15-min (60 mL) linear gradient (0–70%) was used. The alkaline hydrolysis fragment ALK 1 was detected by UV absorbance (215 nm, absorbance range 0.5) and eluted at 32 mL after the start of the gradient.

ALK 1: EIMS (70 eV) *m/z* (relative intensity) 246 (3, M), 217 (8), 200 (9), 110 (13), 94 (26), 82 (20), 81 (4), 80 (6), 44 (32), 43 (30), 38 (20), 36 (58), 32 (74), 31 (100), 29 (51); HREIMS 217.1106 (calcd for $C_{13}H_{15}NO_2$, 217.1103), 200.0861 (calcd for $C_{13}H_{12}O_2$, 200.0837), 110.0598 (calcd for C_6H_8NO, 110.0606), 94.0436 (calcd for C_6H_6O, 94.0419), 82.0635 (calcd for C_5H_8N, 82.0657); ^1H NMR (Me_2SO-d_6) δ 8.34 (2 H, br), 7.90 (2 H, br), 7.83 (1 H, s), 7.14 (2 H, d), 6.92 (2 H, d), 6.60 (1 H, s), 4.88 (2 H, s), 4.04 (2 H, br s), 2.94 (2 H, m), 2.76 (2 H, t); ^1H NMR (D_2O) δ 7.71 (1 H, s), 7.32 (2 H, d), 7.08 (2 H, d), 6.70 (1 H, s), 5.05 (2 H, s), 4.22 (2 H, s), 3.27 (2 H, t), 2.97 (2 H, t); calcd for $C_{14}H_{18}N_2O_2$, M_r 246.1368; found: M_r 246.1351 (HREIMS).

Resolution of D- and L-Glutamate by GC. Methanofuran (1 mg) was hydrolyzed in 6 N hydrochloric acid at 110 °C for 20 h in an evacuated, sealed tube. The hydrolysate was dried in a vacuum oven in the presence of sodium hydroxide pellets. Trifluoroacetyl methyl esters prepared from the amino acids in the hydrolysate and from standard L-glutamic acid (L-Glu) and D-Glu were analyzed by GC using a capillary column filled with 10% *N*-lauroyl-*N'*-*tert*-butyl-L-valinamide on 60–80-mesh Chromosorb WAW as described by Pandey et al.[17] The injector temperature was 150 °C, the detector 270 °C, and the carrier gas was helium (1 mL/min) with the column temperature isothermal at 110 °C for 5 min, followed by a gradient (110–140 °C, 5 °C/min), followed by isothermal at 140 °C. The derivatives of D- and L-Glu eluted at 24.3 and 25.3 min, respectively, while the Glu in the hydrolysate comigrated with L-Glu, as it did when co-injected with L-Glu.

Acetyl Derivative of F1. A sample of F1 (1 mg) was treated with acetic anhydride in anhydrous pyridine for 30 min at room temperature and the product was dried under a stream of nitrogen: ^1H NMR ($CDCl_3$) δ 7.38 (1 H, s), 6.27 (1 H, s), 5.75 (1 or 2 H, t or m), 4.92 (2 H, s), 4.42 (2 H, d), 2.25 (1 H, s), 2.15 (2 H, s), 2.05 (3 H, s), 2.00 (3 H, s).

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Registry No. Methanofuran, 89873-36-9.

Fluorine Recognition at the Active Site of (*N*-(4-Fluorophenyl)-*N*-phenylcarbamoyl)-α-chymotrypsin

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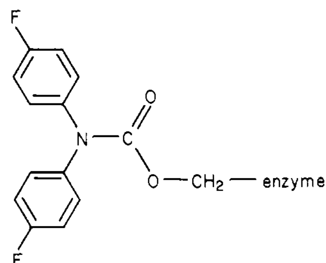
Abstract: *N*-(4-Fluorophenyl)-*N*-phenylcarbamoyl chloride reacts with α-chymotrypsin to give a covalenty modified protein which is devoid of catalytic activity. The fluorophenyl ring of this protein may be found in two magnetically distinct environments; fluorine chemical shift effects suggest that one of these is the substrate specificity pocket at the active site. A competition exists between the fluorophenyl ring and the unsubstituted aromatic ring for residence at each environment, and the NMR observations indicate that the fluorophenyl ring is found preferentially in the active site pocket, a result consistent with the greater hydrophobicity of this group and the nature of the pocket. The kinetics of rotation of the diphenylamino group in both the native and denatured forms of the modified protein have been examined by NMR line shape methods, with results very similar to those obtained with (bis(4-fluorophenyl)carbamoyl)-α-chymotrypsin.

