Photophysical Study of the Probes Acrylodan (1-[6-(Dimethylamino)naphthalen-2-yl]prop-2-en-1-one), ANS (8-Anilinonaphthalene-1-sulfonate) and Prodan (1-[6-(Dimethylamino)naphthalen-2-yl]propan-1-one) in Aqueous Mixtures of Various Alcohols

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In this work, we examined in depth the photophysical behavior of the probes acrylodan (1-[6-(dimethylamino)naphthalen-2-yl]prop-2-en-1-one), ANS (8-anilinonaphthalene-1-sulfonate), and prodan (1-[6-(dimethylamino)naphthalen-2-yl]propan-1-one) in several pure solvents and aqueous mixtures of MeOH, i-PrOH, EtOH, and 2,2,2-trifluoroethanol between 0–90% (*v*/*v*). Although these probes have been widely used to study biological systems, the properties that govern their $\tilde{\nu}_{max}$ emission remain somewhat obscure. These alcohols are soluble in all proportions in H_2O, and are frequently used in peptide and protein studies as denaturing agents or because of their ability to substantially increase secondary structure or alter the folding-unfolding kinetics of proteins. The aim here was to rationalize the interpretation of $\tilde{\nu}_{max}$ emission of these probes in terms of specific and general effects of the solvent. To this end, we used the mixture parameters SA (solvent acidity), SB (solvent basicity), and SPP (solvent polarity/polarizability). The results suggest that it is incorrect to categorize these compounds purely as probes of polarity, when using these solvents.

Introduction. – Proteins exhibit intrinsic fluorescence that arises largely from tyrosine and tryptophan residues, and that has been used in studies of kinetic mechanisms, catalytic mechanisms at active sites, conformational changes, and folding-unfolding processes [1][2]. But the information that can be derived from this luminescence is very limited in many cases because of the difficulty involved in accurately interpreting the $\tilde{\nu}_{max}$ emission of the tyrosine and tryptophan groups [3]. For this reason, fluorescence probes are generally used in several protein studies [4][5]. Generally, the probes are chosen in terms of their sensitivity to the phenomenon of interest.

The biological usefulness of the probes acrylodan, ANS, and prodan relies on the hypothesis that they are purely probes of polarity, and, hence, that their fluorescence depends solely on this property. This has never been irrefutably demonstrated; rather, the probes have provided results that are difficult to interpret in the light of this assumption [6–10].

Acrylodan exhibits a fluorescence-quantum yield that is very low in polar solvents and very high in nonpolar solvents, which has been attributed to the presence of a

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double bond conjugated to the C=O group [6–13]. Fluorescence of ANS is quenched on hydrophilic environments, but substantially enhanced in hydrophobic ones, which is reflected in a large shift in the $\tilde{\nu}_{max}$ emission [14–16]. It has been used to identify structural changes in proteins [17–26]. Prodan is a probe with a high spectral sensitivity to its environment [27–32]. Several authors have ascribed it to the high dipole moment of the compound in the excited state [3][5][33], but others indicate that it is produced by specific interactions with the solvent [31].

To clarify all of this, we assume that the solvent molecules in the microenvironment of a physical process is often a noninert medium that can play a major role in solution chemistry, and solvent molecules can interact specifically with solute. So, the relationship between intrinsic solvent properties and the $\tilde{\nu}_{max}$ emission for our fluorescence probes will shed some light in the resolution of this subject.

Catalán et al. [31] examined the spectroscopic behaviour of prodan in 38 pure solvents, quantifying the photophysics of this compound in terms of the SA (solvent acidity), SB (solvent basicity), and SPP (solvent polarity/polarizability); where, according to *Taft* and *Kamlet* [34], acidity represents the capacity of the solvent to act as the H-bond donor and basicity represents the capacity of the solvent to act as the H-bond acceptor, in H-bonds between solvent and solute molecules.

Catalán et al. have also applied the probe/homomorph method [35–38] for calculating these parameters in solvent mixtures that has allowed them, recently, to rationalize the kinetics of *t*-BuCl hydrolysis in 20 pure and 127 mixed solvents [39], the decarboxylation kinetics of benzisoxazole-3-carboxylic acids in 24 pure solvents and 34 DMSO mixtures [40], and a new contribution to the problem of preferential solvatation [41].

