Solvent Polarity and pH Effects on the Magnetic Properties of Ionizable Nitroxide Radicals: A Combined Computational and Experimental Study of 2,2,5,5-Tetramethyl-3-carboxypyrrolidine and 2,2,6,6-Tetramethyl-4-carboxypiperidine Nitroxides

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The modulation induced by different solvents and by pH on the nitrogen isotropic hyperfine coupling constants (A_N) of 2,2,5,5-tetramethyl-3-carboxypyrrolidine (3-carboxy-PROXYL,CP) and 2,2,6,6-tetramethyl-4-carboxypiperidine (4-carboxy-TEMPO,CT) nitroxides is studied through ab initio quantum mechanical computations and by EPR spectroscopy. The geometry of the neutral and deprotonated forms and the corresponding dissociation constants are computed by using the hybrid PBE0 functional. With these structures, accurate A_N values are obtained by an integrated post-Hartree−Fock/PBE0 approach. Specific solvent effects are modeled by including a few solvent molecules (strongly bound to the solute), whereas bulk contributions are taken into account by the polarizable continuum model (PCM). Both EPR spectra and quantum mechanical computations point out that A_N increases with increasing solvent polarity and, especially, H-bond ability. The A_N values obtained by our computational model both for the neutral and deprotonated forms in aqueous solution are in good agreement with EPR values. On the other hand, the computed pK_a values (4.97 and 5.25 for CP and CT, respectively) are significantly larger than the value reported in the literature for CP (3.4). New potentiometric and spectroscopic measurements lead to values (4 and 4.30 for CP and CT, respectively) in better agreement with quantum mechanical computations and chemical intuition. Also the calculated magnetic titration curves in aqueous solution are in quite good agreement with our new EPR curves.

1. Introduction

Nitroxide radicals are widely used as spin-labels and spin-probes in electron spin resonance (EPR) spectroscopy studies of complex systems.[1,2] As a matter of fact, the stability under ordinary conditions[3] and the strong localization of the unpaired electron on the NO moiety[4] allow us to use nitroxides to evaluate a number of properties of the embedding medium mainly through the nitrogen isotropic hyperfine coupling constant (A_N), which is highly sensitive to micropolarity, hydrogen bonding power, pH, etc.[5−7] As an example, nitroxides can be used successfully as spin-labels for a detailed characterization of microstructured microdomain systems, such as micelles, vesicles, bilayers, liquid crystals, etc.[8] An additional possibility is to use nitroxide radicals as pH indicators[9] to measure with high accuracy the pH in cells or to monitor the pH gradient across lipid and cellular membranes.[9,10] The presence in the molecule of a hydrogen acceptor or donor site makes a nitroxide radical sensitive to the environmental pH due to the different A_N values assumed by the protonated and unprotonated forms: the measured A_N depends then on the pK_a of the nitroxide radical and on the nature of the embedding medium.

A correct interpretation of the results of the experimental investigations requires these factors to be clearly analyzed and understood. To this aim, computational studies have been shown to be extremely helpful and have already allowed to gain a qualitatively and, sometimes, a semiquantitatively correct insight on the different factors influencing the A_N value.

However, the usefulness of a computational approach would be greatly increased if it would be able to provide estimates of structural, thermodynamic, and magnetic parameters of open shell systems with an accuracy fully comparable with experiments. Any lack of computational accuracy would indeed prevent theory from giving reliable prediction of the properties of new compounds, reducing the usefulness of computations for the novo design of new spin labels and spin probes. Furthermore, it would be much more difficult to give a reliable explanation of the experimental results in terms of physico-chemical effects, because the A_N values result from a subtle balance between intrinsic and environmental effects, often weak and acting in opposite directions.

At the same time the adopted methodology should be computationally effective because nitroxides commonly used as spin probes contain at least 20 atoms and the size of the system increases even more when biological systems as proteins and polypeptides are spin labeled and/or solvent effects have to be taken into account. However, we think the recent methodological and technical advances of the computational chemistry already allow us to get an almost quantitative agreement with experiments, without losing the possibility of interpreting the computational results in terms of well-defined

