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NMR Study of the Inclusion Complexes of Carboxy-Phenoxathiin Derivatives with β-Cyclodextrin

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Abstract

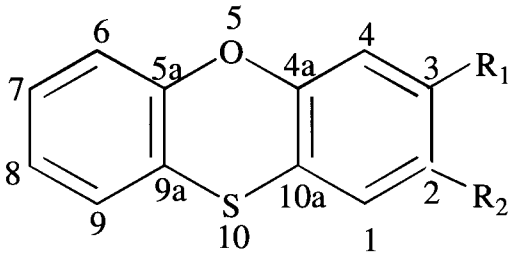
The inclusion complexes of the carboxylate forms of 3-carboxy- (**I**) and 2-carboxy-phenoxathiin (**II**) with β-cyclodextrin were studied by both one- and two-dimensional NMR spectroscopy. The analysis of the induced chemical shifts of the guests in the presence of different amounts of the host indicates the formation of complexes with 1:1 stoichiometry and association averaged pK values of 3.75 (**I**) and 4.4 (**II**). The qualitative analysis of cross peaks in the ROESY spectra support the inclusion of the guests in the cavity with the substituted phenyl ring, the COO^- group being in the proximity of the primary rim.

Introduction

The high sensitivity of the spectrofluorimetric method enhances its use in the study of biopolymer – ligand interactions. During previous investigations [1] on some derivatives in the phenoxathiin class we have found that 3-formyl, 3-acetyl and 3-carboxy-phenoxathiin (**I**) have adequate fluorescence properties to be used as potential biological markers for proteins. As a first step for using these derivatives for this purpose we were interested in the sensitivity of their emission to the presence of local hydrophobic regions in an aqueous medium. This condition is reached in the presence of cyclodextrins, already used as models for protein-ligand interactions.

Cyclodextrins are cyclic oligosaccharides [2–4] composed of 6, 7 or 8, α-1,4-linked glucose residues and characterized by a truncated cone shape. In their cavity, the cyclodextrins can accommodate a wide class of organic molecules leading to inclusion complexes with various stoichiometries. Taking into account the dimensions of the phenoxathiin derivatives, we have chosen as the host β-cyclodextrin (seven glucose residues), which seems appropriate for the formation of 1:1 complexes.

Our previous work [5] was focused on the spectral (steady-state fluorescence spectroscopy), thermodynamical and theoretical characterization of the inclusion complexes of some phenoxathiin derivatives with cyclodextrins. We found that there is a significant interaction between the formyl- and acetyl-phenoxathiin derivatives and cyclodextrin, the association constant being in the range 6000–8000 M^{-1}. For 3-carboxyphenoxathiin (**I**) and 2-carboxyphenoxathiin (**II**) (Figure 1), both the absorption and the fluorescence spectra in aqueous medium reflect a pH-dependence rationalized in terms of an acid-base equilibrium, with a pKa value around 4.5; this value is characteristic of the dissociation constants for related aromatic carboxylic acids.



I: R_1= COOH; R_2=H

II: R_1= H; R_2= COOH

Figure 1. The structures of the investigated compounds.

$$\underset{(\mathbf{a})}{\text{R-COOH}} \rightleftharpoons \underset{(\mathbf{b})}{\text{R-COO}^-} + \text{H}^+.$$

Considering the two molecular species, the non-dissociated and dissociated forms, hereafter labeled as (**a**) and (**b**), respectively, we have found a larger interaction constant for the carboxylate ion, i.e., K(**Ib**) = 7474 M^{-1} > K(**Ia**) = 1518 M^{-1}. In the case of **II** the weaker emission properties prevent the study of the inclusion process by steady state fluorescence measurements.

