Chemical Synthesis of a Proposed Enzyme-Generated "Reactive Intermediate Analogue" Derived from Thiamin Diphosphate. Self-Activation of Pyruvate Dehydrogenase by Conversion of the Analogue to Its Components[1]

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Abstract: The reaction of thiamin diphosphate (TDP) and methyl acetylphosphonate in aqueous sodium carbonate produces racemic methyl 2-hydroxy-2-(2-thiamin diphosphate)ethylphosphonate (**1**). Pyruvate dehydrogenase lacking TDP reacts with **1** to produce TDP. Since TDP remains bound to the enzyme, activation of the enzyme results. The observed kinetics are consistent with both enantiomers of **1** binding to the apoenzyme but only one being converted to TDP. Dialysis of enzyme inhibited by methyl acetylphosphonate restores most activity compared to a control. Thus, material proposed to be an enzyme-generated "reactive intermediate analogue" can be synthesized chemically, presented to the enzyme, and converted to the required products. This confirms the proposed mechanism of inhibition and provides information about the enzyme's active site.

Enzymic catalysis results from the combination of an enzyme having functional groups that bind a substrate well and its having functional groups that catalyze reactions of the bound substrate. The chemical basis of the enzyme's success at catalysis is an important mechanistic and structural problem.

An approach to defining the basis of the binding function is finding molecules that differ in some way from the substrate but which bind to the enzyme without undergoing a reaction. Furthermore, if such an analogue of the substrate can undergo part, but not all, of an enzyme's multistep reaction process, defining characteristics for the catalytic function can be obtained as well. Methyl acetylphosphonate is an analogue of pyruvate that appears to bind and undergo partial reaction with the enzyme pyruvate dehydrogenase.[2]

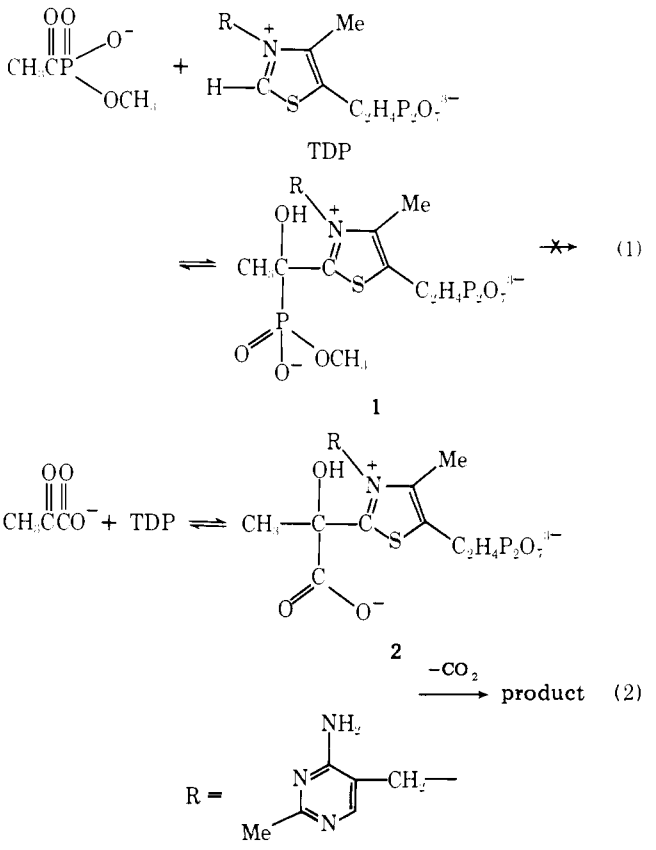
Methyl acetylphosphonic acid is functionally similar to pyruvic acid. Both are α-ketomonoprotic acids of the same acid chain length. The conjugate base of the phosphonic acid is a potent competitive inhibitor against pyruvate ($K_i = 5 \times 10^{-8}$ M) for pyruvate dehydrogenase from *Escherichia coli*.[2] We proposed that the inhibition is powerful because methyl acetylphosphonate adds to enzyme-bound thiamin diphosphate (TDP, eq 1). The normal catalytic process involves addition of TDP to pyruvate and subsequent loss of carbon dioxide[3] (eq 2).