In this work, we rationalize the photophysical behavior of acrylodan, ANS, and prodan in pure solvents of different polarities, as well as in different mixtures of H_2O with MeOH, EtOH, i-PrOH, or 2,2,2-trifluoroethanol. We make no assumptions regarding specific interactions of the solvent with the excited state.

The aim of this work is to contribute to an accurate interpretation of the results obtained with acrylodan, ANS, and prodan in biological systems.

Experimental. – Acrylodan (=1-[6-(dimethylamino)naphthalen-2-yl]prop-2-en-1-one), ANS (=8-anilinonaphthalene-1-sulfonate), and prodan (=1-[6-(dimethylamino)naphthalen-2-yl]propan-1-one) were obtained from *Molecular Probes, Inc.* (Eugen, OR). All solvents used were supplied by *Sigma Chemical Co.* (St. Louis, MO) and were of the highest available purity, verified by GC (MeOH > 99.0%, EtOH > 99.9%, i-PrOH > 98.0%, 2,2,2-trifluoroethanol > 99.5%).

Fluorescence measurements were obtained with a *Perkin-Elmer LS-50B* fluorimeter equipped with a calcite prism and *Polaroid HNP'B* film polarizers. The emission monochromator was calibrated with an *Oriel 6035* Hg (Ar) spectral-calibration lamp. Slit widths were 4 nm for both monochromators (emission and excitation), and a quartz cell of 1-cm light path was used throughout. All emission spectra were obtained with excitation wavelength at the corresponding maximum absorption wavelength of the probe in the corresponding solvent. All solns. were air-equilibrated. The temp. was adjusted at $25.0 \pm 0.1°$ with the aid of an external bath.

The parameters SA, SB, and SPP for each of the 45 H_2O/alcohol mixtures were calculated from the solvatochromism of various probe/homomorph couples according to *Catalán et al.* [35–38]. The SA is evaluated from the solvatochromism of the probe *O'*-(*tert*-butyl)stilbazolium betaine dye and its homomorph *O*,*O'*-di(*tert*-butyl)stilbazolium betaine dye [37]. The SA of solvents more acidic than MeOH (SA = 0.6) is evaluated by applying the solvatochromic comparison method [42] to solvatochromism measurements of the probe 3,6-diethyl-1,2,4,5-tetrazine [38]. The SB is calculated from the solvatochromism of the probe 2,3-dihydro-5-nitroindole and its homomorph 2,3-dihydro-*N*-methyl-5-nitroindole [36]. The SPP is characterized from the solvatochromism of the probe 2-(dimethylamino)-7-nitrofluorene and its homomorph 2-fluoro-7-nitrofluorene [35].

To relate the changes in fluorescence measurements with the properties of the solvent, we use the MINITAB program [43], which fits the $\tilde{\nu}_{max}$ emission of the different probes and provides *Eqn. 1*

$$\tilde{\nu}_{max}\ \text{Emission} = (\tilde{\nu}_{max}\ \text{emission})_0 + a\ \text{SA} + b\ \text{SB} + c\ \text{SPP} \quad (1)$$

where SA, SB, and SPP are typical of the mixture, and coefficients *a*, *b*, and *c* pertain to the property in question, $\tilde{\nu}_{max}$ emission.

Results. – The general solvent effect on the $\tilde{\nu}_{max}$ emission of a probe can be described with the *Lippert* equation *(Eqn. 2)* [44]:

$$\tilde{\nu}_{max}\ \text{emission} = -(\mu^* - \mu)\,\mu^*\,\frac{2}{hca^3}\,\Delta f - (\mu^{*2} - \mu^2)\,\frac{2}{hca^3}\,f + \tilde{\nu}_0\ \text{absortion} \quad (2)$$

where μ^* and μ are the dipole moments for the ground and excited states, respectively, $\Delta f = \frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}$ (ε is the dielectric constant and n the refraction index) is the orientation polarizability, h is *Planck*'s constant, c is the speed of light, a is the radius of the cavity in which the fluorophore resides, $f = \frac{n^2 - 1}{2n^2 + 1}$ is the polarizability, and $\tilde{\nu}_0$ absorption is the frequency corresponding to the vacuum transition.

This equation only holds with solvents that exhibit nonspecific effects. If a chromophore does not obey this equation, it is assumed to be subjected also to specific interactions between solvent and solute molecules (*i.e.*, H-bonds) that are not included in *Eqn. 2*.