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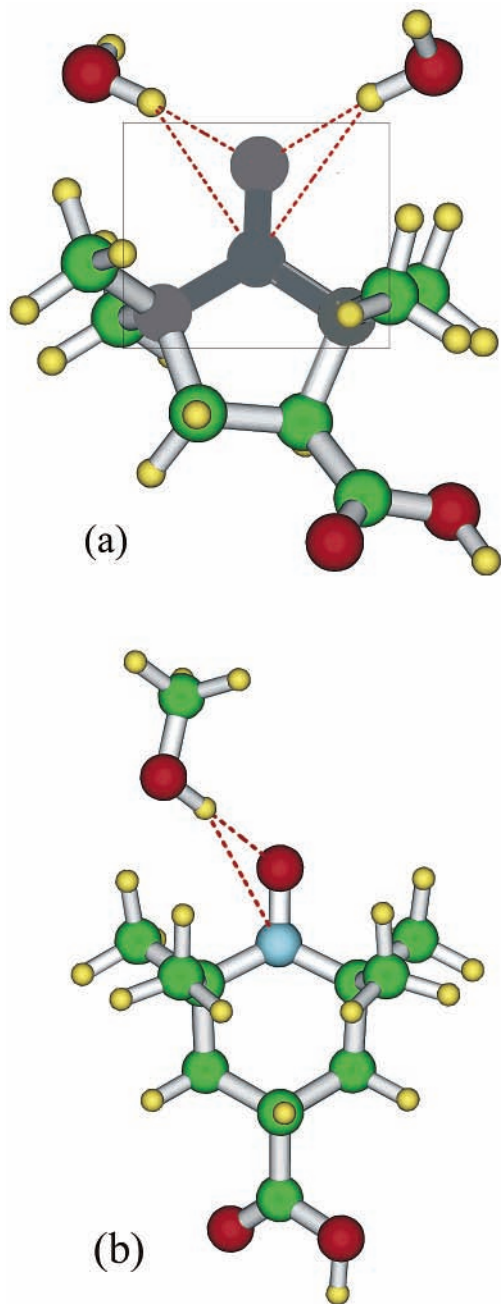‡ Istituto di Biostrutture e Bioimmagini-CNR.
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Figure 1. CP−2(H₂O) (a) and CT−CH₃OH (b) adducts. The shaded region of CP−2(H₂O) corresponds to the small model in composite computations (see text for details).

physicochemical effects. This should be evident when the results of the present paper are examined, where we present a combined experimental and computational study of solvent effects on the structure and magnetic properties of the cyclic nitroxides 2,2,5,5-tetramethyl-3-carboxypyrrolidine (3-carboxy-PROXYL,CP) and 2,2,6,6-tetramethyl-4-carboxypiperidine (4-carboxy-TEMPO,-CT) (see Figure 1).

In the first part of the study we will analyze the influence of the surrounding medium on the A_N value in terms of polarity and hydrogen bonding power for several different solvents with a dielectric constant sweeping on a broad range. Next, we will deal with the effect of pH building completely ab initio magnetic titration curves of CP and CT in aqueous solution. Quite surprisingly, although the computed A_N values are in agreement with experiments, our pK_a value for CP nitroxide is significantly different from the value reported in the literature.[6] Because our

procedure for the pK_a computation provided accurate values for a number of carboxylic acids, we decided to perform a new experimental determination of the CP pK_a.

2. Methods

2.1. Experimental Section. *Materials.* 3-carboxy-PROXYL (CP, 99.5% purity) and 4-carboxy-TEMPO (CT, 97% purity) nitroxides were purchased from Aldrich and used as received. Carbon tetrachloride (99.5%), chloroform (99.8%), tetrahydrofuran (>99% purity), dichlorometane (99.5%), acetone (99.5%), ethanol (99.5%), methanol (>99.9%), nitrobenzene (99%), dimethylformamide (99.8%), and dimethyl sulfoxide (99.8%) were purchased from Sigma Chemicals Co. and used without further purification. Water was degassed and doubly distilled. All solutions were prepared by weight.

EPR Measurements. CP and CT ($C = 1.0 \times 10^{-3}$ mol dm⁻³) EPR spectra in all the above-mentioned solvents were recorded at 20 °C with a Bruker ER200D spectrometer equipped with a TE₀₁₁ cavity, using the following parameters: sweep width 50 G, resolution 8192 points, modulation amplitude 0.2 G, time constant 5 ms, receiver gain 1 × 10⁴, microwave power 2 mW, scan time 41 s. For the EPR measurements, capillary quartz tubes (0.5 mm i.d.) were used. The nitrogen hyperfine coupling constants (A_N) have been measured as the average splitting between the three hyperfine EPR lines of the spectra. The experimental error on the A_N is estimated to be 0.02 G.

In aqueous solutions, EPR spectra of CP and CT were acquired with pH ranging from 1 to 7.5. The desired pH values were obtained by using chloride (1 < pH < 2.5), phthalate (2.5 < pH < 4), and phosphate (4 < pH < 7.5) buffers.

