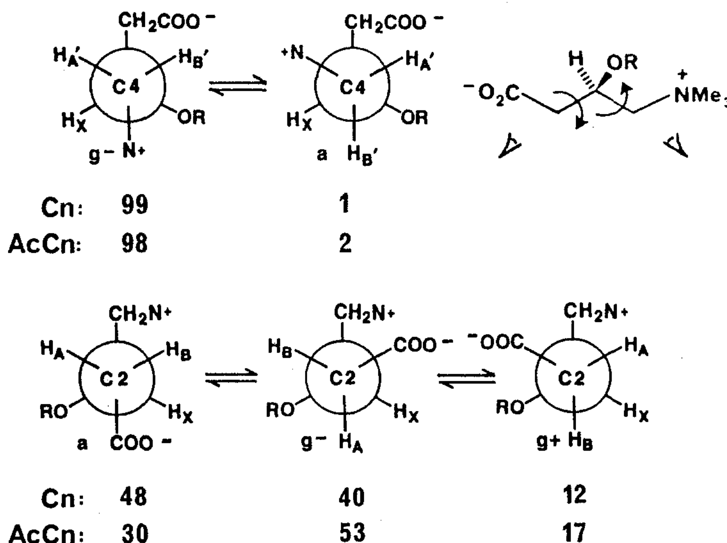


Fig. 4. Proportions of conformations of carnitine (Cn) and acetylcarnitine (AcCn) determined by MM2 computation from ref. [16]. Top line of Newman projections have C4 in front and C3 at the back. g^- and a refer to the torsion angle between C4—N$^+$ and C3—OR. Bottom line of Newman projections have C2 in front and C3 at the back. a, g^-, and g^+ refer to the torsion angle between C2—COO$^-$ and C3—CH_2N^+.

especially when used in tandem have resulted in considerable progress in conformational analysis.

My student, Dr. William J. Colucci, with the help of Dr. Steven Jungk, has developed an equation (Eq. 3) for determining conformations about C—C bonds. His equation [17] like those of Pachler [18] and of Altona [19] is a modified Karplus expression [20]. Rather than using electronegativities to account for substituent effects, Colucci's equation employs empirically derived substituent constants. The ΔS_i term is a group substituent effect. The equation is a classical free-energy relationship such as the Taft equation.

$$^3J(\mathrm{HCCH}) = A + B\cos\theta + C\cos 2\theta + \sum_{i=1}^{4} \Delta S_i \cos\theta \cos\phi \; \mathrm{HX}_i \qquad (3)$$

The question is: how do we get the values for ΔS_i? We have measured or taken from the literature coupling constants for monosubstituted ethanes. Because all conformations are populated due to free rotation, an average coupling constant is measured. Integrating the equation gives the simple result that the average coupling constant for a monosubstituted ethane is simply the average coupling constant for ethane, A, minus 0.25 times ΔS_i. A, B, and C were obtained from calculations on ethane [21].

We measured the average coupling constants for the appropriate ethyl compounds at the same pH as we measured the spectra for carnitine and acetylcarnitine [16]. This illustrates the simplicity of Colucci's equation because the values of ΔS_i are determined in the medium of choice and thus any solvent effects on the substituent effects are accounted for. The results of the ^{1}H NMR study are shown in Figure 5. 'Folded' is favored for acetylcarnitine and 'extended' for carnitine.

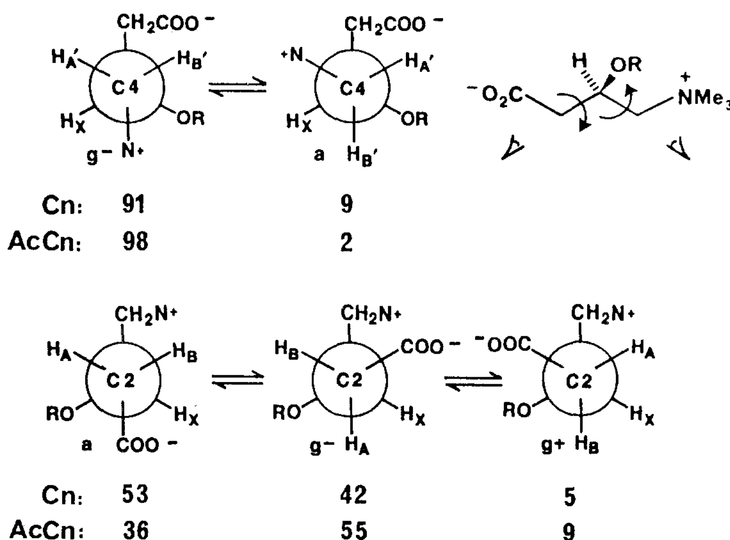


Fig. 5. Proportions of conformations of carnitine and acetylcarnitine determined in solution by ^{1}H NMR, from ref. [16]. Notation is the same as in Figure 4.