The phosphonate adduct **1** resembles the reactive intermediate α-lactyl-TDP (**2**), which is assumed to form during the normal catalytic cycle of the enzyme.[4] What we call phosphalactyl-TDP (**1**) will not undergo a reaction analogous to the decarboxylation in eq 2 because the necessary leaving species would be methyl metaphosphate, and that certainly involves a large energy barrier.[5]

This analysis suggests the enzyme is responsible for catalyzing formation of an analogue (**1**) of a reactive intermediate (**2**). The analogue of a reactive intermediate in an enzymic reaction should bind tightly with the enzyme.[6-8] The formation of enzyme-generated "reactive-intermediate analogues" has been offered as an explanation for the action of other enzymic inhibitors that possess sites to which nucleophiles can add but which lack leaving groups.[2,9-11] However, proof of this analysis has been difficult to obtain.[12]

A necessary consequence of our explanation of the strong affinity of methyl acetylphosphonate for pyruvate dehydrogenase[2] is that the enzyme must also be capable of converting the reactive intermediate analogue back to substrate analogue and nucleophile (the reverse reaction in eq 1). Dialysis is the usual way to reverse the action of an inhibitor associated with an enzyme. However, restoration of activity does not establish whether inhibition involves formation of a covalent bond, as proposed in eq 1.

Therefore, a further test for the hypothesis is necessary. The test we devised involves synthesis of the proposed adduct (**1**) and monitoring the effect of the enzyme (lacking TDP) upon it. The reverse reaction in eq 1 states that phosphalactyl-TDP can be converted to TDP and methyl acetylphosphonate. The ability of the enzyme to perform this task gives information both about the nature of the inhibition in the forward direction and the means by which the enzyme produces the adduct.



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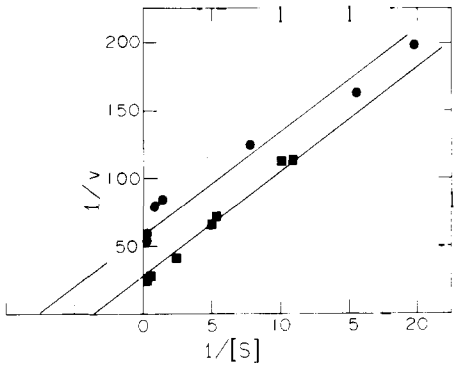


Figure 1. The measurement of the activity of phosphalactyl-TDP and TDP as cofactors with pyruvate dehydrogenase. The initial rate of increase in absorbance at 340 nm of solutions containing sodium phosphate buffer (50 nM, pH 7.7), magnesium chloride (1.0 mM), cysteine (3 mM), sodium pyruvate (2 mM), NAD^+ (1.9 mM), TDP (■) or phosphalactyl-TDP (●), and enzyme and coenzyme A (0.092 mM) was measured. All reaction mixture components, except coenzyme A, were preincubated at 25 °C for 5 min. The reaction was initiated by the addition of coenzyme A. v is in units of change in absorbance at 340 nm/s. [S] is μM.

Experimental Section

Materials. Thiamin diphosphate and chromatographic media were obtained from the Sigma Chemical Co. Pyruvate dehydrogenase (*E. coli*) was a gift from Professor P. A. Frey. Methyl acetylphosphonate was prepared by our published procedure.[2]

All ^1H NMR data for aqueous solutions are relative to internal 4,4-dimethyl-4-silapentane-5-sulfonate (DSS).

Phosphalactyl-TDP [methyl 2-hydroxy-2-(2-thiamin diphosphate)ethylphosphonate chloride] was prepared by reaction of TDP (2 g, 4.3 mmol) and sodium methyl acetylphosphonate (4 g, 25 mmol) in 30 mL of water for 2 h under argon. Sodium carbonate (1 g, 9.4 mmol) was added, and the mixture was kept at room temperature for 3 days. The reaction was followed by ^1H NMR spectroscopy. After 3 days, the signals at δ 7.2 (due to product) and that at δ 8.1 (unreacted TDP) were of equal intensity. Hydrochloric acid was added to make the solution acidic, and the resulting solution was lyophilized. The pale yellow residue was taken up in 25 mL of methanol and filtered free of white solid, and the solution was evaporated to a pale yellow syrup.

A solution of 800 mg of this syrup in 5 mM aqueous phosphate, brought to pH 7 with sodium hydroxide, was added to a 2.5 × 42 cm column of DEAE-cellulose that had been equilibrated with 5 mM phosphate buffer (pH 7.0). The column was eluted with a linear gradient of sodium phosphate, 5 to 50 mM at pH 7 (1 L of 5 mM phosphate (pH 7.0) in the mixing vessel and 1 L of 50 mM phosphate (pH 7.0) in the reservoir). Fractions of 20 mL were collected and those whose UV spectrum showed peaks at λ_{max} 230, 272 (TDP, λ_{max} 234, 268, elutes separately) were pooled (no. 61–76), brought to pH 3.5 with hydrochloric acid, and lyophilized, giving 923 mg of a white solid. Based on spectra of related compounds (hydroxyethylthiamin[13] and phosphalactylthiamin[13]), the spectrum of a solution of the solid indicated it was 10% phosphalactyl-TDP (the remaining part is inorganic phosphate).