Potentiometric Titrations. Aqueous solutions of CP and CT ($C \sim 0.01$ mol dm⁻³) were prepared by weight. Nitroxide solubilization was favored by gentle warming (∼35 °C) and stirring. These solutions were titrated with a NaOH solution ($C^{\circ}_B = 0.0997$ mol dm⁻³); the pH was measured at 25 °C by a Radiometer pH meter, model PHM240 (experimental error on pH determination ±0.01) equipped with a double-junction reference electrode. Carbon dioxide solubilization was prevented by bubbling nitrogen into the solution during the whole titration. The pH meter was standardized frequently before and after measurements. The pK_a value was evaluated by fitting to the experimental data the relation

$$C_B = [OH^-] - [H_3O^+] + CK_a/(K_a + [H_3O^+])$$

in wich C_B and C are the stoichiometric concentration of NaOH and of the nitroxide, respectively. For each nitroxide, the pK_a value was obtained as the mean value of three independent titrations. In the case of CT, the potentiometric titrations were performed in the range $3.5 \leq pH \leq 6$; further addition of the NaOH solution caused pH variations difficult to be interpreted. Work is in progress is our laboratory to clarify this experimental evidence. However, it has to be noted that for pH > 6 the spectroscopic parameters of CT are constant, so that in this pH range this nitroxide is not interesting as a pH indicator.

2.2. Computational Details. All geometries have been optimized at the density functional theory (DFT)[11] level using the PBE0[12] functional. Bulk solvent effects have been modeled by the polarizable continuum model (PCM),[13] in which the solute molecule is embedded in a molecular-shaped cavity surrounded by an infinite dielectric characterized by the dielectric constant ϵ of the solvent. Here we use the UATM parameters[14] for building the cavity and the IVC-PCM[15] (implicit volume charge) version in which the contribution of

the electron density escaping from the solute cavity is properly taken into account. Post Hartree−Fock computations have been performed by the quadratic configuration model including single and double excitations (QCISD)[16] using the basis set developed by Chipman for the computation of magnetic properties.[17] All the calculations have been performed by a development version of the Gaussian package.[18]

3. Computational Methodology

3.1. Calculation of the A_N Values. The absolute value of A_N is underestimated by DFT calculations,[19,20] whereas the QCISD method[16] provides an accurate estimate for this observable.[21] On the other hand, the high reliability of nitroxide radical geometries optimized by DFT methods allows us to avoid QCISD geometry optimizations, which are computationally too expensive. A_N calculation has been thus made on the geometries of the CP and CT radicals optimized at the PBE0/6-31G(d) level, employing the following mixed approach:[22]

$$A_N = A^{PBE0}_{Nwhole} + (A^{QCISD}_{Nsmall} - A^{PBE0}_{Nsmall}) + \Delta A^{vibr}_N$$

where the subscript "whole" refers to the nitroxide radical molecule (including, when needed, some explicit solvent molecules, vide infra) and the subscript "small" refers to a dimethyl nitroxide molecule whose geometry is frozen to that assumed by the C_2−NO moiety in the same nitroxide. Methyl hydrogens are added following the recipe used in the methods of the ONIOM[23] family. The basis set optimized by Chipman[17] for computation of the isotropic hyperfine coupling constant has been used in QCISD calculations. The term ΔA^{vibr}_N refers to the A_N variation induced by the low-frequency inversion motion of the nitrogen atom according to the pyramidalization degree of the nitroxide moiety.[21] This contribution, calculated in a previous work, is ≈ 1 G[24,25] for five-membered ring nitroxides (CP), whereas it is negligible in six-membered ring nitroxides (CT).

3.2. Calculation of the pK_a. For an acid species AH the pK_a defined as minus the logarithm of the dissociation constant of the reaction

$$AH_{aq} \rightarrow A^-_{aq} + H^+_{aq}$$

is given by the well-known thermodynamic relation:

$$pK_a = \Delta G_{aq,AH}/2.303RT$$

The Gibbs energy variation of the deprotonation reaction in aqueous solution has been calculated by adding an aqueous solution contribution to a gas-phase contribution[26] as follows:

$$\Delta G_{aq,AH} = \Delta G_{gas,AH} + \Delta\Delta G_{solv,AH}$$

in which

$$\Delta G_{gas,AH} = G_{gas,A^-} + G_{gas,H^+} - G_{gas,AH}$$

$$\Delta\Delta G_{solv,AH} = \Delta G_{solv,A^-} + \Delta G_{solv,H^+} - \Delta G_{solv,AH}$$

according to the thermodynamic cycle reported in Figure 2.

Note that it is also necessary to take into account the transformation of the concentration units from atm in the gas phase to mol dm^{-3} in aqueous solution. This term $\{-RT \ln(24.46)\}$ has been enclosed in ΔG_{gas}.

Looking at the components contributing to the dissociation process, we can identify two main sources of error: (1) the computational level of the model used in describing the gas-
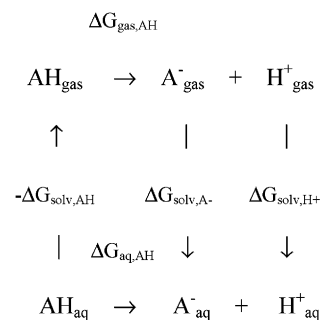

Figure 2. Thermodynamic cycle for a generic acid AH.

phase process can be insufficient; (2) description of the solute−solvent interactions and of the structural changes induced by them can be inappropriate. So we have devised a new procedure, referred to as P02, for the computation of absolute pK_a values.