Molecular modeling of the inclusion complexes was performed by both Molecular Mechanics (MM) and semiempir-

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ical quantum chemistry (AM1) methods with GAMESS and hyperchem softwares, considering the two possible ways the guest penetrates the cavity, either with the unsubstituted phenyl ring (model A) or with the substituted moiety (model B). The results show a significant binding energy accounting well for the values of the association constants. For the carboxylic compounds, the molecular modeling outlined the role of the hydrogen bonding interaction; it was found that whatever way the guest enters the cavity (A or B), it will be involved in hydrogen bonds. In approach A, the hydrogen bond driving force pushes the compound through the cavity to reach a position in which the COO^- group is hydrogen bonded with the HO^- groups of the secondary wide rim. In approach B, the compound enters the cavity as far as the carboxylate ion could be hydrogen bonded with the primary hydroxyl groups. However, the molecular modeling failed to give an unambiguous answer to the most likely way, A or B, the guests penetrates the cavity. The MM calculations favor approach A while the AM1 calculations predict a more stable complex in the geometric arrangement B, but with only 6.27 kJ mol^{-1}.

As one of the most powerful experimental tools to obtain more pertinent information about the geometry of complexes is NMR spectroscopy, we have used 2D-ROESY [6] experiments to better characterize the inclusion complexes. The aim of the present paper is to present the results of one and two dimensional (ROESY) NMR spectroscopy, to estimate the association constants and the NOE dipolar coupling interactions. These host–guest complexes present a suitable case for this purpose; the β-cyclodextrin contains only aliphatic protons, the compound only aromatic ones and, consequently, the NMR signals are well separated.

Experimental

Materials

The phenoxathiin derivatives were synthesized as previously described [7]. The β-cyclodextrin from Aldrich (M = 1135) was used without further purification. The solutions were prepared in sodium tetraborate 0.01 M, Fluka buffer solution, pH = 9.18. The deuterated solvents, dimethylsulfoxide-d_6, and acetone-d_6 were purchased from SDS.

A stock solution of each guest of about 5 mM was prepared in a pH = 9.18 buffer. From this stock solution, host-guest mixtures were prepared following the methods of Orstan and Ross [8] and Dodziuk *et al.* [9]. The stock solution was separated into two parts; in one of them a weighed quantity of cyclodextrin was added to obtain a concentration of 10^{-2} M. Different volumes of these two solutions were mixed yielding the same concentration of the guest compound and variable concentration of the host.

NMR measurements

All ^{1}H and ^{13}C NMR experiments were recorded at 298 K on a Bruker-AMX 400 operating at 400.13 and 100.61 MHz, respectively. The proton and carbon resonances of the uncomplexed phenoxathiin derivatives were measured using DMSO-d_6 as an internal reference; the proton shifts of the complexed compounds were obtained using acetone-d_6 as an external lock.

ROESY [10, 11] spectra were recorded on a Bruker DPX 500, at 500.13 MHz and 300 K. The experimental parameters were as follows: acquisition time of 0.51123 s, spectral width of 4006.41 Hz digitized with 4 K complex points in F2; 32 scans per t_1 increment, 1024 t_1 increments, 500 m s spinlock time; presaturation of H_2O signal during relaxation delay [12, 13]. The data were zero-filled to 2 K in F_1 and proccesed with a $\pi/2$-shifted Q-sine window in both dimensions.

The association constant was evaluated using Macomber's [14] formula (1) for fast exchange, considering a 1:1 stoichiometry.

$$\delta = \delta_g - \left(\frac{\Delta\delta}{2}\right)(b - \sqrt{b^2 - 4R}) \tag{1}$$

where

$$b = 1 + R + \frac{1}{(K[H]_0)}$$

$$R = \frac{[G]_0}{[H]_0}$$

$$\Delta\delta = \delta_g - \delta_c$$

and $[G]_0$ = the guest concentration; $[H]_0$ = the host concentration; δ = the observed chemical shift; δ_g = the initial chemical shift of the compound; δ_c = the complex chemical shift; K = the association constant.

Results and discussion

^{1}H and ^{13}C NMR study in DMSO-d_6

The ^{1}H and ^{13}C NMR spectra and the two-dimensional COSY, HMBC and HMQC spectra allow the complete analysis of the chemical shifts and the coupling constants for the uncomplexed compounds. The results are listed in Table 1. For both compounds the solvent (DMSO) was sufficiently basic to produce a large amount of the dissociated forms and therefore the carboxylic protons were not observed.