This material was used directly in the enzyme kinetic experiments. A sample of this material, on which to collect ^1H, ^{13}C, and ^{31}P NMR spectra, was further purified by titrating an aqueous solution to pH 7.5 with barium hydroxide, filtering free of precipitate, adjusting to pH 3 with hydrochloric acid, and lyophilizing free of water. About 80% of the inorganic phosphate could be removed by this procedure, as estimated from the decrease in intensity of the peak at δ −2.6 in the ^{31}P NMR spectrum, with no substantial loss of product.

The absence of thiamin diphosphate was demonstrated by the lack of a peak at δ 8.1 in the ^1H NMR spectrum, which is characteristic of the 6′ hydrogen of TDP.[14] We also observed that the material did not activate the apoenzyme of pyruvate decarboxylase from brewer's yeast.[15] (The apoenzyme lacks TDP. Methyl acetylphosphonate does not inhibit the holoenzyme.)

The ^1H NMR spectra of TDP and phosphalactyl-TDP are principally distinguished in signals of structural features which they share by the 6′-proton signal in TDP being a singlet at δ 8.1 while that signal in phosphalactyl-TDP is at δ 7.3. In addition, the adduct has peaks due to the methyl ethanephosphonate moiety (δ 2.0, d, $^3J_{PH}$ = 12.9 Hz, CCH_3; 3.7, d, $^3J_{PH}$ = 10.2 Hz, OCH_3). The ^{13}C NMR (^1H decoupled) spectrum of phosphalactyl-TDP (D_2O, downfield from external Me_4Si) shows the additional presence of the methyl ethanephosphonate moiety (δ 77.1, d, $^1J_{PC}$ = 156.1 Hz, CP; 26.5, s, CH_3C; 55.2, d, $^2J_{PC}$ = 6.4 Hz, OCH_3). The ^{31}P NMR spectrum of the adduct, in addition to the pyrophosphate signals at δ −15 which are also present in TDP (relative to internal trimethyl phosphate in D_2O), has an absorbance at δ 10.53 due to the phosphonate. Full spectra are reported elsewhere.[13]

Enzyme Kinetics. Materials for enzyme assays (sodium pyruvate; nicotinamide adenine dinucleotide, reduced form (NADH), oxidized form (NAD^+); acetyl phosphate; alcohol dehydrogenase) were purchased from the Sigma Chemical Co. Dihydrolipoamide was prepared by the procedure of Reed et al.[16] Spectrophotometric assays were done at 25.0 °C.

Pyruvate dehydrogenase was kept in a frozen solution (specific activity, 43.5 μmol of NADH min^{-1} mg^{-1}). Enzyme activity of the pyruvate dehydrogenase complex was determined by the procedure described by Maldonado et al., in which pyruvate is converted to acetyl-coenzyme A, while NAD^+ is converted to NADH, giving a change in absorbance at 340 nm.[17]

We have previously reported that methyl acetylphosphonate is a powerful competitive inhibitor toward pyruvate with this enzyme.[2] The reversibility of this inhibition can be demonstrated by dialysis. The initial rate of the reduction of NAD^+, catalyzed by pyruvate dehydrogenase complex, was measured in two sets of solutions, one with and one without 5 μM methyl acetylphosphonate in a total volume of 1.0 mL. The contents of the reaction mixtures were then dialyzed against a buffered solution containing phosphate (50 mM, pH 7.7), magnesium chloride (1.0 mM), and TDP (0.18 mM). After 3 h, 700 μL of the dialyzed solution was added to a cuvette with NAD^+, pyruvate, and coenzyme A, so that the final concentration of each was the same as before dialysis. The rate of reduction of NAD^+ was again measured.