In particular G_{gas,H^+} and $\Delta G_{solv,H^+}$ have been fixed to their most recent experimental values of 6.28[27] and −264 kcal/mol,[28] respectively. Next, all G_{gas} values have been calculated by single point energy evaluations at the PBE0/6-311+G(2d,2p) level using geometries and harmonic frequencies computed at the PBE0/6-31+G(d,p) level. The G_{gas} values obtained in this way for a training set of eight carboxylic acids are in very good agreement with both experimental and refined post-Hartree−Fock computations (G2, G3, and CBS procedures).[26] Solvation free energies ΔG_{solv} have been estimated at the PCM/HF/6-31+G(d,p) level using geometries optimized in aqueous solution at the PBE0/6-31+G(d,p) level. Standard UATM[14] parameters have been used to build the cavities occupied by the solute in the solvent and to evaluate nonelectrostatic contributions. The only modification concerns the coefficient $\gamma_{q-}(O)$ (changed from −0.3 to −0.44) and is related to the change of reference solvation energies of ionic species brought by the new value of $\Delta G_{solv,H^+}$ and by the new improved procedure for taking into account the so-called escaped charge. The P02 procedure is very effective from a computational point of view and provides remarkable pK_a values for a number of test cases.

4. Results

The presentation of computational and experimental results will be organized in three sections:

1. Intrinsic effects on the magnitude of A_N.

2. Solvent effects on A_N for neutral nitroxides.

3. Experimental and computational determination of the titration curve for CP and CT.

4.1. Intrinsic Effects on the A_N Value. The comparison between the calculated and experimental A_N values in apolar/aprotic solvents such as CCl$_4$ is a good benchmark to assess the reliability of the computational methodology sketched above (see first row of Table 1).

It is thus gratifying that our results are close to the experimental ones, the discrepancy between experiments and calculations (≈ 0.3 G both for CP and CT) being just $\approx 2\%$ of the total value. For comparison, the error obtained by the calculations of ref 29 is $\approx 40\%$. Our methodology can well reproduce the difference in the A_N values found for CP and CT: 1.37 G according to the experiments and 1.29 G according to our calculations. This is a quite important result because this indicates that PBE0/6-31G(d) calculations are accurate enough to correctly reproduce the geometry of the nitroxide under study, also concerning quite subtle details such as the degree of pyramidalization of the nitroxide moiety, which is indeed the

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TABLE 1: Values of ϵ and E_T for the Different Solvents Used in the Present Study and Corresponding Experimental and Computed Values of A_N (Gauss) for CP and CT[a]

| solvent | CP | | CT | | e | E_T |
	exp	calc	exp	calc		
CCl$_4$	14.081	13.80	15.450	15.09	2.240	32.5
CHCl$_3$	14.697	14.05	15.947	15.31	4.90	39.1
		14.61[b]		15.71[b]		
		15.25[c]				
THF	14.252	14.14	15.528	15.4	7.58	37.4
CH$_2$Cl$_2$	14.363	14.17	15.789	15.42	8.93	41.1
CH$_3$COCH$_3$	14.287	14.27	15.601	15.52	20.70	42.2
CH$_3$OH	15.198	14.30	16.252	15.54	32.60	55.1
		15.29[b]		16.17[b]		
Ph$-$NO$_2$	14.471	14.30	15.731	15.54	34.85	46.3
DMF	14.500	14.30	15.716	15.55	36.71	43.8
DMS	14.605	14.31	15.823	15.56	46.70	45.0
H$_2$O	16.050	14.32	17.060	15.54	78.40	63.0
		15.25[b]		15.54[b]		
		16.12[c]		16.74[c]		

[a] Gas-phase results: 13.35 G (CP); 14.80 G(CT). [b] Calculations including one explicit solvent molecule. [c] Calculations including two explicit solvent molecules.

geometric parameter most influencing the A_N value (see the discussion paragraph).

4.2. Solvent Effect on the A_N Value. In Figure 3 are plotted the values of A_N measured in different solvents as a function of the dielectric constant ϵ of each solvent.

Two different trends are evident, relative to aprotic and protic solvents, respectively. As a matter of fact, A_N values measured in water, methanol, and chloroform are fitted quite well by a line with a slope larger than that connecting all the remaining solvents. The only exception concerns dichloromethane, which is in an intermediate position between the two straight lines. The A_N values of CP and CT measured in various solvents show instead a fair linear correlation with the E_T parameter ($A_N = 11.8 + 0.065E_T$ with $R = 0.926$ and $A_N = 13.55 + 0.052E_T$ with $R = 0.904$ for CP and CT respectively).

