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Investigating the Role of the Geminal Dimethyl Groups of Coenzyme A: Synthesis and Studies of a Didemethyl Analogue

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Abstract—An analogue **2** of coenzyme A (CoA) has been prepared in which the geminal methyl groups are replaced with hydrogens. An NMR titration study was conducted and shifts in frequency of protons in the pantetheine portion of the molecule upon titration of the adenine base were observed as has been previously reported with CoA. These studies indicate that the geminal dimethyl groups are not essential for adoption of a partially folded conformation in solution. Based on ^{1}H–^{1}H coupling constants, the distribution of conformations about the carbon–carbon bonds in the region of the methyl deletion were estimated. The results suggest that the conformer distribution is similar to that of CoA, but with small increases in population of the *anti* conformers. A simple model compound containing the didemethyl pantoamide moiety was prepared and subjected to similar conformational analysis. The coupling constants and predicted conformer distribution were almost identical to that of the CoA analogue, indicating that the conformer distribution is controlled by local interactions and not influenced by interactions between distant parts of the CoA molecule. The acetyl derivative of **2** was a fairly good substrate for the acetyl-CoA utilizing enzymes carnitine acetyltransferase, chloramphenicol acetyltransferase, and citrate synthase, with 1.3- to 10-fold increased K_m values and 2.5- to 11-fold decreases in V_{max}. The combined results indicate that the geminal dimethyl groups of CoA have modest effects on function and minimal effects on conformation.

Introduction

Coenzyme A (CoA) **1** and its acyl derivatives play a central role in various aspects of metabolism, being used by about 4% of all enzymes.[1] CoA is a fairly complex molecule, though only the thiol group is modified in the reactions involved in the biological function of CoA. The remainder of the molecule provides a handle for recognition and binding by CoA-utilizing enzymes but more specific roles for individual functionality are unclear. One especially interesting feature of the CoA molecule is the geminal dimethyl groups at C2″. The toxicity of α-ketobutyrate was suggested to be due to the incorporation of α-ketobutyrate into the pantoic acid domain of CoA to form a disfunctional monodemethyl analogue, based on the observation that the toxicity could be overcome with pantetheine.[2] Jencks and co-workers demonstrated that the functionality of the pantoate moiety of CoA, which includes these geminal dimethyl groups, destabilizes the covalent CoA–enzyme intermediate in the reaction of the enzyme 3-oxoacid-CoA transferase but greatly stabilizes the transition state leading to and from this intermediate.[2,3] This results in a large reduction in the energy of activation for this reaction. NMR has been used to study the conformation of CoA both in solution[4–7] and in enzyme complexes[8,9] and the conformation in the region of the geminal dimethyl groups has received substantial attention. The structures of several CoA-utilizing enzymes complexed with CoA or a CoA derivative have been solved by X-ray crystallography[10] and NMR.[11] These studies have shown that CoA exists both in solution and in enzyme complexes with primarily a gauche conformation about the C1″–C2″ bond (see Fig. 1 for numbering). The geminal dimethyl groups might be expected to induce or at least reinforce this conformation due to the geminal-dialkyl effect.[12,13] However, an analogue of CoA having these methyl groups deleted has not been available for direct study of the influence of the methyl groups on the conformation and enzymatic activity.

We report here the synthesis of the analogue of CoA **2** in which the geminal methyl groups are replaced with hydrogens. An NMR titration study indicated shifts in frequency of protons in the pantetheine portion of the

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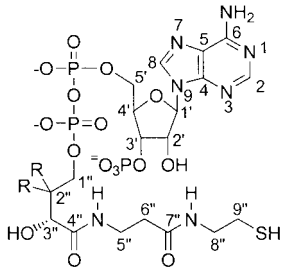


1, R = CH_3 (CoA)
2, R = H (didemethyl-CoA)

Figure 1. Structures of coenzyme A and a didemethyl analogue.

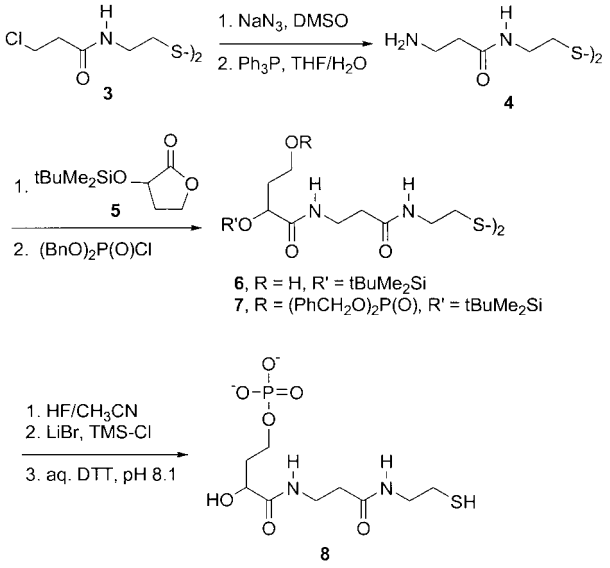
molecule upon titration of the adenine base, as has been previously reported with CoA.[4,5] These studies indicate that the geminal dimethyl groups are not essential for adoption of a partially folded conformation in solution. ^1H–^1H coupling constants were measured to analyze the conformer distribution about the carbon–carbon bonds in the region of the methyl deletion. The results suggest that the conformer distribution is similar to that of CoA, but with a small increase in population of the *anti* conformers. Conformational analysis of a simple model compound containing the didemethyl pantoamide moiety gave almost identical results to the didemethyl CoA analogue, indicating that the conformer distribution is controlled by local interactions. The acetyl derivative of **2** was a fairly good substrate for three acetyl-CoA utilizing enzymes. The role of the geminal dimethyl groups in CoA is discussed in light of these results.

Results

Synthesis of a didemethyl analogue of CoA

The CoA analogue **2** having the geminal pair of methyl groups replaced with hydrogens was prepared using the last two enzymes of CoA biosynthesis to perform the final steps. The didemethyl analogue of phosphopantetheine **8**, which served as the substrate for these final two enzymatic steps was prepared nonenzymatically as shown in Scheme 1. The disulfide of *N*-thioethyl-3-chloropropionamide **3**[14] was reacted with sodium azide in DMF[15] followed by reduction of the azide with triphenylphosphine in aqueous THF[16] to form the amine **4**.[17] The reaction with triphenylphosphine also reduced the disulfide to the thiol, which was oxidized back to the disulfide upon exposure to air. Reaction of **4** with the racemic *t*-butyldimethylsilyl-protected α-hydroxy-γ-butyrolactone **5**[18] gave the protected pantetheine analogue **6**. Reaction of **6** with dibenzyl phosphorochloridate gave the dibenzyl phosphate triester **7**.[19] Reaction with HF removed the *t*-butyldimethylsilyl protecting group and was followed by reaction with trimethylsilyl chloride and lithium bromide to convert the phosphate benzyl esters to the trimethylsilyl esters.[20] Removal of the trimethylsilyl groups upon addition of water and reduction of the disulfide with dithiothreitol formed the phosphopantetheine analogue **8**.