For compound **I** the three protons in the substituted ring are more deshielded than those of the other ring and therefore well separated. The lowest deshielded is proton H-1, situated in a *meta* position with respect to the substituent. The following pattern is seen: an asymmetric doublet of doublets for the H-2 signal, due to a strong coupling with H-1 and a weak coupling with H-4, and two doublets, one asymmetric, one symmetric belonging to H-1, and H-4 respectively. In the case of compound **II**, H-1 and H-3 are also significantly deshielded while the H-4 signal overlaps with those of the protons of the other ring. We can observe that

Table 1. ^{1}H and ^{13}C NMR chemical shifts, δ (ppm), and proton coupling constants, J(Hz), of compounds **I** and **II**

Chemical shifts δ/ppm					Coupling constants J/Hz			
	Compound **I**		Compound **II**		Compound **I**		Compound **II**	
Position	δ_C	δ_H	δ_C	δ_H				
1	127.1	7.38	128.1	7.76	J_{12}	8.0	J_{13}	1.9
2	125.7	7.63	127.6	–				
3	130.9	–	129.7	7.77	J_{24}	1.6	J_{34}	8.8
4	117.8	7.50	117.8	7.16				
4a	151.0	–	154.5	–	J_{67}	7.7	J_{67}	7.4
5a	150.4	–	150.5	–				
6	117.8	7.11	117.8	7.11	J_{68}	1.2	J_{68}	1.1
7	127.1	7.27	127.1	7.24				
8	125.4	7.13	125.5	7.12	J_{78}	7.8	J_{78}	8.1
9	128.7	7.23	128.5	7.26				
9a	118.1	–	119.5	–	J_{79}	1.6	J_{79}	1.6
10a	125.2	–	118.2	–				
11	166.2	–	166.0	–	J_{89}	7.6	J_{89}	7.8

in this case too the proton in the *meta* position *vs* the COO^{-} group is also the lowest deshielded, the effect being more pronounced than for the former. The assignment of this proton was made considering the H-3 resonance in the COSY spectrum.

For both compounds the protons in the unsubstituted ring have very close resonances which are grouped in two multiplets situated in the range 7.29–7.23 ppm and 7.14–7.10 ppm, respectively. Analysis of the COSY shows that the signals overlap: the first multiplet corresponding to the protons H-7 and H-9, and the second one to H-6 and H-8. The most deshielded protons are protons H-7 and H-9, the separation between them being larger in **I**.

In the ^{13}C spectra the most deshielded carbon atoms are the carboxylic ones, clearly observed at 166 ppm. Considering the molecular structure, the ring carbons be divided in three types, the group of carbons C-1, C-4, C-6 and C-9, the group of carbons C-2, C-3, C-7 and C-8 and the quaternary carbons C-4a, C-5a, C-9a and C-10a. For both compounds, in the first group of carbons the proximity of the sulfur atom determines a larger deshielding effect than the oxygen atom, i.e., 127–128 ppm (C-1 and C-9) as against 117.8 ppm (C-4 and C-6). For the positions further from the heteroatoms, C-2, C-3, C-7 and C-8, the chemical shifts are similar. Concerning the quaternary carbons, the data in Table 1 point out a pronounced difference between C-4a and C-5a, significantly deshielded and the other two, C-9a and C-10a. However, a difference has to be noted between the shift of C-10a in both compounds, the shift being larger in **I** than in **II**. This effect is probably due to a cumulative effect of the sulfur atom and of the carboxylate group in the *para* position.

The complete assignment was performed using the HMQC and HMBC spectra. Both the chemical shifts and the coupling constants (Table 1) are similar to those previously reported for other 2-, and, 3-substituted phenoxathiin derivatives. [15] The similar values of the chemical shifts of the substituted carbon in the two compounds can be explained by the weaker influence of carboxyl groups relative to formyl and acetyl groups.