The linear relationship A_N/E_T (this parameter is based on the solvatochromic shift of the absorption maximum of piridinium N-phenolbetaine)[7] is useful from a practical point of view, but in the absence of a detailed theory of the E_T values, it does not provide a molecular understanding of the solvent effects, except for suggesting that not only the polarity of the solvent but also the possibility of explicit hydrogen bonds critically influence the value of A_N.

The role of nitroxide/solvent hydrogen bonds is particularly evident when one compares the A_N values measured for CP and CT dissolved in two solvents having very similar dielectric constants, such as methanol and nitrobenzene. In the former solvent, which can form H bonds, the A_N value is higher by 0.7 and 0.5 G (for CP and CT, respectively), than in the latter, which has no H-bonding ability.

Before the corresponding computational results are analyzed, it is worth highlighting that, according to experiments, not only traditional H-bonding solvents such as methanol and water but also chloroform and, to a smaller extent, dichloromethane exhibit a protic behavior.

PCM calculations confirm the trends sketched above. When non-hydrogen-bond forming solvents are studied, the inclusion of the bulk solvent effect by means of single point PCM calculations on the gas-phase geometries is sufficient to provide A_N values in good agreement with the experiments.

Inspection of Table 1 shows that A_N rapidly increases up to $\epsilon \approx 8$, stabilizing around the constant values of 14.3 and 15.5 G if $\epsilon > 8$ for CP and CT radical, respectively.

TABLE 2: Interaction Energies (kcal/mol) between CP and One Solvent Molecule (S) and between Two Solvent Molecules (S$_2$)

solvent	CP + S	S$_2$
H$_2$O	−9.65	−5.97
CH$_3$OH	−6.57	−5.38
CHCl$_3$	−5.43	−0.77

For chloroform, methanol, and water bulk effects alone cannot thus reproduce the experimental A_N. In these solvents it is indeed necessary to take into account the formation of long living complex adducts between solvent molecules and the solute, due to strong specific interactions such as hydrogen bonds. To analyze in deeper detail the above feature, we performed for CP a comparative analysis of the interaction energies between the solute and a single solvent molecule (water, methanol, or chloroform) with the interaction energy between two solvent molecules. In fact a solvent molecule−solute interaction energy significantly larger than its solvent molecule−solvent molecule counterpart could indicate a residence time of a solvent molecule around the solute large enough to influence its molecular properties via the formation of specific not "averaged" interactions.

The interaction energies have been calculated in the gas phase at the PBE0/6-31G(d) level on the adducts formed by CP and a solvent molecule optimized at the PBE0/6-31G(d) level in the same solvent. Those energies are compared with those relative to solvent molecule dimers calculated at the same level in Table 2. All the interaction energies are corrected for the basis set superposition error by the counterpoise method.[30]

The energy differences between the two sets of interaction energies are quite large to justify the inclusion of explicit solvent molecules. One additional water molecule can be coordinated to the nitroxide moiety, whereas preliminary geometry optimizations suggest that the simultaneous presence of two methanol or chloroform molecules leads to weaker coordination. Very similar trends have been obtained for CT. As a consequence, the A_N values have been computed on adducts containing two explicit solvent molecules in water and only one solvent molecule in methanol and chloroform. It is noteworthy that the choice of introducing two explicit water molecules and only one methanol molecule is supported also by spectroscopic evidence.[31] Using those supermolecules for the calculation of A_N restores the agreement between experiments and calculations also for the protic solvents (see Table 1).

4.3. Experimental and Computational Determination of the Titration Curves for CP and CT. Under the experimental conditions used in the building of the titration curves, proton exchange rates involving the carboxylic group of the considered nitroxides are fast on the EPR time scale,[1] so that each observed spectrum is a weighted average between that of the protonated and that of the unprotonated forms. The construction of the computational curve thus requires essentially: (i) the determination of A_N both for the protonated and the unprotonated forms; (ii) the calculation of the pK_a for CP and CT.

Our calculations (see Table 3) predict that the neutral form of CP and CT exhibits a smaller value of A_N, because the presence of a negative charge should likely lead to an increase of the spin density at the nitrogen atom (see the discussion paragraph for a possible explanation of this feature). The effect of the protonation state of the carboxyl group is larger for CP than for CT, likely because in the latter compound the carboxyl is closer to the nitroxide moiety.