The probes acrylodan, ANS, and prodan possess well-defined $\tilde{\nu}_{max}$ emission in solvents of widely different polarities (*Table*), or in different H_2O/alcohol mixtures (see *Fig.* for H_2O/2,2,2-trifluoroethanol mixture). As the data did not fit *Eqn. 2*, and since the $\tilde{\nu}_{max}$ emission is highly sensitive to changes in the environment, we tried to relate it to the SA, SB, and SPP parameters obtained for the H_2O/alcohol mixtures used. If the probes should be purely polarity probes, then the $\tilde{\nu}_{max}$ emission should depend only on the SPP of the solvent. Thus, first, we tried to fit the results considering only the SPP, and the equations obtained had a correlation coefficient $r < 0.90$ in the three cases, which means mathematically that the data do not significantly fit the equation. This corroborates our idea that the $\tilde{\nu}_{max}$ emission cannot be represented in terms of only the polarity of the media. If the results could be fit considering the parameters SA, SB, and/or SPP, the equations obtained are: *Eqn. 3* for acrylodan, *Eqn. 4* for ANS, and *Eqn. 5* for prodan:

$$\tilde{\nu}_{max}\ \text{emission}\ [\text{kK}] = 32.7 \pm 0.1 + 1.81 \pm 0.06\ \text{SA} + 0.82 \pm 0.06\ \text{SB} - 16.2 \pm 0.2\ \text{SPP} \quad (3)$$

$r = 0.991$, s (standard deviation) $= 0.13$

$$\tilde{\nu}_{max}\ \text{emission}\ [\text{kK}] = 26.1 \pm 0.1 - 2.10 \pm 0.03\ \text{SA} - 4.9 \pm 0.2\ \text{SPP} \quad (4)$$

$r = 0.995$, $s = 0.67$

Table. *The* $\tilde{\nu}_{max}$ *Emission* [kK] *of ANS, Prodan, and Acrylodan in Solvents of Different Polarity*

	$\tilde{\nu}_{max}$ Emission [kK]		
	ANS	Prodan	Acrylodan
Cyclohexane	23.461	24.632	23.678
Pentane	22.191	22.725	20.124
2-Methylbutan-2-ol	22.594	23.411	21.601
Cyclooctane	22.021	23.023	19.689
p-Xylene	21.356	21.971	17.921
Benzene	–	21.902	18.841
Toluene	21.704	21.001	20.831
Mesitylene	20.992	21.551	17.245
Tetralin	21.367	21.195	20.145
THF	21.045	21.002	20.491
2-Methyl-THF	21.154	21.201	20.015
Et_2O	–	–	22.971
Formamide	–	–	21.152
1,2-Dichlorobenzene	–	–	24.015
AcOEt	–	–	24.012
Methyl benzoate	–	–	23.584
Ethylene glycol	–	–	19.121
Octanol	–	–	24.198
2,2,2-Trifluoroethanol	19.417	19.607	24.158
Propan-1-ol	20.964	–	19.961
i-PrOH	21.391	20.618	20.601
MeOH	20.833	20.408	20.125
2,2,2-Trichloroethanol	–	–	19.912
DMSO	23.171	23.605	23.241
Pentyl acetate	21.783	22.311	18.866
AcOPr	21.537	21.589	18.012

$$\tilde{\nu}_{max}\ \text{emission}\ [\text{kK}] = 27.5 \pm 0.2 - 3.16 \pm 0.07\ \text{SA} - 1.20 \pm 0.07\ \text{SB} - 5.2 \pm 0.2\ \text{SPP} \quad (5)$$

$r = 0.988$, $s = 0.13$

In all three cases, the number of points used in the fit was n, n being equal to 17 pure plus 180 mixed solvents (45 H_2O/MeOH, 45 H_2O/EtOH, 45 H_2O/i-PrOH, and 45 H_2O/2,2,2-trifluoroethanol).

As can be seen, the interpretation of the changes in $\tilde{\nu}_{max}$ emission for the three probes requires consideration of specific interactions between probe and solvent, *i.e.* the presence of H-bonds.

Discussion. – The most salient conclusion from these results is that, contrary to widespread belief, acrylodan, ANS, and prodan are not purely polarity probes, although their dependence on other specific interactions with solvents are not identical. ANS can interact in two different ways (SA and SPP) and prodan and acrylodan in three (SA, SB, and SPP).