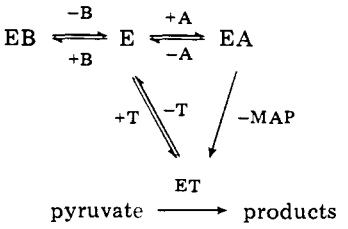
Experiments in which phosphalactyl-TDP was tested for its ability to act as a cofactor in the pyruvate dehydrogenase reaction involved simply substituting it for TDP in the usual assay procedure. Specific conditions are given in the Results section. Enzyme parameters and plots are according to procedures described by Dixon and Webb.[18]

Results

Dialysis. Activity of pyruvate dehydrogenase that has been treated with methyl acetylphosphonate can be recovered by dialysis. Two solutions were prepared. Both contained phosphate buffer (50 mM, pH 7.7), magnesium chloride (1.0 mM), NAD^+ (1.8 mM), cysteine (3 mM), coenzyme A (92 μM), TDP (0.18 mM), pyruvate (1.0 mM), and pyruvate dehydrogenase. One solution contained 5 μM methyl acetylphosphonate. Before dialysis the solution containing phosphonate possessed 2% of the enzymic activity of the solution lacking phosphonate (100%). After dialysis, as described in the Experimental Section, the solution without phosphonate had 22% of its original activity and the solution that had contained phosphonate had 73% of the activity of the other dialyzed solution (or 16% of the original uninhibited solution). Thus, dialysis restores most of the activity of the inhibited sample relative to the control and demonstrates that inhibition is reversible.

Cofactor Activity of Phosphalactyl-TDP. Comparison of pyruvate dehydrogenase activity in the presence of TDP and phosphalactyl-TDP is shown in Figure 1. The experimental conditions are given in the caption. The activity observed when phosphalactyl-TDP is used in place of TDP is about half of that when TDP is used under the conditions of the assay. Contamination of the adduct by TDP could not cause this level of activity, since spectral and enzymic analyses do not detect the presence of TDP in the adduct (an upper limit is 5%). The 2 position of TDP must be available for catalysis of the normal enzymic reaction. Therefore, phosphalactyl-TDP (**1**) must be converted to TDP and methyl acetylphosphonate by the en-

Scheme I



zyme. (The spontaneous conversion is very slow—0.1% in 1 h.) The conversion must be enzyme-catalyzed.[13]

Discussion

The fact that the apparent V and K_m achieved when phosphalactyl-TDP is used are not the same as when TDP is used indicates some properties of the adduct and enzyme. Since phosphalactyl-TDP is a chiral molecule (the carbon bonded to phosphorus is the chiral center), the nonenzymic synthesis from achiral materials must have produced equal amounts of both enantiomers. It is likely that an enzymic reaction produces only one enantiomer of phosphalactyl-TDP from TDP and methyl acetylphosphonate. If only the "correct" enantiomer of **1** bound to the enzyme, then V should be the same as with TDP but the apparent K_m should be twice as large. In fact, however, V decreases and K_m decreases. On the other hand, if the "wrong" enantiomer is a competitive inhibitor, then what is observed can be rationalized, since both enantiomers are present in equal concentrations. A kinetic analysis follows.

Scheme I describes a minimal mechanism. It is assumed that dissociation of TDP or its derivatives is slow relative to catalytic processes, as has been demonstrated elsewhere for this enzyme.[19] To accommodate the observed results, it is assumed that both enantiomers of phosphalactyl-TDP bind (A and B), but only one, A, is converted to TDP and methyl acetylphosphonate (MAP). It is reasonable that enantiomer B can bind and be held, since the enzyme binds a number of TDP derivatives other than the substrate,[19] as well as further derivatives on the catalytic pathway.[20]

The rate equation for the system can be simplified from a general steady-state formulation of Scheme I. When TDP is the source of activity, the enzyme (E) binds TDP (T) reversibly:

$$K_S^T = [E][T]/[ET]$$

The catalytic reaction with pyruvate does not affect this equilibrium, since it regenerates enzyme-bound TDP (ET). Therefore, the relative rate law for the system in terms of TDP concentration is:

$$v = \frac{VT}{K_S^T + T}$$

The line in Figure 1 is plotted as:

$$\frac{1}{v} = \frac{1}{V} + \frac{1}{T}\frac{K_S^T}{V}$$

The K_S^T value for TDP of 0.3 μM agrees with reported values.[21] When phosphalactyl-TDP is the source of enzyme-bound TDP, and little or no TDP is present in the solution, two equilibria are established:

$$E + A \overset{K_S^A}{\rightleftharpoons} ET\ (+M) \qquad K_S^A = [E][A]/[ET]$$

where $[M] \simeq 0$

$$E + B \overset{K_I^B}{\rightleftharpoons} EB \qquad K_I^B = [E][B]/[EB]$$

Since enantiomer A is assumed to be converted to enzyme-bound TDP faster than it is released, the binding of A generates