An inspection of Table 3 shows that the effect of the negative charge on the carboxylate substituent is overestimated in the
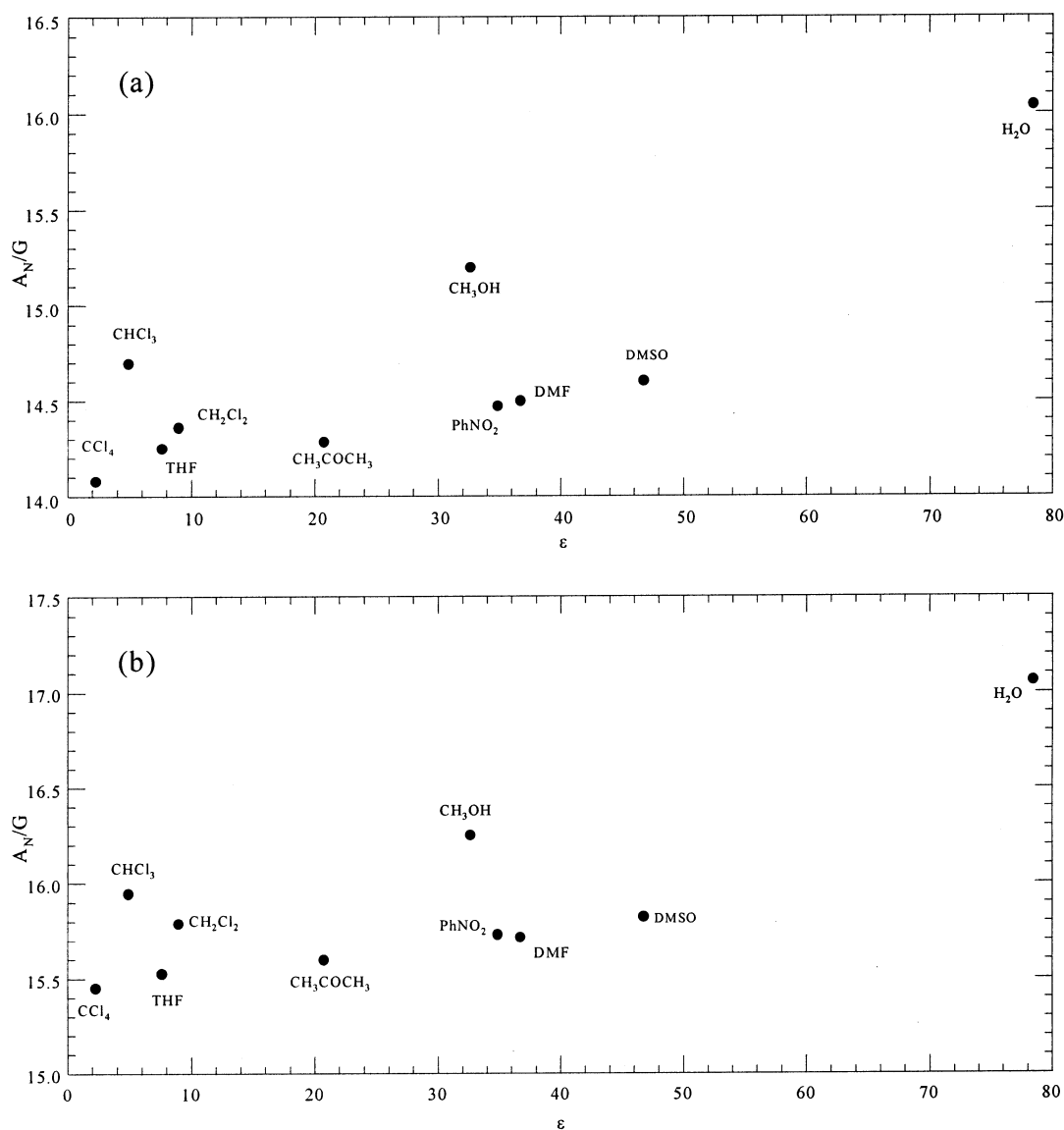
Figure 3. Experimental A_N values of the CP (a) and CT (b) radicals in different solvents as a function of the dielectric constant ϵ of each solvent.

TABLE 3: A_N Values of Protonated and Deprotonated Forms of the Radicals CP and CT with One and Two Explicit Water Molecules, Forming Hydrogen Bonds with Nitroxide Groups, Computed on Geometries Optimized in the Gas Phase and in Aqueous Solution at PBE0/6-31G(d) and PCM/PBE0/6-31G(d) Levels Respectively

nitroxide	R gas phase	R PCM	R + 1S gas phase	R + 1S PCM	R + 2S gas phase	R + 2S PCM	exp
CP	13.45	14.32	14.64	15.25	15.58	16.13	16.03
CP⁻	14.51	14.76	15.78	15.70	16.67	16.56	16.23
CT	14.79	15.55	15.35	16.14	16.05	16.73	17.06
CT⁻	15.03	15.66	15.77	16.26	16.41	16.78	17.13

gas phase, even when explicit solvent molecules are included in the calculations. This result is not surprising because a large number of solvent molecules is required to properly screen a net charge. Preliminary calculations show that the inclusion of one additional explicit water molecule coordinated to the carboxylate group does not improve the agreement with experiments, if bulk effects are not properly taken into account by the PCM.