In summary, our conformational analysis of carnitine and acetylcarnitine shows that carnitine prefers 'extended' or a^-g^- and acetylcarnitine 'folded' or g^-g^-. The important points for inhibitor design are that the C3—C4 bond has a strong preference for g^-, and the C2—C3 bond is equally populated in either the a or g^- conformation. We can lock the C3—C4 bond in this conformation by formation of a ring and not lose much in recognition. We are less certain as to whether or not to lock the conformation of the C2—C3 bond.

5. Inhibitor Design

As shown in Eq. 2, the proposed mechanism involves a direct transfer between carnitine and Coenzyme A. The reaction is specific and for the *R*-enantiomer of carnitine and we have proposed [14] a two-point recognition by the enzyme because the location of the cofactor gives the third point needed for chiral recognition.

Given the need for carboxylate recognition and assuming that the conformation of the bound molecule is 'folded', we can imagine how this mechanism might occur on the enzyme, Figure 6. This leads to two further topographical considerations, the locations of the Coenzyme A site and the acyl site. The carboxylate must be turned away from the oxygen on C3 to allow room for S to attack. This model further suggests a chirality for the tetrahedral intermediate; i.e., the attack must occur away from the trimethylammonium group on the *Re* face of the carbonyl. An additional benefit is the electrostatic catalysis [22] that results from having the developing negative charge on the carbonyl oxygen in close proximity to the trimethylammonium group.

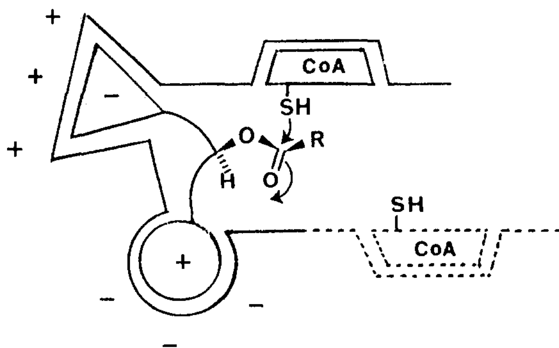
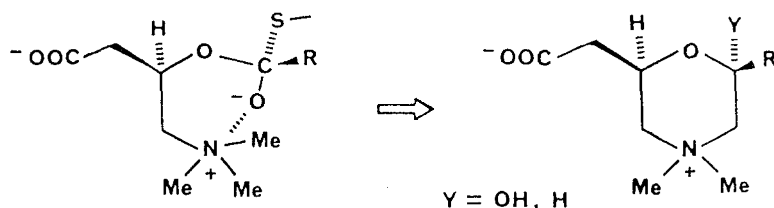


Fig. 6. Mechanism for Coenzyme A attack on acylcarnitine. The carboxylate (triangle with negative charge) is folded back to allow the thiol to approach the carbonyl. We propose the CoA attacks from this side rather than the opposite (shown as dotted lines) because of steric and electrostatic effects (see text).

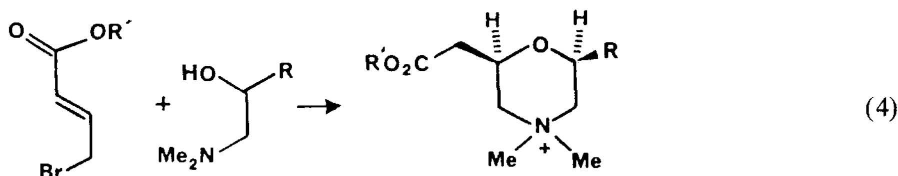
We reason that the electrostatic interaction could be replaced by a covalent bond and that the S could be replaced by an H or OH, see below.



Knowing that the conformation about C3—C4 in carnitine is predominantly g^-, we felt that locking this conformation in a six-membered ring should not detract from binding. Conformational considerations in six-membered rings led us to anticipate that the carboxymethyl and the alkyl chain attached to the anomeric carbon should be *cis*-disequatorial. We approached the syntheses of morpholinium inhibitors from two directions.

6. Morpholiniums

One approach is the reaction of dimethylaminoalcohols with an ester of 4-bromo-2-butenoate, Eq. 4. Cyclization to the morpholinium ring occurs in a separate step and is stereoselective.