In earlier work we showed that bis(4-fluorophenyl)carbamoyl chloride reacts stoichiometrically with the proteolytic enzyme α-chymotrypsin to give a material with no catalytic activity[1] and indications that this reaction takes place at the active site of the enzyme in a manner completely analogous to the corresponding reaction of diphenylcarbamoyl chloride[2] were described. As structure I suggests, the two aromatic rings of the diphenylamino moiety are magnetically nonequivalent. This nonequivalence is borne out in the fluorine magnetic resonance spectrum of I; two broad signals of equal intensity separated by about 3.4 ppm are observed. One of these signals is at the approximate chemical shift expected for a fluorophenyl ring in solution whereas the other shift is downfield of this position. Derivatives of chymotrypsin which have *p*-fluorophenyl rings able to access the active site pocket usually exhibit large, downfield shifts away from the position observed for the same fluorine resonance in the denatured form of the enzyme derivative.[3-6] Thus, it was suggested that one fluorophenyl ring of modified enzyme I, represented by the low-field signal, resides in the specificity pocket at the active site while the other is placed in a second environment that does not exert as strong an effect on the chemical shift. In the earlier work, various NMR experiments were used to determine the kinetics of diphenylamino group rotation in I; this motion interchanges



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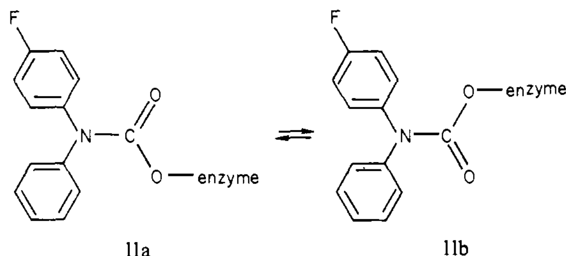
the environments of a given phenyl ring.

Fluorine substitution provides a powerful tool for examination of details of protein structure in that the fluorine NMR spectra of such proteins are often much more highly resolved than corresponding proton or carbon spectra.[3,7,8] Introduction of fluorinated aromatic rings is a common gambit in this regard and biosynthetic incorporation of 3-fluorotyrosine, 4-fluorophenylalanine, and fluorotryptophans has provided a route to useful information about a variety of proteins.[7] Of constant concern in such studies are the possible effects of fluorine substitution on the system under examination. While the covalent radii of hydrogen and fluorine are reasonably similar, the electronic natures of these are not comparable and covalent fluorine may influence local protein structure by dipolar effects, by entering into coordination with metals or by acting as a hydrogen bond acceptor.[9] The diphenylcarbamoylated chymotrypsin system provides a means, albeit imperfect, to compare the interactions between a fluorophenyl ring and its local protein environment with the corresponding nonfluorinated structure and, thus, indicate how seriously fluorine subsitution may alter protein structures.

For the present study, (*N*-(4-fluorophenyl)-*N*-phenylcarbamoyl)chymotrypsin (II) was prepared. As in structure I, there are two magnetically distinguishable environments accessible to the fluorophenyl ring. However, in II the fraction of molecules with this ring in a given environment will be defined by a competition between the phenyl ring and the fluorophenyl ring at each locus. The observations reported below indicate the fluorinated ring of II preferentially resides in the active site pocket and confirm the suggestion that local hydrophobic interactions around aromatic rings in proteins may be stabilized by fluorine substitution.