The probes ANS and prodan have similar dependencies on SA and SPP (the fit equations, *Eqn. 4* and *Eqn. 5*, have same order values of coefficients and identical signs). However, the SB term is almost negligible for some mixtures in the final value for the $\tilde{\nu}_{max}$ emission because of its coefficient (1.2 compared with 5.2 or 3.1), and

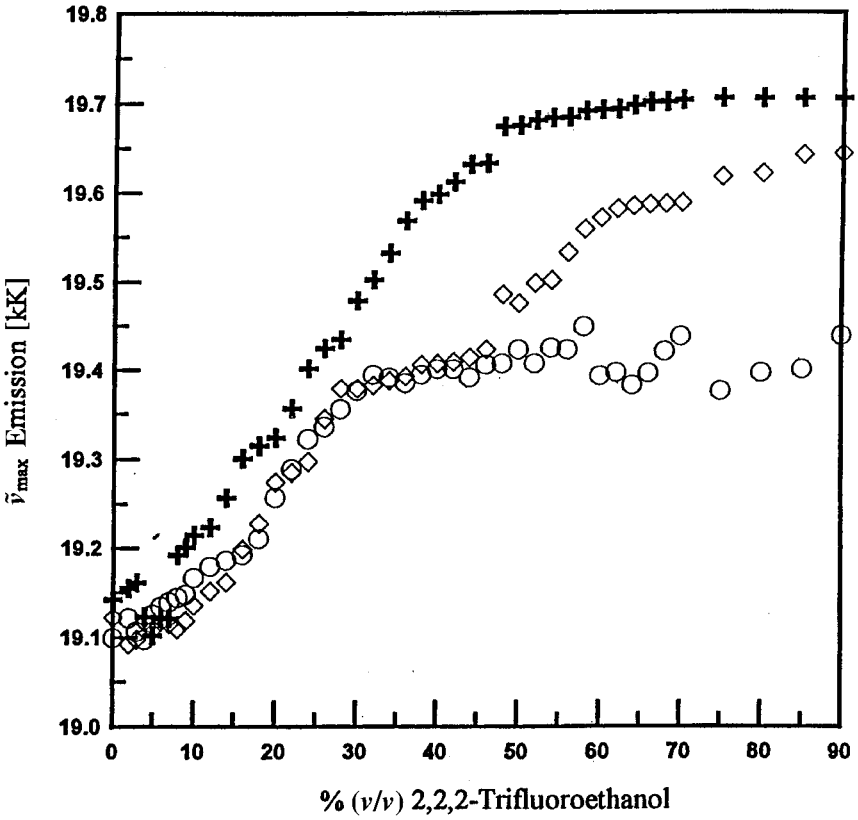

Figure. *Variation of the $\tilde{\nu}_{max}$ emission* [kK] *of acrylodan* (○), *ANS* (+), *and prodan* (◇) *as a function of the alcohol concentration* (% *v/v*) *for the mixture H_2O/2,2,2-trifluoroethanol*

because the SB value for pure H_2O is 0.025 [36]. Consequently, both probes should lead to very similar results in mixtures with a high H_2O content.

The relative contributions of the SA and SPP terms to the $\tilde{\nu}_{max}$ emission of the probes is alcohol-dependent. Thus, *e.g.*, for prodan in aqueous mixtures of 2,2,2-trifluoroethanol, the SA varies from 0.920 in pure alcohol to 1.062 in pure H_2O, and the SPP varies from 0.912 in pure alcohol to 0.962 in pure H_2O. This relationship is different for aqueous mixtures of i-PrOH (SA = 0.283, SPP = 0.848), EtOH (SA = 0.400, SPP = 0.853), and MeOH (SA = 0.605, SPP = 0.857).

One must admit, at least in some cases, that ANS exhibits dual behavior, because changes in its fluorescence may result from changes in a hydrophobic or an acidic environment. This is consistent with the dual behavior observed by several authors in studies about ANS-protein interactions that depend on the particular environment of the binding site [14–16][18–21]. Taking into account that H-acceptor groups (C=O) and H-donor groups (NH) exist in the protein core, and that the side chains of amino acids also possess H-acceptor and donor groups, binding of ANS to proteins can occur in different ways. The dependence of $\tilde{\nu}_{max}$ emission on acidity of the solvent could be

explained on the basis of assumption of specific interactions between the SO_3^{2-} group in ANS molecule and the solvent. *Matulis* and *Lovrien* [19] previously found the sulfite-ion substituent in ANS to interact with proteins and polyamino acids.