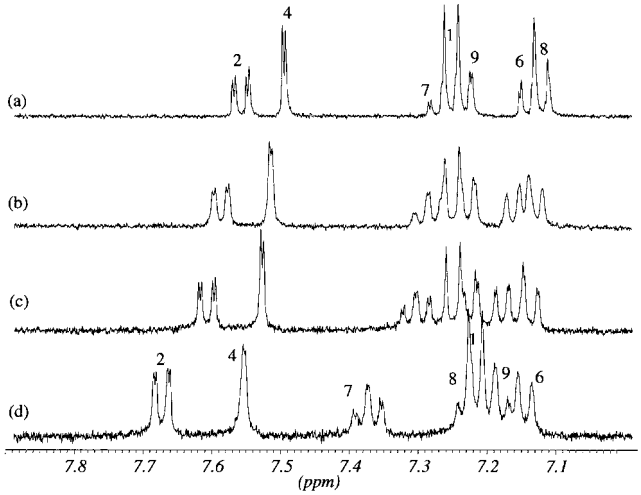

Figure 2. Partial 400 MHz ^{1}H NMR spectra (only guest protons are shown) for solutions of β-cyclodextrin/compound **I** complexes; molar ratios host/guest: (a) 0; (b) 0.29; (c) 0.39; (d) 1.86.

Inclusion complex formation

Addition of different amounts of cyclodextrin to a solution with constant concentration of the guests leads to modification of the NMR spectra confirming the formation of inclusion complexes [16, 17]. A typical example is presented in Figure 2 for compound **I**. The ^{1}H-NMR spectra of the host-guest complexes are characterized by two well separated domains, the domain of the resonances of the host protons (2.4–3.1 ppm) and the domain of the guest aromatic protons (7.1–7.8 ppm).

Considering the cyclodextrin resonances, the most influenced protons are protons H-3 and H-5, which are the protons located in the interior of the cavity. The significant

Table 2. Complexation-induced ^{1}H chemical shifts differences, $\Delta\delta_H$ (ppm), for the two compounds

Compound	$\Delta\delta_{H-1}$	$\Delta\delta_{H-2}$	$\Delta\delta_{H-3}$	$\Delta\delta_{H-4}$	$\Delta\delta_{H-6}$	$\Delta\delta_{H-7}$	$\Delta\delta_{H-8}$	$\Delta\delta_{H-9}$
I	0.034	−0.115	–	−0.057	−0.021	−0.109	−0.093	0.053
II	−0.046	–	−0.092	−0.013	0.011	−0.079	−0.078	0.093

modifications of these protons are usually considered as a support for the complexation process [18, 19].

The complexation induced chemical shifts for the guest protons are listed in Table 2. The following observations can be made:

- The changes in the chemical shifts upon inclusion are lower for compound **II**.
- For both guests the larger downfield shifts are presented by the protons vicinal to the carboxylate group (H-2 for **I** and H-3 for **II**) and by the protons H-7 and H-8 in the unsubstituted ring.
- The other two protons in the *ortho* position to the substituent (H-4 for **I** and H-1 for **II**) are shifted almost to the same extent.

Due to the clear pattern of the NMR spectrum of the guests, the formation of the complexes was quantitatively studied by monitoring the changes of the chemical shifts of the guest-protons in respect with increasing the β-cyclodextrin concentration [20]. It was observed that, in all cases, a limit value for the chemical shift is reached for cyclodextrin concentration above a given threshold corresponding to 100% complexation. The host–guest molar ratio at this threshold value indicates that the stoichiometry of the complexes is 1:1. The data were fitted with the Macomber formula. The results are given in Table 3. We can observe a difference between the association constants of our two guests. This fact was also discussed by Zubiaur [21].

A ROESY spectrum of compound **I** is presented in Figure 3 and the results of a qualitative analysis of cross-peaks [18, 22, 23] between the guests and the β-cyclodextrin protons is presented in Table 4. The relevant cross-peaks confirm the interaction between the inner protons of the host, H-3, H-5 and H-6,6′, with some guest protons. There is no correlation with the outer protons H-2 and H-4 of the β-cyclodextrin. This observation indicates that the guest is included in the β-cyclodextrin cavity. This is in agreement with most of the NMR studies. Another behavior was reported by Zubiaur *et al.* who found also an external interaction [21]. The data in Table 4 show that the protons H-5, H-6 and H-6′ of the β-cyclodextrin interact mainly with H-2 and H-4 of compound **I** and H-3 and H-5 of compound **II**, respectively. The H-3 proton of the β-cyclodextrin interacts more strongly with the protons H-6, H-7, H-8 and H-9 in both compounds.