Experimental Section

Instrumentation and common salts, buffers, organic reagents, water, and deuterium oxide used in the present work were identical with those described previously.[1]

***N*-(4-Fluorophenyl)aniline.** In a 125-mL round-bottom flask equipped with a condenser and magnetic stirrer were placed 6 g of *p*-fluoroacetanilide (0.038 mol, Fairfield Chemical Co.), 4.5 g (0.032 mol) of potassium carbonate, 1.2 g of copper(I) iodide (Alfa), and 17 mL (0.16 mol) of bromobenzene. When the reaction conditions and workup previously described[1] were used this mixture gave *N*-(4-fluorophenyl)-*N*-phenylacetamide, identified by its pmr spectrum, as a dark brown oil. The material obtained was dissolved in 65 mL of 95% ethanol containing 8 g (0.14 mol) of KOH and the solution heated to reflux for 1.5 h, whereupon it was poured into 500 mL of water. The mixture was extracted three times with methylene chloride. The combined extracts were back-extracted three times with water and then dried over magnesium sulfate. Evaporation of the solvent in vacuo produced 5.1 g (62%) of a dark brown oil. The ^{1}H NMR spectrum of this material showed complex patterns centered at 7.0 and 7.05 ppm relative to Me_4Si ($CDCl_3$ solvent).

***N*-(4-Fluorophenyl)-*N*-phenylcarbamoyl Chloride.** A 125-mL three-neck round-bottom flask was charged with 2 g (10 mmol) of *N*-4-fluorophenyl)aniline, 0.84 g (10 mmol) of pyridine, and 10 mL of chloroform. Treatment with phosgene and subsequent workup proceeded as described in ref 1. The product (1.3 g, 55%) was recrystallized twice from ethanol to give white needles, mp 86–87 °C. The ^{1}H NMR of the product showed multiplets centered at 7.2 and 7.4 ppm while the carbonyl group absorption of the material was observed at 1700 cm^{-1} (Nujol mull), midway between the value observed for diphenylcarbamoyl chloride (1685 cm^{-1}) and the difluorinated compound (1720 cm^{-1}).[1] The mass spectrum (EI) showed parent ions at m/e 249 and 251 in the ratio 100:33, a major fragment at m/e 214 (M – Cl), and other fragments consistent with the desired structure. The fluorine NMR spectrum of the material (0.1 M in cyclohexanone/acetone-d_6, 94/6) showed two signals of equal intensity (±1%) at 7.43 and 5.74 ppm downfield from the signal of 4-fluoroacetanalide (0.02 M).

Anhydrous methanol stock solutions of the monofluorinated diphenylcarbamoyl chloride were stable for at least 1 week at room temperature as demonstrated by UV spectroscopy. At 22 °C in 3.2% methanol–water mixtures containing 0.05 M phosphate buffer, the pseudo-first-order rate constant for solvolysis of the chloride was 2×10^{-5} s^{-1} while in 10% methanol the rate constant was 7×10^{-6} s^{-1}. The hydrolysis rates were identical at pH 5.6 and 7.6. The rates are intermediate between those observed for the unsubstituted compound and the difluorinated form,[1] with the reaction becoming progressively slower with increasing fluorine substitution.

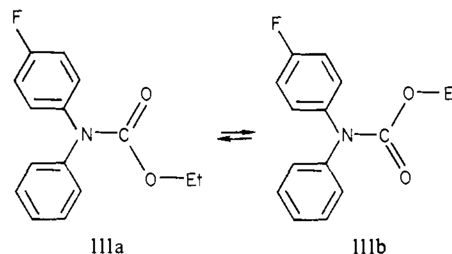
Ethyl *N*-(4-fluorophenyl)-*N*-phenylcarbamate (III) (mp 61–63 °C) was prepared by the procedure described previously for the difluorinated compound.[1] The fluorine NMR spectrum of the product at –60 °C (0.1 M in acetone/acetone-d_6, 94/6) showed two signals of equal intensity at 4.00 and 2.90 ppm downfield from 4-fluoroacetanalide.

Enzyme Modification. Inactivation of the enzyme by the monofluorinated chloride follwed the same protocol as was used for the difluorinated case.[1] The solubility of *N*-(4-fluorophenyl)-*N*-phenylcarbamoyl chloride under these conditions was approximately 60 μM.[10] The apparent first-order rate constant for release of H^+ during the reaction was 2.8×10^{-3} s^{-1} at pH 5.6 and 3.8×10^{-2} s^{-1} at pH 7.6; the modification reaction is thus slightly faster with a single fluorine present in the carbamoyl chloride than when two are present. The modified enzyme appeared to be at least as stable toward deacylation as the difluorinated system.[1]

The methods used to obtain fluorine NMR spectra and to analyze observed line shapes for kinetic data were the same as those mentioned in ref 1. A 0.1 M solution of *p*-fluoroaniline (Aldrich) in methanol sealed in a capillary tube provided an external reference signal.