The didemethyl analogue of phosphopantetheine **8** was converted to the corresponding CoA analogue **2** using

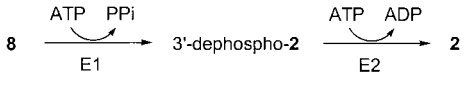


Scheme 1.

ATP and the final two enzymes of CoA biosynthesis as shown in Scheme 2. Prior to preparative conversion of **8** to **2**, kinetic analysis of **8** as a substrate for phosphopantetheine adenylyltransferase was performed in a coupled enzyme assay with pyrophosphate-dependent phosphofructokinase.[21] The V_{max} for racemic **8** was almost 6-fold lower than that for the natural substrate phosphopantetheine, and the K_m value of 5.7 mM was 73-fold higher than that for the natural substrate. Kinetic analysis of the product didemethyldephospho-CoA with the final enzyme of CoA biosynthesis, dephospho-CoA kinase was not performed. The enzymes of Scheme 2 were isolated from *Corynebacterium* (formerly *Brevibacterium*) *ammoniagenes* and immobilized in polyacrylamide gel as described previously.[22,23] The first enzyme, phosphopantetheine adenylyltransferase catalyzed the coupling of **8** with the α-phosphate of ATP to give the 3′-dephospho analogue of **2**. Inorganic pyrophosphatase was included to hydrolyze the pyrophosphate formed in this reaction to make the reaction irreversible. Dephospho-CoA kinase catalyzed the selective phosphorylation of the 3′-hydroxyl group by ATP to give the CoA analogue **2**. Phosphoenolpyruvate and pyruvate kinase were included to regenerate ATP from the ADP formed in this step.[24] The product **2** was purified by ion-exchange chromatography on DEAE cellulose, followed by reverse-phase HPLC.

NMR analysis of 2

Initial comparison of the ^1H NMR spectrum of **2** with that of CoA revealed differences in the protons of the modified pantoate moiety. The singlets at 0.71 and



E1 = phosphopantetheine adenylyltransferase
E2 = dephosphocoenzyme A kinase

Scheme 2.

0.84 ppm for the diastereotopic methyl groups at C2″ of CoA are replaced with complex multiplets of the C2″ protons of **2** at 1.63 and 1.96 ppm (numbering is shown in Fig. 1). Based on coupling constants and relative chemical shift, the resonance at 1.63 ppm was assigned to the pro-*S* proton (H2″$_S$) and the resonance at 1.96 to the pro-*R* proton (H2″$_R$) (see discussion section). H3″, which for CoA gives a singlet at 3.94 ppm, appeared near 4.1 ppm and overlapped with the C-5′ protons of the ribose moiety. The protons of C-1″, which give distinct signals at 3.80 and 3.54 ppm for CoA, appeared as an overlapping multiplet at 3.9 ppm for **2**.

^{1}H NMR spectra of **2** were obtained in D_2O at 13 different pD values ranging from pD 2.35 to 13.43. Protons of the pantetheine moiety exhibited changes in chemical shift as the pD crossed the pK_a of the adenine base (pK_a = 4.3), the 3′-phosphate (pK_a = 6.3) and the thiol group (pK_a = 9.8), as was observed previously with CoA.[4,5] Table 1 shows the differences in chemical shift of the protons at C5″, C6″, C8″ and C9″ on going from pH 3.62 to 5.40, along with the previously reported changes in chemical shift of the equivalent protons of CoA. As with CoA, the same protons exhibited smaller changes in chemical shift (0.003–0.007 ppm) upon titration of the 3′-phosphate group and the protons at C8″ and C9″ exhibited additional upfield shifts upon ionization of the thiol group. All of these results are very similar to the NMR titration results previously reported with CoA.[5]

Analysis of the ^{1}H–^{1}H coupling constants in the region of the modified pantoate moiety of **2** was performed. Analysis was hampered by the fact that the resonances for the protons at C1″ overlapped with each other and proton H3″ overlapped with protons of the ribose ring. However, the protons at C2″ were well separated from each other and from other protons in the molecule. In the fully coupled spectrum, the multiplicities of these protons were too complex to analyze, thus decoupling experiments were performed. When the C1″ protons were decoupled, the signals for the C2″ protons were each simplified to a doublet of doublets. Both exhibited a large coupling constant (14.4 Hz), which is attributed to the geminal coupling. The smaller splittings correspond to coupling by H3″ and give coupling constants of about 2.7 Hz and 9.0 Hz for the C2″ protons at 1.96 and 1.63, respectively.

Analysis of the C2″ protons when H3″ was decoupled was made more complex by the presence of three coupling constants for each proton, due to splitting by both C1″ protons in addition to the geminal splitting. The decoupled spectrum showed an apparent doublet of triplets for each proton. Again, the large coupling constant is attributed to geminal coupling of about 14.4 Hz. The remaining triplet structure for each proton indicates that each proton has approximately equal coupling constants with both C1″ protons. For the proton at 1.63 ppm, these coupling constants are about 4.8 Hz while for the proton at 1.96 ppm they are about 7.3 Hz. The coupling constants for the didemethylpantoate moiety of **2** are summarized in Table 2.

Table 1. Changes in chemical shift of protons of the pantetheine moiety of **1** and **2** upon titration of the adenine base

	H5″	H6″	H8″	H9″
Δδ **1**[a]	0.034	0.035	0.031	0.021
Δδ **2**[b]	0.035	0.032	0.022	0.019

[a]Data from ref 5.
[b]Difference in chemical shift at pD 3.62 versus pD 5.40.

Synthesis and NMR analysis of a model for the modified pantoate moiety of 2

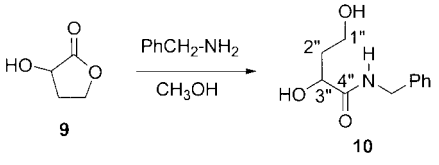
N-Benzyl 2,4-dihydroxybutanamide **10** was prepared by reaction of benzyl amine with racemic 2-hydroxy-γ-butyrolactone **9** (Scheme 3). Similarly to **2**, the protons at C2″ in the ^{1}H NMR spectrum of **10** were well separated (numbering analogous to CoA). As with **2**, coupling constants between H3″ and the C2″ protons were determined by decoupling the protons at C1″ while coupling constants of the C2″ protons with the protons at C1″ were determined by decoupling H3″. The coupling constants for **10** are also given in Table 2 and are very similar to the analogous coupling constants in **2**.