The $\tilde{\nu}_{max}$ emission of acrylodan is a function of SA, SPP, and, to a lesser extent, SB (*Eqn. 3*). However, the dependence on SPP is the most marked, and the sign of the dependence on SA is opposite of that for ANS and prodan (*Eqns. 4* and *5*). This probe must reflect the polarity of an environment with greater accuracy than either prodan or ANS, because the $\tilde{\nu}_{max}$ emission of the probe is essentially dependent on the SPP term. In fact, the fluorescence emission appears to be closely related to the dielectric constant of the medium, ε [45], that reflects the polarity/polarizability of the solvent according to *Eqn. 1*.

The only structural difference between acrylodan and prodan is the presence of unsaturation in the former. Acrylodan has been reported to bind covalently to SH groups to become prodan, the $\tilde{\nu}_{max}$ emission of which depends on the three solvent parameters in a radically different way from that of acrylodan. Therefore, it should be used with caution in environmental studies as it exhibits two different types of photophysical behavior depending on whether or not it binds covalently to a protein. Both acrylodan and prodan can interact *via* the C=O group with acidic sites (H-donor groups). Thus, it is not possible to make unequivocal conclusions about the polarity of the probe's environment, accounting for the disparate results provided by these probes [6].

Consequently, the only way to derive accurate information about the environment of each probe is to use pairs of probes that give different information and then cancel the dependence of $\tilde{\nu}_{max}$ emission on one or more solvent parameters. Thus, the $\tilde{\nu}_{max}$ emission for ANS, according to *Eqn. 4*, depends on SA and SPP, the $\tilde{\nu}_{max}$ emission for prodan (*Eqn. 5*) in the same environment must depend on SA, SPP, and SB; in both cases there is a fluorescence shift due to the acidity or polarity of the surroundings, but the shift for prodan must include also information about basicity of the environment. Comparing the shifts for the two probes allows one to draw reliable conclusions about other types of interactions and consequently other types of solvent properties different from those derived from polarity.

This research was funded by Spain's *DGICYT Projects* BQU 2000/0787, PB96-0596, and PB96-0667. *F. M.* acknowledges award of a fellowship from Spain's *Ministry of Science and Education*, *S. C.* one from the *Complutense University* of Madrid (Spain), and *C. D.* postdoctoral grant from the *Comunidad de Madrid.*

REFERENCES

[1] F. Moreno, S. Corrales, F. García-Blanco, M. G. Gore, K. Rees-Milton, J. E. Churchich, *Eur. J. Biochem.* **1996**, *240*, 435.
[2] F. Vanzi, B. Madan, K. Sharp, *J. Am. Chem. Soc.* **1998**, *120*, 10748.
[3] J. R. Lakowicz, B. P. Maliwal, H. Cherek, A. Batier, *Biochemistry* **1983**, *22*, 1741.
[4] J. R. Lakowicz, 'Principles of Fluorescence Spectroscopy', Polonium, New York, 1983, Chapt. 9.
[5] J. R. Lakowicz, 'Topics in Fluorescence Spectroscopy: Biochemical Applications', Polonium, New York, 1992, Chapt. 7.
[6] M. P. Mims, C. B. Sturgis, J. T. Sparrow, J. D. Morrisett, *Biochemistry* **1993**, *32*, 9215.
[7] A. W. Yem, D. E. Epps, W. R. Mathews, D. M. Guido, K. A. Richard, N. D. Staite, M. R. Deibel, *J. Biol. Chem.* **1992**, *267*, 3122.