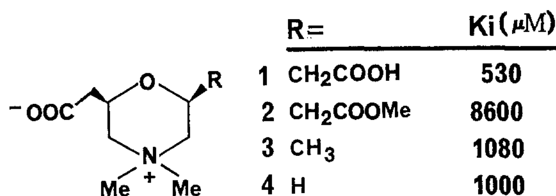
(4)

These compounds are conformationally rigid analogs of the tetrahedral intermediate proposed for acetyl transfer in CAT. CAT binds both enantiomers of carnitine equally well, but only the (*R*)-enantiomer is active. These analogs must have the same relative configuration as (*R*)-carnitine, if CAT stereoselectively binds them as it binds the tetrahedral intermediate. To verify that pigeon breast CAT recognizes only one configuration of our inhibitors, we have devised a unique approach [23], that utilizes only racemic compounds and a prochiral molecule with an achirotopal plane.

We made (*meso*)-2,6-bis(carboxymethyl)-4,4-dimethylmorpholinium, **1**, in two steps from condensation of sodium (*R*)-norcarnitine [24] and methyl-(*E*)-4-bromo-2-butenoate. Only one pair of enantiomers, presumably the *cis*-diastereomer, of **2** was present by ^{1}H NMR, indicating that the ring formed stereoselectively. Hydrolysis of **2** yielded the anticipated diequatorial *meso*-diacid, whose structure was verified by single crystal X-ray analysis. The solid-state structure of **1**, in fact, displays *meso*-symmetry, with a crystallographic mirror plane containing the O and N atoms of the ring. Hence, we call it a 'Siameso' inhibitor because of its morphological similarity to Siamese twins. Compounds **3** and **4** were prepared by similar reactions of the appropriate dimethylamino alcohol and the bromoalkenoate.

We have measured the K_is of **1–4** with pigeon breast CAT (Figure 7). Of this series **1** binds most strongly, with a K_i half that of the racemic compounds, **3** and **4**. This is because every molecule has one side with the correct configuration of (*R*)-carnitine. This two-fold improvement in binding for **1** suggests that CAT is selectively binding one configuration of these inhibitors. Compound **2** does not bind well because of the increased size of the ester or the polarity change from acid to ester.

The key features of these inhibitors are their rigidity and their similarity to the tetrahedral intermediate. Rigidity in the inhibitor reduces binding because only the enzyme can adjust, but rigidity is essential for identifying the topographical arrangement of recognition points on the enzyme, as well as the conformation of the substrate fragment of the tetrahedral intermediate. For example, the N—C—C—O torsion angle in the inhibitors is locked in the g^- conformation, which is predicted for carnitine bound to CAT [16].

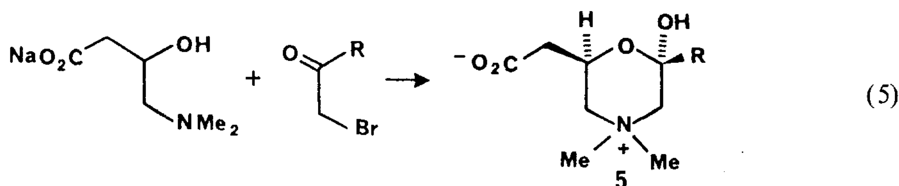


	R=	Ki (μM)
1	CH_2COOH	530
2	CH_2COOMe	8600
3	CH_3	1080
4	H	1000

Fig. 7. Inhibition constants for morpholinium derivatives.

7. 2-Hydroxymorpholiniums

2-Hydroxymorpholinium derivatives designed as mimics of choline have shown biological activity [25]. They are prepared by the reaction of 2-(dimethylamino)-ethanol, and a halomethyl ketone. In extending this approach to carnitine, large quantities of norcarnitine are required. We have developed a large-scale procedure for demethylating carnitine in high yield [24]. Sodium norcarnitine and the corresponding bromomethyl ketone produce the 2-hydroxymorpholinium analogs, **5**, Eq. 5.



We have prepared the carnitine analogs (hemiacylcarnitiniums) **5**, with R=CH_3(HAC) and $(CH_2)_{14}CH_3$(HPC) from the sodium norcarnitine and the corresponding bromomethyl ketone. The ring closure is highly favored because of

the strong electron withdrawing effect of the quaternary ammonium ion. We have only seen one isomer formed and rationalize the preference for an axial OH as arising from the anomeric effect, the gauche effect, and steric effects.