Fitting of coupling constants with rotamer populations

For analysis of the conformation about the C1″–C2″ and C2″–C3″ bonds of **2** and **10**, coupling constants for each proton pair in each low energy staggered conformation were predicted for 2,4-dihydroxybutanamide-4-phosphate using Macromodel.[25] The rotamer designations as used previously are shown in Figure 2.[6,7] The predicted coupling constants between H3″ and the pro-*S* and pro-*R* protons at C2″ are given in Table 3. Likewise, the predicted coupling constants between each of the C1″ protons and each of the C2″ protons are given in Table 4. The populations of each low-energy rotamer about the C1″–C2″ and C2″–C3″ bonds which best fit the predicted and measured coupling constants were then calculated,[26] with the results shown in Table 5. Shown for comparison are the rotamer populations for CoA **1** as predicted by Wu et al.[7]

Table 2. Measured coupling constants for **2** and **10**

	2		**10**	
	H2″$_S$	H2″$_R$	H2″$_S$	H2″$_R$
H1″$_S$	4.8	7.3	5.5	7.3
H1″$_R$	4.8	7.3	5.5	7.3
H3″	9.0	2.7	8.5	3.6



9 **10**

Scheme 3.

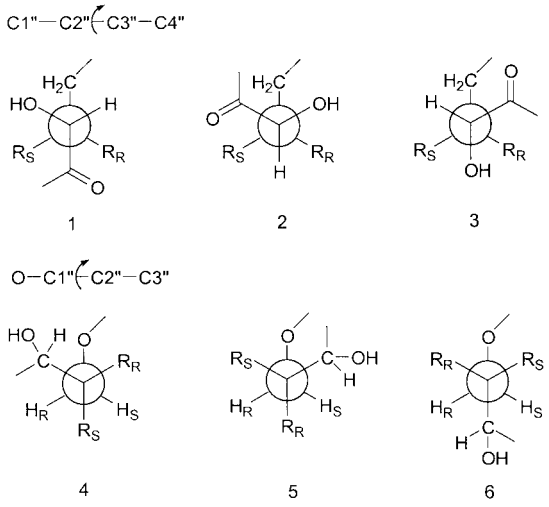


Figure 2. Newman projections of the low-energy rotamers about the C2″–C3″ and C1″–C2″ bonds of coenzyme A.

Table 3. Predicted coupling constants for H3″ with H2″$_S$ and H2″$_R$

	Rotamer		
	1	2	3
H2″$_S$	11.5	2.5	3.7
H2″$_R$	1.9	3.5	11.5

Table 4. Predicted coupling constants for H1″$_S$ and H1″$_R$ with H2″$_S$ and H2″$_R$

	Rotamer					
	4		5		6	
	H1″$_S$	H1″$_R$	H1″$_S$	H1″$_R$	H1″$_S$	H1″$_R$
H2″$_S$	2.7	1.1	3.7	12.0	11.5	2.3
H2″$_R$	1.9	11.8	11.8	3.1	0.7	3.4

Table 5. Calculated conformer distributions for **1**, **2**, and **10** (%)

	C1″–C2″–C3″–C4			O–C1″–C2″–C3		
	1	2	3	4	5	6
1[a]	36	62	2	64	28	8
2	72	24	4	49	11	40
10	59	27	14	39	14	47

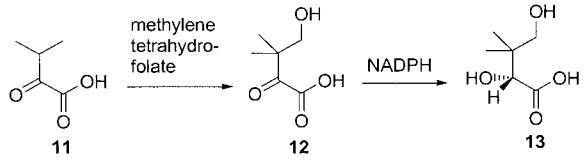
[a]Data from ref 7.

2 as a substrate for CoA-utilizing enzymes

2 was converted to the acetyl derivative by reaction with thiophenyl acetate. The resulting acetyl-CoA analogue was tested as a substrate for the acetyl-CoA-utilizing enzymes carnitine acetyltransferase, chloramphenicol acetyltransferase, and citrate synthase. The resulting K_m and V_{max} values are given in Table 6. With carnitine acetyltransferase, the V_{max} was 9% of that observed with acetyl-CoA while the K_m value was increased only slightly. With chloramphenicol acetyltransferase, the V_{max} for the analogue was about 40% that with acetyl-CoA while the K_m was increased about 8-fold. With citrate synthase, the V_{max} was 11% of that with acetyl-CoA while the K_m was about 10-fold higher.

Discussion

It is unclear why CoA contains the *gem*-dimethyl functionality in the pantoate moiety, but a possible issue is biosynthetic accessibility. The biosynthetic pathway for pantoic acid **13** (Scheme 4)[27] illustrates that the methyl groups originate from α-keto isovaleric acid **11**, which is derived from the transamination of valine or in bacteria by the pathways of branched-chain amino acid biosynthesis.[28] Reaction of **11** with methylene tetrahydrofolate produces ketopantoate **12** which is followed by reduction to form **13**. If pyruvate were substituted for **11** in this pathway and the subsequent steps of CoA biosynthesis, the product would be the didemethyl analogue **2** while substitution by α-ketobutyrate would result in a product with a single methyl group at this position.[29] α-Ketobutyrate is an intermediate in the threonine to isoleucine biosynthetic pathway, and a byproduct in the homocysteine/serine to cysteine pathway while pyruvic acid plays a role in many cellular processes, thus both would be expected to be present in cells.[28] Thus, the inclusion of these methyl groups is not imposed by the availability of biosynthetic building blocks. A possible role of the methyl groups in prebiotic synthesis of CoA has also been discussed.[30]



Scheme 4.

Table 6. Comparison of acetyl-CoA and acetyl-**2** as substrates for acetyl-CoA utilizing enzymes[a]

	Carnitine acetyltransferase		Chloramphenicol acetyltransferase		Citrate synthase	
	K_m (μM)	rel. V_{max}	K_m (μM)	rel. V_{max}	K_m (μM)	rel. V_{max}
Acetyl-CoA	60 (5)	1	32 (3)	1	7 (1)	1
Acetyl-**2**	77 (7)	0.09 (0.02)	250 (30)	0.41 (0.1)	68 (8)	0.11 (0.03)

[a]Numbers in parentheses are standard error.

A likely role of the geminal methyl groups is that they would be expected to influence the conformation of the molecule. Geminal dialkyl groups have been shown to have large effects on conformation. A classical illustration of this effect is in the observed rates of reaction of 3,3-disubstituted glutarate monoesters. The 3,3-dimethylglutarate monoester **14b** cyclizes 19-fold faster than the unsubstituted glutarate monoester **14a** (Fig. 3).[31] The rate enhancement is attributed to an increase in the concentration of the gauche conformation of the C2–C3 and C3–C4 bonds required for cyclization. The effect of the methyl groups is even more striking in the equilibrium for lactone formation from pantoic acid **16b** and its demethylated equivalent 2,4-dihydroxybutyric acid **16a**. While the K_{eq} for lactone formation from pantoic acid **16b** in aqueous solution is 270, the K_{eq} for 2,4-dihydroxybutyric acid **16a** is only 0.67 under the same conditions (Fig. 3).[30]

The CoA analogue lacking the geminal dimethyl groups was prepared to study the effect of deletion of these methyl groups on the conformation and function of CoA. The final two steps of the synthesis were performed using the enzymes phosphopantetheine adenylyltransferase and dephosphocoenzyme A kinase, which catalyze the final two steps of CoA biosynthesis. These enzymes have been used in the synthesis of other CoA analogues in this lab.[23,32] The phosphopantetheine analogue **8** used for these final two steps was racemic. Phosphopantetheine adenylyltransferase has been shown to be highly selective for the natural *R*-enantiomer of a phosphopantetheine analogue,[32] thus it is expected that the 'natural' *R*-enantiomer of **8** was converted preferentially to the dephospho-CoA analogue and subsequently to the CoA analogue **2**. The actual K_m value for the *R*-enantiomer of **8** is expected to be 2.8 mM or one-half the K_m value measured for the racemic compound. Though the yield of the final two steps was low, apparently due to inefficient conversion of **8** to **2**, enough product was obtained for several studies to be performed.