[8] S. S. Lehrer, Y. Ishii, *Biochemistry* **1988**, *27*, 5899.
[9] R. Wang, S. Sun, E. J. Bekos, F. V. Bright, *Anal. Chem*. **1995**, *67*, 149.
[10] J. D. Jordan, R. A. Dunbar, F. V. Bright, *Anal. Chem.* **1995**, *67*, 2436.
[11] W. J. Dong, H. C. Cheung, *Biochem. Biophys. Acta*. **1996**, *1295*, 139.
[12] S. W. Reid, E. K. Koepf, L. D. Burtnick, *Arch. Biochem. Biophys.* **1993**, *302*, 31.
[13] J. S. Lundgren, M. P. Heitz, F. V. Bright, *Anal. Chem*. **1995**, *67*, 3775.
[14] L. Stryer, *J. Mol. Biol*. **1965**, *13*, 482.
[15] P. M. Mulqueen, M. J. Kronman, *Arch. Biochem. Biophys.* **1982**, *215*, 28.
[16] M. Engelhard, P. A. Evans, *Protein Sci*. **1995**, *4*, 1553.
[17] E. Bismuto, G. Irace, I. Sirangelo, E. Gratton, *Protein Sci*. **1996**, *5*, 121.
[18] G. V. Semisotnov, N. A. Rodionova, O. I. Razgulyaev, V. N. Uversky, A. F. Gripas, R. I. Gilmanshin, *Biopolymers* **1991**, *31*, 119.
[19] D. Matulis, R. Lovrien, *Biophys. J.* **1998**, *74*, 422.
[20] M. V. Encinas, J. A. Evangelio, J. M. Andreu, H. Goldie, E. Cardemil, *Eur. J. Biochem*. **1998**, *255*, 439.
[21] I. Sirangelo, E. Bismuto, S. Tavassi, G. Irace, *Biochem. Biophys. Acta* **1998**, *1385*, 69.
[22] N. Poklar, J. Lah, M. Salobir, P. Macek, G. Vesnaver, *Biochemistry* **1997**, *36*, 14345.
[23] A. Stevens, R. C. Augusteyn, *Eur. J. Biochem.* **1997**, *243*, 792.
[24] R. H. Smulders, W. W. Jong, *FEBS Lett*. **1997**, *409*, 101.
[25] A. R. Prasad, R. F. Luduena, M. Horowitz, *Biochemistry* **1986**, *25*, 3536.
[26] M. Mazumdar, P. K. Parrack, K. Mukhopadhyay, B. Bhattacharyya, *Biochemistry* **1992**, *31*, 6470.
[27] G. Weber, F. J. Farris, *Biochemistry* **1979**, *18*, 3075.
[28] J. B. Massey, H. S. She, H. J. Pownall, *Biochemistry* **1985**, *24*, 6973.
[29] R. B. McGregor, G. Weber, *Nature* **1986**, *319*, 70.
[30] P. L. G. Chong, *Biochemistry* **1988**, *27*, 399.
[31] J. Catalán, P. Pérez, J. Laynez, F. García-Blanco, *J. Fluoresc.* **1991**, *4*, 215.
[32] J. Zeng, P. L. G. Chong, *Biophys. J.* **1995**, *68*, 567.
[33] C. E. Bunker, T. L. Bowen, Y. P. Sun, *Photochem. Photobiol.* **1993**, *58*, 499.
[34] R. W. Taft, M. J. Kamlet, *J. Am. Chem. Soc*. **1976**, *10*, 2886.
[35] J. Catalán, V. López, P. Pérez, R. Martín-Villamil, J. G. Rodríguez, *Liebigs Ann. Chem.* **1995**, 241.
[36] J. Catalán, C. Díaz, V. López, P. Pérez, J. L. G. de Paz, J. G. Rodríguez, *Liebigs Ann. Chem.* **1996**, 1785.
[37] J. Catalán, C. Díaz, *Liebigs Ann. Chem*. **1997**, 1941.
[38] J. Catalán, C. Díaz, *Eur. J. Org. Chem.* **1999**, *2*, 351.
[39] J. Catalán, C. Díaz, F. García-Blanco, *J. Org. Chem*. **1999**, *64*, 6512.
[40] J. Catalán, C. Díaz, F. García-Blanco, *J. Org. Chem*. **2000**, *65*, 3409.
[41] J. Catalán, C. Díaz, F. García-Blanco, *J. Org. Chem*. **2000**, *65*, 9226.
[42] M. J. Kamlet, R. W. Taft, *J. Am. Chem. Soc*. **1977**, *98*, 377.
[43] Program Minitab, Inc., v. 9, State College PA; USA.
[44] E. Lippert, *Z. Elektrochem*. **1957**, *61*, 962.
[45] I. Tinoco, K. Sauer, J. C. Wang, 'Physical Chemistry: Principles and Applications in Biological Sciences, 3rd edn., Prentice-Hall, Inc., Englewood, 1978.

Received April 9, 2001