Results

Ethyl *N*-(4-Fluorophenyl)-*N*-phenylcarbamate. The carbamoyl group is presumably attached to the enzyme at a serine residue and the ethyl carbamate (III) provides a reasonable model for the behavior of this linkage. At room temperature, the fluorine



spectrum of III in acetone-d_6 is a single (averaged) peak, but as the sample temperature is lowered to –60 °C two resonances of equal intensity (±1%) emerge with a chemical shift difference of 1.09 ppm. Analysis of the fluorine NMR line shapes at intermediate temperatures gave rate constant data for rotation about the carbonyl carbon–nitrogen bond in this compound, and these are summarized by the activation parameters given in Table I. These were identical within experimental error to the corresponding data observed with the bis(4-fluorophenyl) system.

Denatured (*N*-(4-Fluorophenyl)-*N*-phenylcarbamoyl)chymotrypsin. A solution of the modified enzyme (~1 mM) in 8 M urea exhibited a fluorine spectrum at 94 MHz which consisted of a single line about 15-Hz wide at half-height and 15.2-ppm downfield from the 4-fluoroaniline reference signal. Such solutions remained fluid to about –15 °C, and near this temperature the spectrum changed into a set of strongly overlapping lines of equal intensity (Figure 1). Analysis of the line shapes observed at temperatures between –13 and 25 °C employing the assumptions regarding the denatured enzyme indicated in ref 1 produced the

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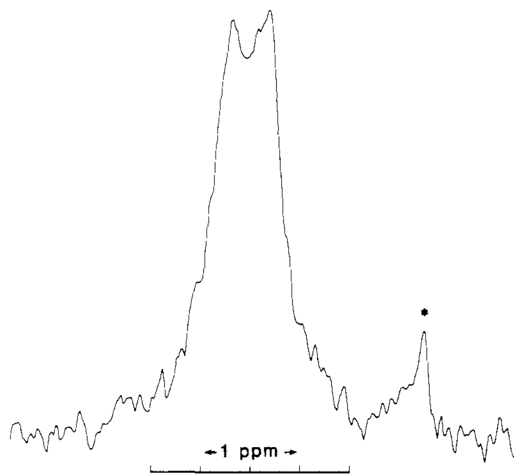

Figure 1. Fluorine NMR spectrum at 94 MHz of (*N*-(4-fluorophenyl)-*N*-phenylcarbamoyl)-α-chymotrypsin dissolved in 8 M urea containing 0.02 M NaCl at −13 °C. The small upfield peak (*) appeared in some spectra and is presumed to arise from contaminants in the protein used in preparation of the carbamoylated enzyme.

activation parameters given in Table I. Again, the appearance of the spectra (two equally intense resonances, their separation and position relative to the reference) is very similar to what was observed with the difluorinated case. However, the rotation kinetics appear to be somewhat different, and rotation at 25 °C in the monofluorinated case is about three times more rapid than that with denatured I. The temperature dependence of the rate of rotation appears to be less steep in the former case.

A minor, temperature-dependent resonance at about 14.5 ppm was noted in the spectrum of the denatured enzyme. Previous work with (4-(trifluoromethyl)benzene)sulfonyl fluoride indicates that commercial chymotrypsin contains several minor contaminants that can react with this reagent,[11] and presumably reaction of these active protein contaminants with the carbamoyl chloride is responsible for this extra signal. No attempt was made to purify the protein samples used in the current work.