Several NMR studies of the conformation of CoA have been reported. Lee and Sarma and subsequently Kiere et al. reported the effects of pH on the ^{1}H NMR spectrum of CoA, dephospho-CoA, and some CoA thioesters and disulfides.[4,5] These studies showed an upfield shift in protons of the pantetheine moiety of CoA as the pH was increased above the pK_a of the adenine amino group (pK_a = 4.3) and the 3′-phosphate (pK_a = 6.3). These pH effects were observed in CoA, dephospho-CoA as well as thioesters and disulfides of CoA, but not in phosphopantetheine. These distant effects were attributed to intramolecular interactions between the pantetheine moiety and the adenine base in a folded conformation of CoA in which the pantetheine tail is coiled around the adenine base. The increased shielding at higher pH was attributed to a decrease in the population of this folded conformation. The intramolecular interaction responsible for these effects has not been addressed. Interaction between protonated adenine and the pyrophosphate group might be possible, though the chemical shift effects are observed in parts of the pantetheine moiety quite distant from the pyrophosphate group. Lee and Sarma estimated that no more than 30% of CoA exists in the folded conformation on a time-average basis.[4] The folded and 'open' conformations were postulated to interconvert via rotation about the pyrophosphate moiety. A similar folded conformation was proposed previously for benzoyl-CoA.[33] Studies reported here of the pH dependence on chemical shift of protons of the modified pantetheine moiety of **2** gave very similar results to those observed with CoA. While the pD range studied pushes the limits of accurate pH meter readings, the inflection points are all within the range of reliable measurements. Of the data included in Table 1, the differences in Δδ for H5″, H6″, and H9″ are certainly within experimental error and the difference for H8″ may also be. This suggests that deletion of the methyl groups of CoA has no significant effect on the existence of the proposed folded conformation of CoA in solution.

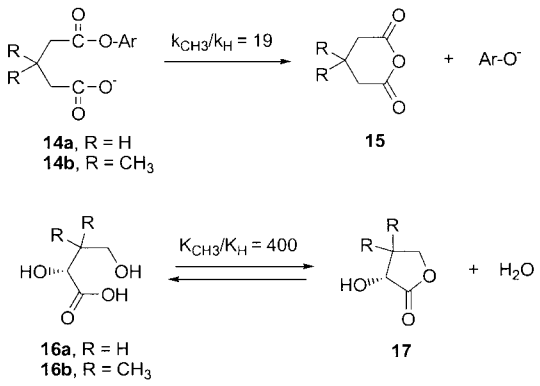


Figure 3. Effects of geminal dimethyl groups on cyclization reactions.

Coupling constants between the protons of the modified pantoate moiety of **2** were determined to analyze the conformation of **2** and for comparison of its conformation with that of natural CoA. The coupling constants between H3″ and the C2″ protons were easily determined upon decoupling of the C1″ protons. The coupling constants were initially used to tentatively assign the H2″$_R$ and H2″$_S$ chemical shifts. The coupling constant of 2.7 Hz between H3″ and the proton at 1.96 ppm indicates that these two protons are almost exclusively gauche to each other. The coupling constant of 9.0 Hz between H3″ and the proton at 1.63 ppm indicates that these protons are largely *anti* to each other. It has been shown that CoA exists as a mixture of conformers 1 and 2 about the C2″–C3″ bond (Fig. 2), with conformation 3 estimated to represent only 2% of the dynamic population.[7] If **2** is assumed to also exist primarily as a mixture of conformers 1 and 2, with little contribution of conformer 3, the signal at 1.96 ppm must be assigned to the pro-*R* proton. This proton is gauche to H3″ in both of the populated conformers while the pro-*S* proton is gauche and *anti* to H3″ in the two populated conformers and thus has the larger coupling constant. The opposite assignment would require that conformers 2 and 3 be predominant, with little population of conformer 1. Such a dramatic change in rotamer populations appears especially unlikely given the fairly good

activity of the acetyl derivative of **2** with the three enzymes tested. Furthermore, loss of the geminal dialkyl effect would appear to favor an increase in population of rotamer 1 for **2** relative to CoA, thus a dramatic decrease in the population of rotamer 1 appears very improbable. This assignment is further supported by comparison of the chemical shifts of the C2″ protons with those of the corresponding methyl groups of CoA. The more upfield methyl signal in the ^{1}H NMR spectrum of CoA has been assigned to the pro-*S* methyl group while the more downfield signal has been assigned to the pro-*R* methyl group.[7] It is thus consistent that the pro-*S* proton at C-2″ of **2** is more upfield than the pro-*R* proton.

The relative rotamer populations estimated based on coupling constants (Table 5) suggest that for **2** as with CoA, the population of rotamer 3 about the C2″–C3″ bond is very small. This can be seen more simply and conclusively in that H2″$_S$ and H3″ must be almost exclusively gauche to each other, as in rotamers 1 and 2 to give the small coupling constant of 2.7 Hz. Any significant population of rotamer 3 would give rise to larger coupling. The data also suggests that for **2**, there is a greater population of the *anti* conformation 1 than is observed with CoA. The coupling constant of 9.0 Hz measured between H2″$_S$ and H3″ is almost large enough to be consistent with the *anti* rotamer 1 being the exclusive conformation, though the significant population of rotamer 2 gives a better fit with the coupling constants predicted by Macromodel. The data suggests that the conformation of **10** about the C2″–C3″ bond is similar to that of **2**, though with a moderately greater population of conformer 3 relative to 1 for **10**.

Regarding the conformation about the C1″–C2″ bond, for CoA the population of the *anti* conformation 6 has been determined to be small while the population of the gauche conformation 4 is estimated to be more than double that of the other gauche conformation 5. Estimates for **2** indicate that the population of the *anti* conformation 6 is somewhat greater than that observed in CoA while as with CoA, the gauche conformation 4 is more populated than the other gauche conformation 5. While the ratios of conformers 4 to 5 are different between **1** and **2**, it is not clear if this difference is significant given the potential error. The estimated conformer distribution for **10** is again very similar to that for **2**.