The hemiketal carbon of **5** and the hemiorthothioester carbon of the proposed tetrahedral intermediate are chiral. CAT and CPT may prefer one configuration at this center, just as they prefer one configuration of the chiral center of acetylcarnitine. The preferred absolute configuration of the tetrahedral intermediate is *R*, because the most likely approach of the thiol group is on the *Re* face of the acetoxyl group. Therefore, the 2*S* configuration of **5** has the same relative configuration as the proposed tetrahedral intermediate, and only (2*S*,6*R*)-**5** of the four possible diastereomers is expected to have activity.

Because the hydroxy is more stable in the axial position, there is complete asymmetric induction when the hemiketal is formed and only one pair of enantiomers, (2*R*,6*S* : 2,6*R*)-**5** is produced. This stereoselectivity prevents the formation of (2*S*,6*S* : 2*R*,6*R*)-**5** in which the hydroxy is equatorial. The intriguing possibility of determining the chirality of the reaction center by comparing (2*S*,6*R*)-**5** activity with that of (2*R*,6*R*)-**5** is unfortunately not possible with this inhibitor.

Racemic [26] and chiral HAC are good inhibitors of CAT. The chiral material is 3.6-fold better than the racemic, demonstrating chiral recognition of the inhibitor. This is relevant because for this enzyme the *S*-enantiomers of carnitine and acetylcarnitine bind as well as the natural substrates. Because HAC is a competitive inhibitor of both, we suggest that it occupies the same site as carnitine and acetylcarnitine, but that the enzyme is in a conformation that recognizes the chirality, presumably adopted during its catalytic activity. HAC is a good inhibitor, binding a factor of 6 better than acetyl carnitine and a factor of 2 better than carnitine. (Table I).

Table I. Binding constants for selected inhibitors and substrates of CAT and CPT.

Inhibitors (K_i, μM)	CAT	CPT
HAC (2*R*,6*S* : 2*S*,6*R*)	212[a]	—
HAC (2*R*,6*S*)	59.5[a]	—
HPC (2*R*,6*S* : 2*S*,6*R*)	—	5.1[b], 1.6[c]
Substrates (K_m, μM)		
(*R*)-Carnitine	120[d]	200[e]
(*R*)-Acetylcarnitine	350[d]	—
(*R*)-Palmitoylcarnitine	—	14[e]

[a] ref. [27]; [b] ref. [28], vs. (*R*)-carnitine; [c] ref. [28], vs. (*R*)-palmitoylcarnitine; [d] ref. [29]; [e] ref. [11].

Racemic-HPC, a strong inhibitor of CPT, [28] binds 9-fold better than palmitoylcarnitine. The chiral material promises to be even better. HAC competes for the carnitine site in the short-chain, but not the long-chain transferase. In summary, the success of these inhibitors attests to the design rationale that the g^- conformation

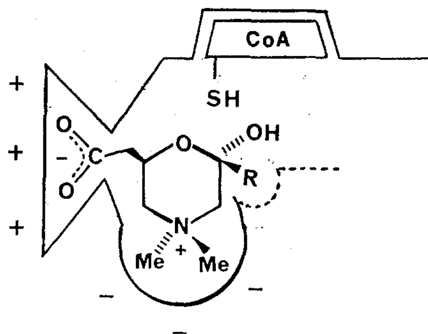


Fig. 8. Proposed fit of 2-hydroxymorpholinium in catalytic center of acyltransferase. Compare with Figure 6.

about C3—C4 can be locked. Their rigidity allows us to speculate on the relative locations of the recognition sites in each enzyme (Figure 8).

In this view of a carnitine acyltransferase, we propose that CoA is located below the inhibitor away from the quaternary ammonium. This view emphasizes the *cis*-diequatorial configuration of the alkyl (*R*) and carboxymethyl groups.

8. Summary and Conclusions

After structural studies on carnitine and acetylcarnitine as well as an analysis of a possible mechanism for acyl transfer, we have designed and synthesized effective inhibitors of CAT and CPT. These morpholinium derivatives provide a rigid framework from which to anchor the molecular fragments of the reaction. Consequently, we can map the topography of the active site by measuring the relative binding strength of stereoisomeric inhibitors. Our first inhibitors are very competitive, which suggests that they have the correct stereochemistry. HPC is currently the best inhibitor of purified CPT. We are working to verify that the stereochemistry of HAC and HPC are optimal by preparing other stereoisomers. The eventual goal is to add a CoA fragment to the framework in order to determine its recognition site relative to the others.