The actual ROESY results predict that the COO^- group protrudes from the cavity such that the protons in the unsubstituted ring, H-6, H-7 and H-8 are found in the proximity of the H-3 cyclodextrin proton. A qualitative representation of the complex is given in Figure 4. These experimental findings support the previous AM1 calculations, which predict a more stable structure for the case in which the guest penetrates the cavity with the substituted ring. Some differences are found concerning the exact position of the carboxylate group; the MO calculations predict that the COO^- group is inside the cavity, implied in hydrogen bonds with the primary hydroxyl groups, while ROESY results point out that the carboxyl protrudes from the cavity.

Literature data on NMR experiments coupled with molecular modeling of the inclusion complexes of carboxylic acids and carboxylate ions with cyclodextrin have reported different results concerning the way the guest penetrates the cavity. Using the ROESY experiments for the inclu-

Table 3. Calculated values of log K for different protons of the investigated compounds

Compound	Proton	pK
I	H-2	3.6 ± 0.1
	H-7	3.9 ± 0.1
II	H-3	4.3 ± 0.1
	H-7	4.5 ± 0.1

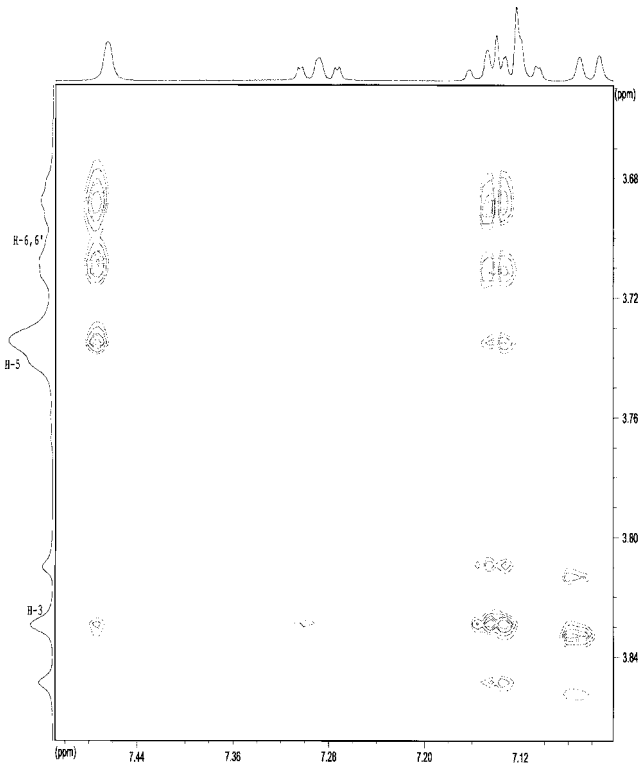

Figure 3. Partial 500 MHz ROESY spectrum of the complex formed between the β-cyclodextrin and compound **I**.

Table 4. Summary of intermolecular ROE cross-peaks* between protons of β-cyclodextrin and compounds **I** and **II**

	$H_{\mathbf{I}}$-1	$H_{\mathbf{I}}$-2	$H_{\mathbf{I}}$-4	$H_{\mathbf{I}}$-6	$H_{\mathbf{I}}$-7	$H_{\mathbf{I}}$-8	$H_{\mathbf{I}}$-9
$H_{\beta-CD}$-3	+	+	+	++	++	++	+
$H_{\beta-CD}$-5	+	++	++	++	+	0	+
$H_{\beta-CD}$-6,6′	+	+	++	+	+	0	+
	$H_{\mathbf{II}}$-1	$H_{\mathbf{II}}$-3	$H_{\mathbf{II}}$-4	$H_{\mathbf{II}}$-6	$H_{\mathbf{II}}$-7	$H_{\mathbf{II}}$-8	$H_{\mathbf{II}}$-9
$H_{\beta-CD}$-3	+	+	+	++	+++	+++	+++
$H_{\beta-CD}$-5	++	++	+	+	+	+	++
$H_{\beta-CD}$-6,6′	++	+	+	+	0	0	+

* The relative strength of cross-peaks is indicated by: +++ (strong); ++ (medium); + (weak) and 0 (no effect).