The estimated rotamer populations of Table 5 are subject to errors in both the measured and predicted coupling constants. While there is thus some uncertainty in the accuracy of the estimated conformer distributions, some definite conclusions can be made. First, the coupling constants and the estimated rotamer populations for **2** and the simple model compound **10** are very similar. This indicates that the conformation of the pantoate moiety is controlled by short-range interactions, with possible long-range interactions such as those predicted between the adenine and pantetheine moieties not having a major influence on the local conformation of the pantoate moiety. The second conclusion is that the structure of **2**, like CoA, is not dominated by a single conformation. While the coupling constants could perhaps be rationalized based on a predominantly *anti* conformation about the C2″–C3″ bond, the coupling constants are clearly not consistent with a single conformation about the C1″–C2″ bond.

The greater estimated population of *anti* conformers (1 and 6) about the C2″–C3″ and C1″–C2″ bonds for **2** relative to CoA is consistent with the expectation that the methyl groups at C2″ of CoA would tend to stabilize the gauche conformations about these bonds. However, the differences in estimated conformer populations are very modest and appear to be substantially smaller than the effects observed in other compounds, such as the classical glutarate monoesters. It is likely that other factors are important such as the gauche effect, which tends to favor the positioning of polar groups gauche to a neighboring C–C bond and *anti* to a C–H bond as has been extensively studied with fluorinated alkanes.[34] This effect in **2** and **10** could mirror the conformational effect of the methyl groups of **1**, thus helping to rationalize the similarities in predicted rotamer populations. Dipole–dipole interactions, especially involving the polar C–O bonds at C1″ and C3″ may also play a role in the conformer distribution and in the interplay between conformations about the C2″–C3″ and C1″–C2″ bonds, as has been studied in some detail for 1,3-difluoropropane.[35]

The differences in V_{max} and K_m for acetyl-**2** relative to acetyl-CoA with three acetyl-CoA utilizing enzymes as shown in Table 6, and for **8** relative to phosphopantetheine with phosphopantetheine adenylyltransferase may be attributed in part to the influence of the methyl groups on the relative population of the conformation of CoA recognized by the enzymes. Another factor could be the favorable transfer of the geminal dimethyl groups from aqueous solution to a more hydrophobic environment of an enzyme active site. The simple kinetic data does not permit clear distinction of possible effects. However, conformational effects would probably only influence the K_m by affecting the concentration of the active conformation. The only slight increase in K_m for acetyl-**2** versus acetyl-CoA with carnitine acetyltransferase further suggests that the methyl groups do not have a major effect on the conformation of CoA, or at least on the population of the conformer of acetyl-CoA bound by carnitine acetyltransferase. Other factors including hydrophobic effects might influence both K_m and V_{max} by differential effects on binding in the ground state and transition state complexes. The hydrophobic effect of a methyl group was predicted by Pauling to provide a maximum 0.9 kcal/mol of binding energy based on the energy of transfer of a methyl group from an aqueous to hydrophobic environment,[36] though effects of up to 3.6 kcal/mol have been observed in the aminoacyl-*t*RNA synthetases.[37]

The largest effect of deletion of the methyl groups on enzyme activity was observed in the utilization of **8** as a substrate for phosphopantetheine adenylyltransferase in the synthesis of **2**. This might be expected based on the proximity of the reaction to the site of modification.

Further analysis of the recently reported crystal structure of the phosphopantetheine adenylyltransferase[38] may provide further insight into the basis for the 6-fold decrease in V_{max} and 36-fold increase in K_m (assuming only the *R*-enantiomer of **8** is a substrate). For carnitine acetyltransferase, there is no crystal structure or other information regarding the conformation of CoA in the enzyme complex with which to compare the kinetic results. For both citrate synthase and chloramphenicol acetyltransferase, the *anti* conformation 1 about the C2″–C3″ bond and the gauche conformation 5 about the C1″–C2″ bond is observed in the crystal structures of the enzyme complexes with substrate and substrate analogues.[6] Interestingly, NMR analysis of the substrate complex of chloramphenicol acetyltransferase has suggested that the gauche conformation 3 about the C2″–C3″ bond may be important.[8] The data of Table 5 predict a modest increase in the population of conformer 1 and a similar decrease in the population of conformer 5 in **2** relative to CoA. In simplest terms, this would predict approximately equal populations of the 1,5 conformation for **2** versus CoA, though this does not consider that the conformations about these adjacent C–C bonds may influence each other. It thus appears that conformational effects resulting from deletion of the methyl groups are not a major factor in the differences in kinetic data for acetyl-CoA versus acetyl-**2**. Thus, both conformational and enzyme kinetic studies suggest that the geminal dimethyl groups do not play a major conformational role in the function of CoA.

Summary

The studies of the didemethyl analogue of CoA **2** reported here do not support a major conformational role of the geminal dimethyl groups of CoA. The pH-dependent chemical shifts of protons of the pantetheine moiety support a folded conformation for **2**, similar to CoA. Conformational populations estimated based on coupling constants suggest only modest increases in *anti* relative to gauche conformations of the pantoate moiety upon deletion of the geminal dimethyl groups. While the methyl groups of CoA may have a small effect on the conformation which may result in a similar effect on its activity in enzymatic reactions, other effects such as hydrophobic interactions are probably of greater importance.

Experimental

General experimental

Enzymes and enzyme substrates and cofactors were obtained from Sigma. Other chemicals were obtained from Aldrich. Dichloromethane, toluene, and acetonitrile were distilled from calcium hydride. Tetrahydrofuran was distilled from sodium benzophenone radical. NMR experiments were conducted on Varian Gemini 200, Varian XL-400, and Varian Inova 500 and 600 MHz spectrometers. In ^{1}H NMR experiments, TMS (0 ppm) and HOD (4.8 ppm) were used as internal reference in $CDCl_3$ and D_2O, respectively. Mass spectral analysis was performed at the University of California, Riverside Mass Spectrometry facility, Riverside, CA.

***N*-Mercaptoethyl-3-aminopropionamide disulfide (4).**[17] To a solution of **3**[14] (10.1 g, 30.4 mmol) in dimethyl sulfoxide (120 mL) was added sodium azide (5.9 g, 91 mmol) and the reaction mixture was stirred at 50 °C overnight. Water (100 mL) was added and the precipitate was collected via filtration, rinsed with water, and dried in vacuo overnight to yield a white solid (5.3 g). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.55 (s, 1H), 3.63 (overlapping triplets, 4H), 2.85 (t, 2H), 2.5 (t, 2H). The solid was dissolved in THF (110 mL) and triphenylphosphine (8.08 g, 30.8 mmol) and water (0.67 mL, 37.2 mmol) were added. The reaction mixture was stirred under nitrogen at room temperature overnight. THF was evaporated in vacuo, and the residue was redissolved in methylene chloride (300 mL). The organic layer was extracted with water (3×100 mL) and the aqueous layers were combined and lyophilized to yield a white solid (3.88 g, 13.2 mmol, 43% yield): ^{1}H NMR (200 MHz, D_2O): δ 3.51 (t, 2H), 2.93 (t, 2H), 2.85 (t, 2H), 2.42 (t, 2H).