Native (*N*-(4-Fluorophenyl)-*N*-phenylcarbamoyl)chymotrypsin. The fluorine NMR spectrum of the native, modified enzyme displays two resonances of unequal intensities at sample temperatures below 35 °C; the resonance at low field (17.9 ppm from the external reference) account for 70% of the total signal intensity. The relative intensities of the signal were not detectably dependent on the sample temperature at those temperatures where separate signals could be observed (Figure 2). With the difluorophenyl system (I) the absolute fluorine chemical shifts as well as the chemical shift difference between the two resonances are strongly temperature dependent; in that case a study of the chemical shifts as a function of radiofrequency was carried out in order to separate the changes in the chemical shift difference due to chemical exchange from other factors. In the monofluorinated system (II) virtually identical behavior as regards both the chemical shift difference between the two signals and the absolute position of each resonance relative to a reference signal were observed at 94 MHz. The line widths of the corresponding signals in the two systems were also essentially identical.

Given the close similarity of the spectra from systems I and II, it was assumed in analyzing the line shape for kinetic information that the temperature dependence of the nonexchange chemical shift difference revealed in studies of I would also apply to protein II. With use of this assumption, the line shapes observed at each temperature were simulated with the program described previoulsy[1] and the rate data for exchange between the two fluorine environments obtained were used to compute the activation parameters given in Table I. Comparison to the data in ref 1 shows that the rotation processes in both I and II are characterized by similar $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ values. Diphenylamino group rotation

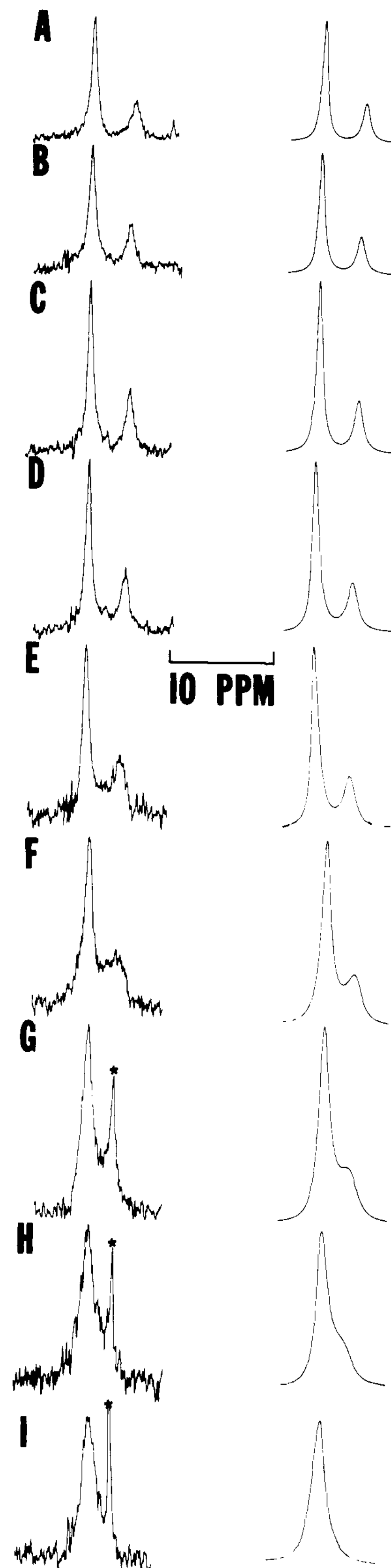

Figure 2. Fluorine NMR spectra at 94 MHz of native (*N*-(4-fluorophenyl)-*N*-phenylcabamoyl)-α-chymotrypsin at various temperatures. The smooth curves were calculated by assuming a two-site exchange situation using the general approach and assumptions about the temperature dependence of the chemical shift difference outline in ref 1. The sample temperatures from top to bottom were 1.5, 14, 17, 25, 30, 34.5, 38, 41, and 45 °C. The sharp resonance (*) appeared after extended heating of the sample above 35 °C and likely represents non-native protein.