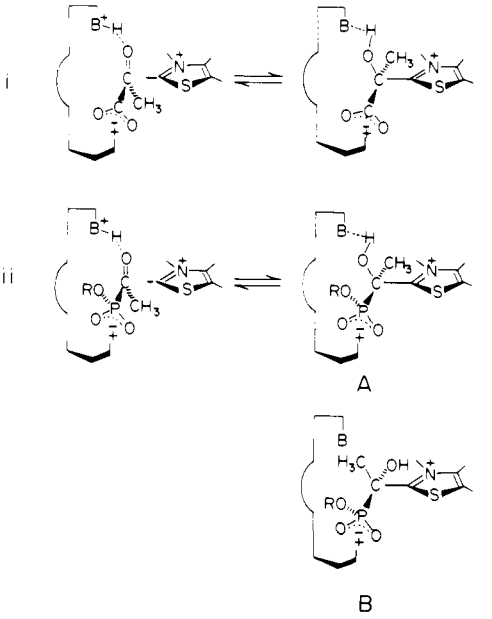


Figure 2. Binding and reaction of TDP and pyruvate (i), and TDP and methyl acetylphosphonate (ii). Phosphalactyl-TDP isomers A and B are as discussed in the text. Absolute stereochemistry is unknown. TDP and the TDP portion of its derivatives are tightly and specifically held. The interaction of the anionic functional groups and the cationic site is also strong. Hydrogen bonding of the carbonyl group is responsible for recognition of pyruvate, but hydrogen bonding is a relatively minor feature in binding of lactyl-TDP, since the two other points dominate.

ET and methyl acetylphosphonate (M) at very low concentrations. If TDP is released it is greatly exceeded by the amount of A and B in solution. If enantiomer B binds, the inactive complex, EB, is formed. Since A and B both bind to the TDP site, B is formally a competitive inhibitor toward A. Furthermore, at all times $[A] \simeq [B] = [S]/2$, where [S] is the concentration of racemic phosphalactyl-TDP that has been added.

The relative rate equation includes a maximal rate term, V, which is the same quantity as in the equation when added TDP is the source of coenzyme:

$$v = V/([A] + K_S^A(1 + [B]/K_I^B))$$

Substituting $[S]/2$ for [A] and [B] and inverting gives:

$$1/v = (1/V)(1 + K_S^A/K_I^B) + (1/[S])(2K_S^A/V)$$

The plot in Figure 1 for this experiment will have an intercept on the $1/v$ axis of $(1/V)(1 + K_S^A/K_I^B)$. This is greater than the intercept when TDP is used, by a factor of $(1 + K_S^A/K_I^B)$. Since the observed intercept is higher by a factor of about two than in the TDP case, $K_S^A \simeq K_I^B$. Since the K_S^A term involves dissociation of TDP rather than A, no absolute conclusion about relative affinities is possible from this information.

The slope in Figure 1 for this experiment is the coefficient of $(1/[S])$, $2K_S^A/V$. Since the slope is the same for the TDP experiment, $K_S^T = 2K_S^A$. Since both K_S terms involve the same dissociation of TDP, this suggests that TDP associates with the enzymes more slowly than does A by a factor of two, if conversion of A to TDP is fast compared to its dissociation.

Finally, the apparent K_S value for phosphalactyl-TDP (K_S^P) is obtained from the $(1/[S])$ intercept:

$$1/K_S^P = (1 + K_S^A/K_I^B)/(2K_S^A)$$

In Figure 1, $K_S^T \simeq 2K_S^P$. We have said that $K_S^A \simeq K_I^B$, then $K_S^P = K_S^A$, and, again, $K_S^T = 2K_S^A$.

The relative values of K_S terms and K_I^B are reasonable in view of our description of the system. However, further kinetic

experiments will be necessary to evaluate in detail the meaning of the apparent values. The conclusions that both enantiomers A and B bind and that one is converted to enzyme-bound TDP appear to be a necessary consequence of any analysis. This confirms the central point of this study.

Consequences for the Structure of the Active Site. The inhibition of pyruvate dehydrogenase by methyl acetylphosphonate also involves catalysis by the enzyme of formation of the covalent adduct with TDP, phosphalactyl-TDP. The pyruvate binding site of the enzyme to which TDP binds must recognize the common features of the substrate and inhibitor with regard to monoanionic state, α-keto group, and the methyl group adjacent to the carbonyl group. This is based on a survey of other potential inhibitors and substrates.[2]

It is reasonable that a monocation serves as the binding site for the substrate anionic center and a hydrogen bond donor associates with the keto group. The donor also can act as a general acid catalyst in the step in which TDP adds to pyruvate (see i and ii in Figure 2).