By use of the P02 procedure, the computed pK_a's of CP and CT radicals are 4.97 and 5.25, respectively. The computed pK_a value of the CP radical shows a severe discrepancy with the

corresponding experimental value reported in the literature (3.4).[6] Thus we decided to carry out an experimental study to determine the pK_a's of both CP and CT radicals.

The plots relative to the variation of A_N in CP and CT with the pH of the embedding medium are shown in Figure 4.

In agreement with our computations, for both nitroxides A_N is predicted to increase with the pH of the embedding medium, following a sigmoid trend: at pH < 2.5 A_N assumes a constant value [A_N(CP) = 16.04 G and A_N(CT) = 17.06 G] characteristic of the protonated form of the nitroxide acids; for 2.5 < pH < 5 A_N gradually increases; at pH > 5 A_N assumes another constant value [A_N(CP⁻) = 16.23 G and A_N(CT⁻) = 17.13 G] relative to the completely deprotonated form of the acids. The difference between the two constant values is significantly higher for CP than for CT (ΔA_N(CP/CP⁻) = 0.19 G and ΔA_N(CT/CT⁻) = 0.07) and it is comparable to that predicted by our computations (0.43 G for CP and 0.05 G for CT, respectively). The pK_a can be determined from the experimental data as the pH at which the protonated and the unprotonated forms of the acids are present in equal concentration, so that A_N = (A_N(CP⁻) + A_N(CP))/2 = 16.13 G and A_N = (A_N(CT⁻) + A_N(CT))/2 = 17.095 G. The obtained pK_a are pK_a(CP) = 3.89 and pK_a(CT) = 4.03, respectively. These values are in good

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Figure 4. Experimental EPR titration curves of CP (a) and CT (b).

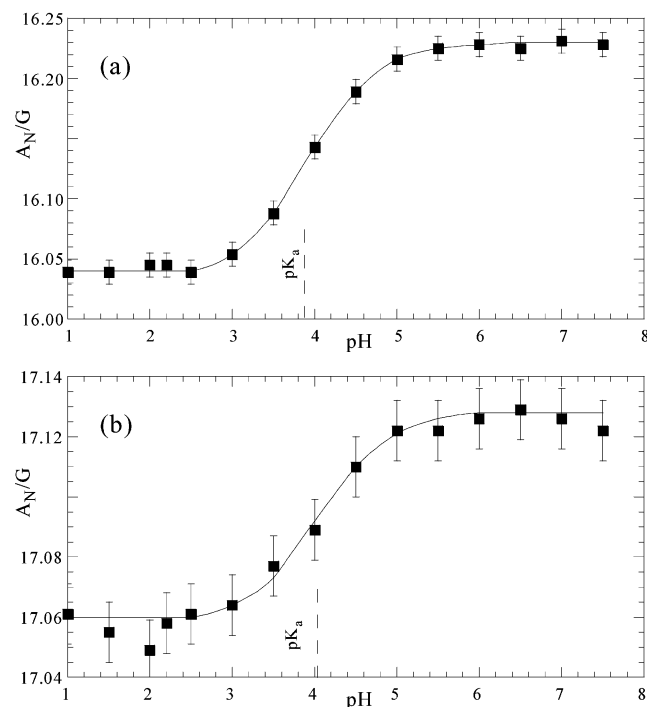**Figure 5.** Comparison between experimental (continuos lines) and computed (broken lines) EPR titration curves of CP (a) and CT (b).

agreement with those obtained from the potentiometric titrations [pK_a(CP) = 4.00 ± 0.01 and pK_a(CT) = 4.30 ± 0.02], whereas there is a large difference with respect to the experimental value of 3.4 ± 0.2 previously reported in the literature for CP.

The computed values are thus overestimated by ∼1 pH unit with respect to the experimental ones for both radicals. A pK_a unit corresponds to 1.36 kcal/mol, which is a reasonable error on the computed free energies involved in the thermodynamic cycle used for the pK_a calculations. However, it is important to highlight that the differences between the pK_a computed for CP and CT is 0.28 pH units, in good agreement with those predicted by the experiments (0.14 and 0.30 according EPR and potentiometric titrations, respectively).

By use of the computed pK_a and A_N values, a theoretical titration curve has been finally built for CP and CT (see Figure 5). The computed curves are similar to the experimental ones, but for the above-mentioned pK_a shift.

5. Discussion and Conclusions

In this paper we have presented a thourough characterization, by both computational and experimental standpoints, of two cyclic nitroxides. Our results show that a suitable theoretical procedure can couple accuracy and computational feasibility, giving an almost quantitative agreement with experiments, for what concerns (i) the prediction of the magnetic properties of nitroxides of different geometry, (ii) the determination of the solvent effect on A_N, and (iii) the evaluation of the influence of the variation of the pH of the embedding medium.