Acknowledgement

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References

1. J. D. Dunitz, M. Dobler, P. Sieler, and R. P. Phizackerley: *Acta Crystallogr., Sect. B.* **B30**, 2733 (1974).
2. F. Mathieu, B. Metz, D. Moras, and R. Weiss: *J. Am. Chem. Soc.* **100**, 4412 (1978).
3. D. J. Cram and K. N. Trueblood: *Top. Curr. Chem.* **98**, 43 (1981).

4. R. A. Schultz, D. M. Dishong, and G. W. Gokel: *J. Am. Chem. Soc.* **104**, 625 (1982); G. W. Gokel, D. M. Goli, C. Minganti, and L. Echegoyen: *J. Am. Chem. Soc.* **105**, 6786 (1983).
5. R. D. Gandour, F. R. Fronczek, V. J. Gatto, C. Minganti, R. A. Schultz, B. D. White, K. A. Arnold, D. Mazzocchi, S. R. Miller, and G. W. Gokel: *J. Am. Chem. Soc.* **108**, 4078 (1986).
6. J. P. Long and F. W. Schuler: *J. Pharm. Sci.* **43**, 79 (1954).
7. D. E. Koshland, Jr.: *Proc. Natl. Acad. Sci. USA* **44** 98 (1958).
8. C. L. Hoppel: *Carnitine Palmitoyltransferase and Transport of Fatty Acids* (The Enzymes of Biological Membranes v. 2, Ed. A. Martonosi) pp. 119–143. Plenum Press (1976).
9. I. B. Fritz: *Adv. Lipid Res.* **1**, 285 (1963).
10. J. Bremer: *Physiological Rev.* **63**, 1420 (1983).
11. L. L. Bieber and S. Farrell: *Carnitine Acyltransferases* (The Enzymes v. 16, Ed. P. D. Boyer), pp. 627–644. Academic Press (1983).
12. R. Wolfenden: *Transition-State Affinity as a Basis for the Design of Enzyme Inhibitors* (Transition States of Biochemical Processes, Eds. R. D. Gandour and R. L. Schowen) pp. 555–578 (1978).
13. R. L. Schowen: *Catalytic Power and Transition-State Stabilization* (Transition States of Biochemical Processer, Eds. R. D. Gandour and R. L. Schowen) pp. 77–117 (1978).
14. R. D. Gandour, W. J. Colucci, and F. R. Fronczek: *Bioorg. Chem.* **13**, 197 (1985).
15. W. J. Murray, K. W. Reed, and E. B. Roche: *J. Theor. Biol.* **82**, 559 (1980).
16. W. J. Colucci, R. D. Gandour, and E. S. Mooberry: *J. Am. Chem. Soc.* **108**, 7141 (1986).
17. W. J. Colucci, S. J. Jungk, and R. D. Gandour: *Mag. Reson. Chem.* **23**, 335 (1985).
18. K. G. R. Pachler: *J. Chem. Soc., Perkins Trans. 2*, 1936 (1972).
19. C. A. G. Haasnoot, F. A. A. M. de Leeuw and C. Altona: *Tetrahedron* **36**, 2783 (1980).
20. M. Karplus: *J. Phys. Chem.* **30**, 11 (1959).
21. G. E. Maciel, J. W. McIver, N. S. Ostlund, and J. A. Pople: *J. Am. Chem. Soc.* **92**, 4497 (1970).
22. G. Asknes and J. E. Prue: *J. Chem. Soc.*, 103 (1959).
23. W. J. Colucci, R. D. Gandour, F. R. Fronczek, P. S. Brady, and L. J. Brady: *J. Am. Chem. Soc.* **109**, 7915 (1987).
24. W. J. Colucci, S. P. Turnbull, Jr., and R. D. Gandour: *Analyt. Biochem.* **162**, 459 (1987).
25. B. Collier and F. C. MacIntosh: *Can. J. Physiol. Pharmac.* **47**, 127 (1969).
26. R. D. Gandour, W. J. Colucci, T. C. Stelly, P. S. Brady, and L. J. Brady: *Biochem. Biophys. Res. Commun.* **138**, 735 (1986).
27. R. D. Gandour, W. J. Colucci, P. S. Brady, and L. J. Brady: unpublished.
28. R. D. Gandour, W. J. Colucci, T. C. Stelly, P. S. Brady, and L. J. Brady: *Arch. Biochem. Biophys.* **267**, 515 (1988).
29. J. F. A. Chase and P. K. Tubbs: *Biochem. J.* **99**, 32 (1966).