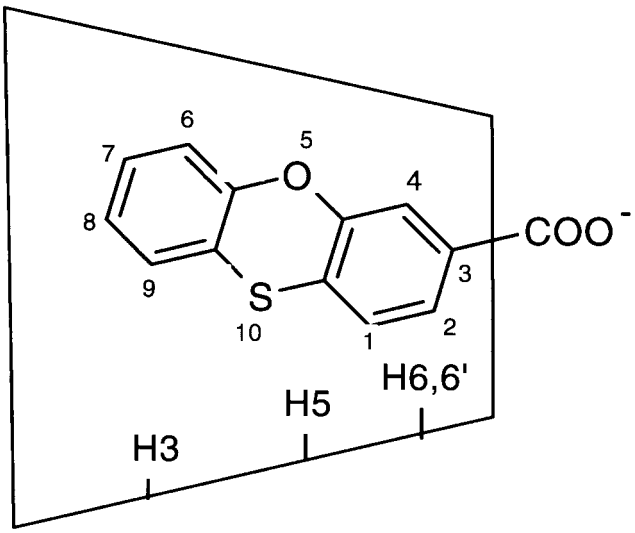

Figure 4. Presumed geometry for the compound **I** and β-cyclodextrin inclusion complex deduced from the ROE experiments.

sion complexes of cyclodextrin with substituted cyclohexancarboxylic and phenylalcanoic forms, Gadre *et al.* [24] found that all complexes seem to be encapsulated simultaneously with both possible geometries. The Molecular Mechanics calculations of the possible geometry of the inclusion complex of benzoic acid with β-cyclodextrin [18] showed that the two ways the guest could penetrate the cavity, with the phenyl or with the carbonyl group, are almost isoenergetic. This was also supported by the very low association constant found by NMR measurements ($K = 48$) and the minor line-shape changes of the signals upon complexation indicating a fast exchange between the free and complexed compound. However, a comparison with the host–guest distances determined by NOESY experiments favor the complex with the carboxyl group embedded in the cavity and directed toward the primary rim. The ROESY results of Hirai *et al.* [25] on the conformation of two β-cyclodextrin-aromatic carboxylate anions (the benzoate and the 4-biphenyl carboxylate anions) indicated that the carboxylate group is located at the primary hydroxyl side of the cyclodextrin. In their NMR study of the interaction of (+)- and (−)-flurbifen with β-cyclodextrin Salvadori *et al.* [11] found that both enantiomers led to 1:1 complexes; at high concentration the same stoichiometry was retained but the dynamic behavior indicated the formation of larger aggregates. In the reported structures the COO^- group is also located in the proximity of the primary rim.

Conclusions

The analysis of the changes in the chemical shifts of the protons of the two carboxylic derivatives of phenoxathiin with the β-cyclodextrin concentration determined the formation of complexes with 1:1 stoichiometry. The determined complexation constants are of the same order for the two guests and are in agreement with the literature data [26] for this type of carboxylic acids. The dipolar interactions between the guest and host protons are visible in the ROESY spectra of the complexes. The qualitative analysis of the intensity of the cross-peaks confirms the disposition of the guest molecules inside the β-cyclodextrin as predicted by molecular modeling.

The relative rigidity of phenoxathiin structures will induce a restrained dynamics complexation process. This is favourable for extraction of distances between protons of the guest and β-cyclodextrin from the quantitative analysis of intermolecular ROE interactions. These measurements complemented by molecular modeling and molecular dynamics are in progress in order to obtain the three-dimensional structure of the supramolecular aggregates formed.

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