3-*O*-*tert*-Butyldimethylsilyl-2,2-didemethylpantethine (6). A solution of **4** (3.07 g, 10.44 mmol) and **5**[18] (4.54 g, 21 mmol) in methanol (90 mL) was stirred at room temperature overnight. The reaction mixture was concentrated in vacuo, and the remaining oil was purified on silica gel with 3:1 ethyl acetate/methanol. Fractions containing **6** were combined and concentrated under reduced pressure and the residue was dissolved in ethyl acetate, filtered to assure removal of silica gel, and concentrated to yield a yellow oil (6.12 g, 81% yield): ^{1}H NMR (200 MHz, $CDCl_3$): δ 4.25 (dd, 1H), 3.7 (t, 2H), 3.5 (m, 4H), 2.8 (t, 2H), 2.45 (m, 2H), 1.9 (m, 1H), 1.7 (m, 1H), 0.9 (s, 9H), 0.12 (s, 3H), 0.10 (s, 3H).

3-*O*-*tert*-Butyldimethylsilyl-2,2-didemethylpantethine-1-*O*-dibenzylphosphate (7). To a solution of *N*-chlorosuccinimide (5.33 g, 39.9 mmol) in toluene (100 mL) was added dibenzyl phosphite (8.7 mL, 39.5 mmol). The solution was stirred for 2 h under nitrogen, during which time the reaction flask was cooled in an ice bath as needed to prevent refluxing. The resulting dibenzyl phosphorochloridate solution was used immediately in the next reaction.

Compound **6** (3.1 g, 4.2 mmol) was dissolved in pyridine (25 mL) and the solvent was evaporated in vacuo to remove residual water. The residue was redissolved in dry pyridine (50 mL) and cooled in an acetonitrile–dry ice bath. The dibenzyl phosphorochloridate solution was added to the reaction flask over 10 min. The reaction mixture was allowed to warm to room temperature and was stirred under nitrogen overnight. Water (30 mL) was added and the resulting mixture was concentrated under reduced pressure to a brown oil. The residue was redissolved in ethyl acetate (200 mL) and washed sequentially with aq sulfuric acid (1 M, 2×50 mL), aq sodium bicarbonate (1 M, 2×50 mL), and saturated aq sodium sulfate (1×50 mL). The organic layer was dried

over $MgSO_4$ and concentrated under reduced pressure to yield a golden oil. The product was purified on silica gel with 3:1 ethyl acetate/methanol. Fractions containing **7** were combined and concentrated under reduced pressure and the residue was dissolved in ethyl acetate, filtered to assure removal of silica gel, and concentrated to yield **7** as a yellow oil (2.22 g, 42% yield): ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.35 (m, 10H), 5.0 (d, 4H), 4.2 (m, 3H), 3.5 (m, 4H), 2.75 (t, 2H), 2.4 (t, 2H), 1.9–2.1 (m, 2H), 0.9 (s, 9H), 0.1 (s, 6H).

2,2-Didemethylpantethine-1-*O*-phosphate (8-disulfide). To a solution of **7** (1.38 g, 1.09 mmol) in acetonitrile (7 mL) was added aqueous hydrofluoric acid (0.18 mL, 48%). The reaction mixture was stirred for 2.5 h at room temp. Chloroform (100 mL) was added, and the organic layer was washed with sat aq NaCl (2×50 mL), dried over $MgSO_4$, and concentrated in vacuo to yield a yellow oil (0.80 g). ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.35 (m, 10H), 5.0 (m, 4H), 4.2 (m, 3H), 3.5 (m, 4H), 2.75 (t, 2H), 2.4 (t, 2H), 1.9–2.1 (m, 2H). To a solution of the resulting oil (0.80 g, 0.79 mmol) in dry acetonitrile (6 mL) was added lithium bromide (0.29 g, 3.29 mmol). Acetonitrile was evaporated in vacuo to remove residual water and the residue was redissolved in dry acetonitrile (6 mL). Chlorotrimethylsilane (2 mL, 15.7 mmol) was added and the reaction mixture was stirred under nitrogen at 50 °C overnight. The mixture was concentrated under reduced pressure, dissolved in water (5 mL), washed with ether, and lyophilized. The resulting mixture containing inorganic salts was purified by preparative C-18 reverse-phase HPLC, monitoring at 270 nm, and using a gradient of methanol in 0.2% aqueous trifluoroacetic acid. Fractions containing **8**-disulfide were lyophilized to obtain a white solid: ^{1}H NMR (400 MHz, D_2O): δ 4.25 (dd, 1H, J = 5.8, 3.3 Hz), 3.96 (m, 2H), 3.49 (m, 4H), 2.82 (t, 2H, J = 6.3 Hz), 2.46 (t, 2H, J = 6.6 Hz), 2.06–2.14 (m, 1H), 1.75–1.84 (m, 1H); ^{13}C NMR (100 MHz, D_2O): δ 175.36, 173.08, 67.06, 61.69, 37.12, 35.62, 34.73, 34.46, 33.45; HRMS (FAB): $[M-H^+]^-$ calcd for $C_{18}H_{35}N_4O_{14}P_2S_2$ m/z 657.1066, found 657.1097.

Determination of kinetic parameters for 8 with phosphopantetheine adenylyltransferase

To a solution of pyrophosphate reagent (Sigma Chemical Company, 1.0 mL) containing added ATP (5 mM) was added phosphopantetheine adenylytransferase (0.01 units) and **8**-disulfide (1.0 to 10.3 mM, preincubated with 1 M DTT at pH 7 for 1 hour to reduce the disulfide) and the decrease in absorbance at 340 nm was observed over time.