Furthermore, the good agreement with the experiments makes more reliable any assessement of the physical effects governing the magnitude of the A_N in nitroxides.

The comparison between the results obtained for CP and CT clearly shows the influence of the geometry of the nitroxide moiety on A_N. As has already been recognized, a partial pyramidalization of the nitrogen atom of the nitroxide moiety allows for a direct contribution to the A_N due to involvment of the nitrogen s atomic orbital in the molecular orbital bearing the unpaired spin electron. On the contrary, when the nitroxide
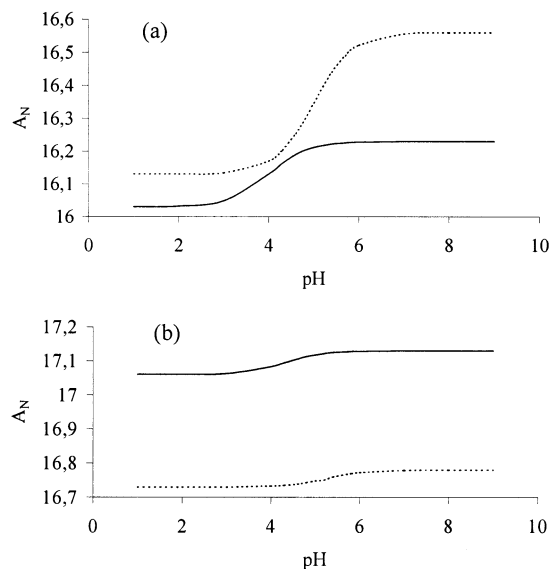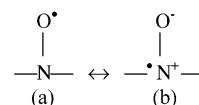
moiety has a planar geometry, only indirect contributions are possible because the nitrogen atom lies in a nodal plane of the p orbital taking part to the SOMO. As a matter of fact, the nitroxide moiety is predicted to be planar in CP and pyramidal (and likely with the right degree of pyramidalization) in CT. From a methodological point of view, the present study shows that the geometries optimized at the PBE0/6-31G(d) level are a reliable starting point for the evaluation of A_N and that few quite simple a posteriori corrections allow us to include the effects not properly treated at the DFT level.

Within the same geometry, the trend of A_N values can be explained in terms of relative weights of the two most important resonance structures of the nitroxide moiety

$$\underset{\text{(a)}}{\overset{\overset{\displaystyle O^\bullet}{|}}{-N-}} \;\leftrightarrow\; \underset{\text{(b)}}{\overset{\overset{\displaystyle O^-}{|}}{-{}^\bullet N^+ -}}$$

As a matter of fact, A_N is increased by the stabilization of the resonance structure (b) with respect to (a). In these terms, it is possible to rationalize a number of experimental results, including both intrinsic and environmental effects. Any polar solvent would indeed increase A_N through the relative stabilization of the "more polar" resonance structure (b). This effect should be relatively more important for a planar geometry of the nitroxide moiety, because the stability of the resonance structure (b), bearing a lone pair on the nitrogen, is larger for a pyramidal arrangement of the NO group. It is then not surprising that the predicted solvent shift is larger for CP than for CT.

Analogously, the presence of a negatively charged group $-COO^-$ will stabilize a partial positive charge on the nitrogen atom of the nitroxide moiety. As a matter of fact, the influence on A_N of the protonation state of the carboxyl group is larger for CP, where the carboxyl group is closer to the nitroxide moiety and, moreover, the ring is planar and any possible delocalization effect would be more effective. The above considerations are indirectly confirmed by the fact that A_N is found to decrease when a positive charge is present in the nitroxide ring.[7,22]

The present results also allow us to evaluate the relative importance of bulk solvent and of explicit hydrogen bond effects in determining A_N (see Table 3). The latter interactions appear

to be more important because, on the average, they contribute ≈80% of the total solvent shift. However, bulk effects cannot be neglected, because the inclusion of only the first solvation shell molecules leads to a nonnegligible error (≈0.5 G). Bulk solvent effects are even more important for the charged residues, showing the effectiveness of continuum approaches in modeling the solvent screening of electrostatic effects in solution.

Finally, the P02 procedure has been shown to be quite reliable for determining the pK_a also in nonstandard compounds as open shell radicals, because the order of magnitude of the error is ≈1 kcal/mol and there is a good prediction of the relative acidities of CP and CT.

This is a quite interesting result, because the experimental evaluation of pK_a values for complex systems can be very difficult and a computational procedure that gives an accurate estimation could be an helpful alternative. Furthermore, a reliable calculation of A_N vs pH curves could allow the prediction of the dependence on the pH also for unstable radicals, not easily accessible by the experiments.

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