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Table I. Chemical Shift Differences and Activation Parameters

system	$\Delta\delta^a$	R^b	k_{25},[c] s^{-1}	ΔG^*, kJ mol^{-1}	ΔH^*, kJ mol^{-1}	ΔS^*, J deg^{-1} mol^{-1}
carbamate III[d]	1.09	50/50	30000	48 ± 0.4	50 ± 6	8 ± 21
native enzyme II[e]	3.35	70/30	93 ± 12[f]	63 ± 1	79 ± 17	46 ± 54
denatured enzyme II[g]	0.21	50/50	350 ± 30	62 ± 1	67 ± 10	26 ± 38

[a] Observed chemical shift difference between the two fluorine signals. [b] Intensity ratios of the observed signals. [c] Extrapolated value for the rotation rate constant at 25 °C. [d] In acetone-d_6. [e] In water containing 10% D_2O and 0.035 M KCl at pH 5.6. The chemical shift given is the observed peak separation at 25 °C. [f] Rate constant for conversion of the less favored isomer to the more favored form. The rate constant for the reverse transformation would be 3/7 of the value shown. [g] In 8 M urea in water, containing 0.02 M KCl and 6% D_2O at pH 5.6. The chemical shift is the inferred slow-exchange shift difference; at 25 °C only an averaged signal is observed.

at 25 °C appears to be about 25% slower in the monofluorinated system, however.

Some exploratory studies of spin–lattice relaxation rates and $\{^{19}F\,^{1}H\}$ nuclear Overhauser effects were carried out at 94 MHz, and to the extent that comparisons are possible, these values were essentially the same for the mono- and difluorinated systems.

Discussion

A conderation of the dynamics of the diphenylamino group rotation in modified chymotrypsin I led to the conclusion that this process likely involves motions at the active site of the enzyme which temporarily unfold the local structure sufficiently that the interchange of the two phenyl rings is feasible. Although the rotation kinetics for II in the native and denatured forms appear not to be as similar as these rates are in both forms of I, the striking similarity of virtually all of the fluorine NMR observations made with the monofluorinated system (II) to those made with the difluorinated case argues strongly that the two systems are closely similar in both their time-averaged and dynamical properties.

The major difference between the two systems is that the fluorophenyl ring of II prefers the environment represented by the low-field signal of the spectrum; this preference correponds to a free-energy difference between the two rotomeric forms IIa and IIb of about 2.1 kJ mol^{-1}. This difference is probably not due to different electronic or steric interactions within the carbamate group itself, for in the model compound III and the corresponding carbamoyl chloride the corresponding rotamers are isoenergetic and contribute signals of equal intensity to their respective fluorine spectra. The preference for the environment represented by the low-field signal therefore must arise from interactions between the phenyl rings and the protein.

It has been suggested previously that one of the phenyl rings of the diphenylamino group can take up residence in the active site pocket and that when this ring bears a *p*-fluoro substituent this active site region produces a characteristic downfield fluorine chemical shift effect. If this suggestion is correct then it appears that the fluorophenyl ring of II has a greater affinity for the active site pocket than it does for the environment which produced the upfield signal. Alternatively, the fluorophenyl ring may be repulsed from this second location by unfavorable interactions. However, van der Waals interactions likely have a dominating influence on fluorine chemical shifts in proteins;[3,12] the chemical shift for this second position has the value expected for an unfolded structure and thus provides no evidence for strongly repulsive interactions.

The active site pocket of chymotrypsin is generally regarded as hydrophobic in nature.[13,14] The Hansch hydrophobicity parameter π_x for the *p*-fluorophenyl ring ranges from 0.14 to 0.30, depending on the system used to evaluate it.[15] These values correspond to an increase in the hydrophobic interaction energy of 3–5 kJ mol^{-1}. That fluorine substitution increases the hydrophobicity of an aromatic ring is consonant with experiment. For example, Bosshard and Berger[16] showed that the binding of tripeptide inhibitors of chymotrypsin is enhanced about 2.5 kJ mol^{-1} when a C-terminal phenylalanine is replaced by *p*-fluorophenylalanine. Thus, the assignment of the low-field fluorine signal to a fluorophenyl ring within the active site pocket and the known hydrophobic propensities of this ring system and the active site are mutually consistent.

Our results signal the possibility that fluorine substitution on aromatic rings in protein structures may enhance hydrophobic interactions and thereby alter the local stability of the structure both in the thermodynamic sense and the dynamical sense. Other factors such as hydrogen bonding to fluorine and dipolar interactions with the carbon–fluorine bond dipole could well alter the situation, and it remains for additional experimentation to reveal how significant these factors are.

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