2″,2″-Didemethyl-coenzyme A (2). To a solution of **8**-disulfide (181 mg, 275 μmol) in HEPES buffer (2 mL, 0.1 M, pH 8.1) was added dithiothreitol (42.3 mg, 275 μmol). The solution was incubated for 1 hour, after which it was added to a solution of HEPES buffer (10 mL, 0.1 M, pH 8.1) containing ATP (231 mg, 400 μmol), $MgCl_2$ hydrate (81 mg, 400 μmol), and phosphoenolpyruvate (83.2 mg, 400 μmol). The soluble enzymes pyruvate kinase (50 units) and inorganic pyrophosphatase (50 units) were added along with the enzymes phosphopantetheine adenylyltransferase (3 units) and dephosphocoenzyme A kinase (3 units), which were partially purified from *Corynebacterium ammoniagenes* and coimmobilized in polyacrylamide gel as described previously.[23] The resulting suspension was slowly stirred at room temperature for 6 days. After 2 days, additional pyruvate kinase (50 units) and inorganic pyrophosphatase (50 units) were added, and after 4 days additional ATP (100 mg, 170 μmol), phosphoenolpyruvate (40 mg, 200 μmol), pyruvate kinase (50 units) and inorganic pyrophosphatase (50 units) were added. The reaction was monitored by analytical reverse phase HPLC (Rainin Microsorb C-18 column, 4.6 mm×25 cm, flow rate 1 mL/min, 5 min at 5% methanol in 50 mM phosphate buffer, pH 4.5 followed by a linear gradient to 60% methanol over 12 min and then maintained at 60% methanol) and after 6 days no more **8** (retention time 12.1 min) was observed. The reaction mixture was centrifuged to remove immobilized enzymes, which were further washed with water (3×20 mL). The combined reaction solution and washes were diluted to 80 mL with water, and the pH adjusted to 2.5 with aqueous HCl. The solution was loaded onto a DEAE cellulose column (2.5×20 cm) and eluted with a linear gradient of LiCl (0–0.2 M) in 3 mM HCl (800 mL total volume) at a flow rate of 0.5 mL/min at 4 °C. **2** eluted between 0.12 and 0.16 M LiCl and was detected by reaction of aliquots of individual fractions with DTNB at pH 8.0. The fractions containing **2** were combined, lyophilized, and further purified by reverse-phase HPLC (5 min at 5% methanol in 10 mM phosphate buffer, pH 4.5 followed by a linear gradient to 40% methanol over 30 min, **2** eluted between 16 and 20 min) to give **2** (12.1 mg, 6%). ^{1}H NMR (400 MHz, D_2O): δ 8.54 (s, 1H), 8.30 (s, 1H), 6.14 (d, 1H, J = 5.6 Hz), 4.53 (s, 1H), 4.15–4.20 (m, 3H), 3.90–4.02 (m, 2H), 3.39 (t, 2H, J = 6.2 Hz), 3.26 (t, 2H, J = 6.4 Hz), 2.54 (t, 2H, J = 6.6 Hz), 2.38 (t, 2H, J = 6.6 Hz), 1.95–2.05 (m, 1H), 1.60–1.72 (m, 1H). ^{31}P NMR (161.9 MHz, D_2O, external H_3PO_4 reference): δ −10.72, −10.33, 0.38. HRMS (FAB): $[MH^+]$ calcd for $C_{19}H_{31}N_7O_{16}P_3S$ m/z 738.076, found 738.077.

pH-Dependent NMR shift studies of 2. The 400 MHz ^{1}H NMR spectrum of a solution of **2** (2.3 mg, 3 μmol) and trimethylsilyl-1-propane sulfonic acid (0.22 mg, 1 μmol) in 700 μL D_2O was recorded with suppression of the HOD signal. Proton shifts were referenced relative to the methyl groups of trimethylsilyl-1-propane sulfonic acid at 0 ppm. The pH was adjusted between spectra with aqueous DCl or NaOD, and pD was taken as the pH meter reading + 0.4. NMR spectra were recorded across the pD range of 2.35 to 13.43.

Synthesis of 10. To a solution of **9** (1.49 g, 11.2 mmol) in THF (26 mL) was added benzyl amine (2.39 g, 22.3 mmol). The solution was stirred at 55 °C for 4 h, then cooled to 4 °C. Aq HCl (10%, 10 mL) was added and the solution was extracted with ethyl acetate (3×20 mL). The combined organic layers were concentrated under reduced pressure and the remaining oil was purified on silica gel with ethyl acetate/hexane (R_f = 0.42 in 4:1 ethyl acetate/hexane) to yield 10 (1.93 g,

9.23 mmol): ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.2 (m, 5H), 5.5 (bs, 1H), 4.3 (d, 2H), 4.0 (m, 1H), 3.5 (m, 2H), 1.9 (m, 1H), 1.6 (m, 1H).

Synthesis of acetyl-2. To a solution of **2** (5 mg, 6.8 μmol) in HEPES buffer (1.0 mL, 0.025 M, pH 8.2) was added a solution of *S*-phenylthioacetate (0.1 mL, 0.75 mmol) in acetonitrile (0.9 mL). The resulting turbid solution was stirred vigorously for 1 hour at room temperature. The mixture was allowed to separate into two phases which were separated and the aqueous layer was extracted with ether (5×4 mL) and adjusted to pH 4.5 with aq HCl. The product was purified by reverse-phase HPLC (5 min at 5% methanol in 10 mM phosphate buffer, pH 4.5 followed by a linear gradient to 45% methanol over 30 min, product eluted between 19 and 24 min) to give acetyl-**2** (2.96 mg, 60%). ^{1}H NMR (400 MHz, D_2O): δ 8.54 (s, 1H), 8.28 (s, 1H), 6.16 (d, 1H, J = 6.0 Hz), 4.57 (s, 1H), 4.15–4.27 (m, 3H), 3.95–4.05 (m, 2H), 3.40 (t, 2H, J = 6.6 Hz), 3.30 (t, 2H, J = 5.8 Hz), 2.92 (t, 2H, J = 6.2 Hz), 2.37 (t, 2H, J = 6.6 Hz), 2.30 (s, 1H), 2.00–2.10 (m, 2H), 1.62–1.72 (m, 1H).

Enzyme assays. Assays of carnitine acetyltransferase were conducted in potassium phosphate buffer (25 mM, pH 7.5), containing 4,4′-dithiopyridine (PDS, 0.2 mM), l-carnitine (0.2 mM), carnitine acetyltransferase (0.03 units, from pigeon breast) and acetyl-CoA or acetyl-2 (20–200 μM) in a total volume of 1 mL by monitoring the increase in absorbance of the thiopyridine anion at 324 nm. Assays of chloramphenicol acetyltransferase were conducted in Tris buffer (10 mM, pH 7.8) containing chloramphenicol (50 μM), DTNB (1 mM), chloramphenicol acetyltransferase (0.03 units, from *Escherichia coli*) and acetyl-CoA (10 μM to 100 μM) or acetyl-**2** (50–500 μM) by monitoring the increase in absorbance of the nitrothiobenzoate dianion at 412 nm. Citrate synthase assays were conducted in Tris buffer (0.1 M, pH 8), containing DTNB (0.1 mM), oxalo-acetate (0.5 mM), citrate synthase (0.03 units, from porcine heart) and acetyl CoA (4–40 μM) or acetyl-**2** (20–200 μM) by monitoring the increase in absorbance at 412 nm.