The addition of TDP to the keto group of the substrate or analogue generates a chiral center. In Figure 2 we have arbitrarily drawn the D-lactyl and D-phosphalactyl adducts for i and ii. When the D,L mixture of A and B isomers of phosphalactyl-TDP binds, presumably the TDP and anionic portions determine the position of binding. However, when isomer B binds (see Figure 2) the general base that removes the hydroxyl proton is in the wrong position to promote the reaction. This picture gives a simple direct relationship between our results and a reasonable minimal functional set at the active site. The site is certainly more complex and we are conducting further studies to obtain more detail.

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References and Notes

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Communications to the Editor

Nonsymmetry of the Hydrogen Bond in 1-Phenylamino-7-phenylimino-1,3,5-cycloheptatriene[1]

Sir:

There is currently considerable interest in intramolecularly hydrogen-bonded systems, such as the enol of malondialdehyde and the title compound, in which it is necessary to distinguish between a truly symmetric hydrogen bond with a single well potential function and a pair of rapidly equilibrating tautomers in which the proton experiences a symmetric double well potential. Several criteria, including X-ray photoelectron spectra,[2] hydrogen–deuterium–tritium chemical-shift isotope effects,[3] and deuteron quadrupole coupling constants,[4] have been successfully applied to this problem. Such systems have also been the subject of several sophisticated quantum mechanical calculations.[5] X-ray and neutron diffraction methods on the other hand have been less successful in that it is difficult to distinguish between a symmetric molecular structure and a symmetric Fourier map which arises from static or dynamic disorder associated with the existence of tautomers.[6] We have recently described[7] a method, based on studies of spin-lattice relaxation times, which can locate the position of a proton in an unsymmetrical hydrogen bond, and we now show that it can be extended to resolve the position(s) of a proton in potentially symmetric situations.

The spin-lattice relaxation method utilizes the strong dependence on internuclear separation ($1/r^6$) of the contribution of the bridging proton to the observed T_1's of neighboring, fully substituted ^{13}C atoms. The precise contribution is found from a comparison of the T_1's for the protio and deuterio species. The relaxation times for various proton bearing carbons provide the necessary knowledge of the rotational diffusion parameters which together with the C–H bond lengths allow calculation of the unknown internuclear distances.

We now report a study of the title compound[8] (Figure 1). An X-ray diffraction structure[9] of the analogous *N,N'*-dimethyl compound was found to be consistent with the existence of a symmetric hydrogen bond or with a disordered structure due to a statistical distribution of tautomers. The diphenyl derivative is symmetric on the ^{13}C chemical shift time scale (26 °C) so that only two ^{13}C resonances, viz., those of C(1), C(7), and ipso pair, provide relaxation data. Furthermore, because of the proximity of the other protons and the nitrogen nuclei, the contribution of the bridging proton to the relaxation times of these positions is relatively small. For this reason we have studied the compound fully labeled with ^{15}N.[10] The pertinent data are presented in Table I.

Table I. ^{13}C and ^{15}N Spin Lattice Relaxation Times (seconds) for 1-Phenylamino-7-phenylimino-1,3,5-cycloheptatriene (0.30 M) in Deuteriobromoform at 26 °C

	δ, ppm[a]	T_1^{obsd} (H)	T_1^{obsd} (D)	T_1^{DD} (H)[b]
2,6	113.9	0.387 ± 0.005	0.387 ± 0.004	
3,5	132.3	0.463 ± 0.006	0.441 ± 0.006	
4	121.0	0.504 ± 0.007	0.540 ± 0.008	
para	122.8	0.391 ± 0.003	0.396 ± 0.004	
1,7	150.3	8.8 ± 0.2	11.1 ± 0.2	40 ± 4
ipso	143.7	9.7 ± 0.1	12.1 ± 0.2	46 ± 4
^{15}N	164.8[c]	4.17 ± 0.04	24.8 ± 0.3	4.71 ± 0.05

[a] The assignments are based on single-frequency off-resonance decoupling experiments and long-range (three-bond) ^{13}CH splittings. [b] Contribution of the labile proton to the relaxation time.[7] [c] Downfield from external 1.0 M aqueous $^{15}NH_4Cl$.

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