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References and Notes

1. Lee, C.-H.; Chen, A. F. In *The Pyridine Nucleotide Coenzymes*; Everse, J., Anderson, B., You, K., Eds.; Academic: New York, 1982; p 189.
2. Primerano, D.; Burns, R. O. *J. Bacteriol.* **1982**, *150*, 1202. La Rossa, R. A.; VanDyk, T. K. *J. Gen. Microbiol.* **1989**, *135*, 2209.
3. Fierke, C. A.; Jencks, W. P. *J. Biol. Chem.* **1986**, *261*, 7603. Whitty, A.; Fierke, C. A.; Jencks, W. P. *Biochemistry* **1995**, *34*, 11678.
4. Lee, C.-H.; Sarma, R. H. *J. Am. Chem. Soc.* **1975**, *97*, 1225.
5. Keire, D. A.; Robert, J. M.; Rabenstein, D. L. *J. Org. Chem.* **1992**, *57*, 4427.
6. D'Ordine, R. L.; Paneth, P.; Anderson, V. E. *Bioorg. Chem.* **1995**, *23*, 169.
7. Wu, W.-J.; Tonge, P. J.; Raleigh, D. P. *J. Am. Chem. Soc.* **1998**, *120*, 9988.
8. Barsukov, I. L.; Lian, L.-Y.; Ellis, J.; Sze, K.-H.; Shaw, W. V.; Roberts, G. C. K. *J. Mol. Biol.* **1996**, *262*, 543.
9. Wu, W.-J.; Anderson, V. E.; Raleigh, D. P.; Tonge, P. J. *Biochemistry* **1997**, *36*, 2211.
10. For reviews of X-ray structures of CoA-utilizing enzymes see: Engel, C.; Wierenga, R. *Curr. Opin. Struct. Biol.* **1996**, *6*, 790. Modis, Y.; Wierenga, R. *Structure* **1998**, *6*, 1345. For more recently reported structures see: Benning, M. M.; Wesenberg, G.; Liu, R.; Taylor, K. L.; Dunaway-Mariano, D.; Holden, H. M. *J. Biol. Chem.* **1998**, *273*, 33572. Wybenga-Groot, L. E.; Draker, K.; Wright, G. D.; Berghuis, A. M. *Structure* **1999**, *7*, 497. Hickman, A. B.; Namboodiri, M. A. A.; Klein, D. C.; Dyda, F. *Cell* **1999**, *97*, 361. Trievel, R. C.; Rojas, J. R.; Sterner, D. E.; Venkataramani, R. N.; Wang, L.; Zhou, J.; Allis, C. D.; Berger, S. L.; Marmorstein, R. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 8931. Clements, A.; Rojas, J. R.; Trievel, R. C.; Wang, L.; Berger, S. L.; Marmorstein, R. *EMBO J.* **1999**, *18*, 3521.
11. Lin, Y.; Fletcher, C. M.; Zhou, J.; Allis, C. D.; Wagner, G. *Nature* **1999**, *400*, 86.
12. Allinger, N. L.; Zalkow, V. *J. Org. Chem.* **1960**, *25*, 701. Wheeler, O. H.; de Rodriguez, E. E. G. *J. Org. Chem.* **1964**, *29*, 1227.
13. Kirby, A. J. *Adv. Phys. Org. Chem.* **1980**, *17*, 183.
14. Airapetyan, G. M.; Zherebchenko, P. G.; Bystrova, V. M.; Benevolenskaya, Z. V.; Kuleshova, N. D.; Linkova, M. G.; Kildisheva, O. V.; Knunyants, I. L. *Bull. Acad. Sci. USSR, Div. Chem. Sci.* **1967**, 317.
15. Howson, W.; Kitteringham, J.; Mistry, J.; Mitchell, M. B.; Novelli, R.; Slater, R. A.; Swayne, G. T. G. *J. Med. Chem.* **1988**, *31*, 352.
16. Knouzi, M.; Vaultier, M.; Carrie, R. *Bull. Soc. Chim. Fr.* **1985**, 815.
17. Oiry, J.; Pue, J. Y.; Imbach, J. L.; Fatome, M.; Sentenac-Roumanou, H.; Lion, C. *J. Med. Chem.* **1986**, *29*, 2217.
18. Dauben, W. G.; Hendricks, R. T.; Pandy, B.; Wu, S. C.; Zhang, X.; Luzzio, M. J. *Tetrahedron Lett.* **1995**, *44*, 519.
19. Shemin, D., Ed. *Biochemical Preparations*; John Wiley & Sons: New York, 1957; Vol. 5, p 1.
20. McKenna, C. E.; Higa, M. T.; Cheung, N. H.; McKenna, M. *Tetrahedron Lett.* **1977**, 155.
21. O'Brien, W. E. *Anal. Biochem.* **1976**, *76*, 423.
22. Martin, D. P.; Drueckhammer, D. G. *Biochem. Biophys. Res. Commun.* **1993**, *192*, 1155.
23. Martin, D. P.; Bibart, R. T.; Drueckhammer, D. G. *J. Am. Chem. Soc.* **1994**, *116*, 4660.
24. Hirschbein, B. L.; Mazenod, G. M.; Whitesides, G. M. *J. Org. Chem.* **1982**, *47*, 3765.
25. Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Kiskamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W. C. *J. Comput. Chem.* **1990**, *11*, 440.
26. Pachler, K. G. R. *Spectrochim. Acta* **1964**, *20*, 581.
27. Teller, J. H.; Powers, S. G.; Snell, E. E. *J. Biol. Chem.* **1976**, *251*, 3780. Aberhart, D. J. *J. Am. Chem. Soc.* **1979**, *101*, 1354.
28. Epelbaum, S.; LaRossa, R. A.; VanDyk, T. K.; Elkayam, T.; Chipman, D. M.; Barak, Z. *J. Bacteriol.* **1998**, *180*, 4056.
29. Ketopantoate hydroxymethyltransferase, the enzyme that catalyzes the addition of the hydroxymethyl group to **11**, has been shown to accept α-ketobutyrate but not pyruvate as a substrate; Powers, S. G.; Snell, E. E. *J. Biol. Chem.* **1976**, *251*, 3786.

30. Miller, S. L.; Schlesinger, G. *J. Mol. Evol.* **1993**, *36*, 308.
31. Bruice, T. C.; Pandit, K. P. *J. Am. Chem. Soc.* **1960**, *82*, 5858. Lightstone, F. C.; Bruice, T. C. *J. Am. Chem. Soc.* **1997**, *119*, 9103.
32. Bibart, R. T.; Vogel, K. W.; Drueckhammer, D. G. *J. Org. Chem.* **1999**, *64*, 2903.
33. Mieyal, J. J.; Webster, L. T.; Siddiqui, U. A. *J. Biol. Chem.* **1974**, *249*, 2633.
34. Kunttu, H.; Raaska, T.; Rasanen, M. *Theochem* **1989**, *184*, 277.
35. Wu, D.; Tian, A.; Sun, H. *J. Phys. Chem.* **1998**, *102*, 9901.
36. Pauling, L. *Festschr. Prof. Dr. Arthur Stoll Siebzigsten Geburstag* **1958**, 597.
37. Fersht, A. R.; Shindler, J. S.; Tsui, W.-C. *Biochemistry* **1980**, *19*, 5520.
38. Izard, T.; Geerlof, A. *EMBO J.* **1999**, *18*, 2021.