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Inclusion Complex Formation of Anthracycline Antibiotics with Cyclodextrins; a Proton Nuclear Magnetic Resonance and Molecular Modelling Study

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Abstract. The inclusion complexation of the anthracyline antibiotics doxorubicin and daunorubicin with cyclodextrins has been studied by proton NMR and molecular modelling. The anthracyclines were found to complex with γ-cyclodextrin, whereby the aglyconic part of the molecule is included in the cyclodextrin cavity. Job ratio plots based on NMR data indicate that the complex has a stoichiometry of 1 : 1. Complex constant values of 345 M^{-1} and 323 M^{-1} (at pH 3) were found for doxorubicin and daunorubicin, respectively.

Key words. Anthracyclines, cyclodextrins, complex formation, complex structure, ^{1}H-NMR, molecular modelling.

1. Introduction

Cyclodextrins (CyDs) are cyclic oligosaccharides built up from glucopyranose units. Commonly, naturally occurring CyDs consist of 6, 7 or 8 glucose units and are named α-, β- and γ-CyD, respectively. The CyD molecule has a conical shape, the inside being moderately non-polar. CyDs are water soluble since all free hydroxyl groups of the glucose molecules are on the outside of the ring. Because of their shape and physicochemical properties, CyDs can form inclusion complexes with various lipophilic compounds in a hydrophilic environment [1–3].

Anthracyclines are a group of antibiotics which show very strong antitumour activity [4, 5]. The two best known members of this class of compounds are doxorubicin (Dx) and daunorubicin (Dr). The structures are characterized by a tetrahydronaphthacenequinone aglycone, glycosidically linked to an aminosugar (Figure 1). Although the anthracyclines are stable in the solid state, they decompose in aqueous media [6–8]. The stability of anthracyclines in aqueous solution is affected by CyDs, indicating the formation of an inclusion complex [9].

The proton nuclear magnetic resonance (^{1}H-NMR) and molecular modelling studies described in this paper were carried out to gain insight into the anthracycline–CyD complex structure. Furthermore, NMR spectroscopy has been used to obtain information about the stoichiometry of the complex and to calculate complex constants.

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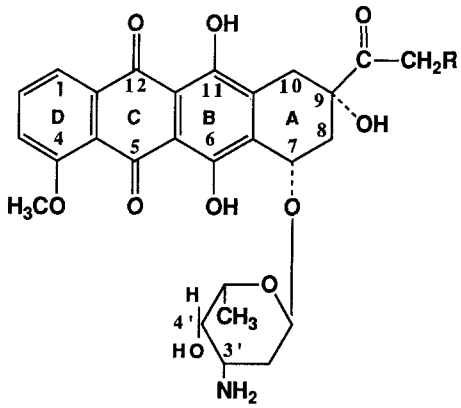

Fig. 1. Chemical structure of the anthracycline antibiotics doxorubicin (R = OH) (Dx) and daunorubicin (R = H) (Dr).

2. Experimental

2.1. CHEMICALS

All CyDs (α-, β- and γ-CyD) were donated by Nihon Shokukin Kako Co. Ltd. (Tokyo, Japan) and freeze-dried before use.

Dx was a gift from the Netherlands Cancer Institute (Amsterdam, The Netherlands) and Dr was kindly provided by Rhône-Poulenc Nederland BV (Amstelveen, The Netherlands). Both anthracyclines were supplied as hydorchloride salts and used as received. Deuterium oxide (D_2O) came from Janssen Chimica (Goirle, The Netherlands). All other chemicals were of analytical grade and deionized water was used throughout.

2.2. PREPARATION OF THE COMPLEX

The complexes were obtained by adding an anthracycline solution in D_2O to variable amounts of freeze-dried CyD. The desired pD value of the anthracycline solution was adjusted with DCl or NaOD, using a glass reference electrode and pH meter (Metrohm, E516 Titrikop, Herisau, Switzerland). The pH values are straight pH meter readings and were not corrected for the deuterium effect.

For the NMR study, the anthracycline concentration was kept constant at 3.7×10^{-3}M. The concentrations of α- and β-CyD were both 3.5×10^{-3}M. The γ-CyD concentration varied between 8.4×10^{-4}M and 1.3×10^{-2}M.

2.3 PROTON NMR STUDY

^{1}H-NMR spectra were recorded at ambient temperature with a Bruker WP 200 instrument at a frequency of 200.13 MHz. Deuterium oxide was used as the solvent and the HOD signal as the internal standard. Changes in chemical shifts were calculated by subtracting the chemical shift value of a proton in the complexed state from that of the same proton in the free state, at the same concentration. The study was performed at ambient temperature.

Phase sensitive, two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) experiments were carried out with a Bruker AM 500 instrument. D_2O was used as the solvent and the HOD signal was presaturated.

2.4 MOLECULAR MODELLING STUDY

The structure used for γ-CyD was taken from the Cambridge Structural Database (CSD) [10, 11]. Structures for α- and β-CyD were built using a procedure described earlier by van Helden *et al.* [12]. Dx was built using some fragments from CSD and the molecular editor available in Quanta [13]. Dx has several chiral centres of which the chiral centre at C_7 (Figure 1) strongly influences the orientation of the sugar ring. Although Arcamone *et al.* [14] have reported that the configuration at C_7 is (*S*); both the (*S*) and the (*R*) configuration were built.

The main degrees of freedom of the Dx molecule result from the two torsion angles in the glycosidic linkage. The energetically favourable conformations were found by a systematic grid search, and were optimized by using the adapted Newton-Raphson minimizer and the CHARMm force field [13]. All minimum energy conformations of Dx were subsequently docked into γ-CyD. This was performed manually by using the docking features of Quanta. Low energy complexes were then fully optimized with a distance constraint. All calculations were performed with the crystal structures of the compounds, so the solvent effect is not taken into account.

3. Results and Discussion

3.1. ^{1}H-NMR STUDY

3.1.1. *Interaction of Dx and Dr with Various CyDs*

The influence of the host (α-, β- or γ-CyD) on the ^{1}H-NMR spectra of the guest (Dx and Dr), and vice versa, were studied in D_2O on the basis of a 1 to 1 molar ratio. Signals were assigned on the basis of previous work [14–16].

After mixing the solution of the guest molecule with either α- or β-CyD, neither the chemical shifts of the guest nor those of the host showed a significant change. This indicates that no inclusion of the anthracycline molecule with either α- or β-CyD occurs. This observation is in good agreement with results obtained during a previous stability study, where both CyDs did not exert any effect on the degradation rate of Dx and Dr [9].

Figure 2 illustrates the Dx-induced ^{1}H-chemical shifts of γ-CyD. Since the signals of the H_5 and H_6 protons of CyD overlap with each other and with Dx signals, quantitative data were only obtained for protons 1 to 4 of the CyD molecule. The signals for these four protons shift upfield. The signal of H_3, which is located in the interior of the CyD cavity, obviously displays the largest shift, indicating that the guest is included in the cavity.

In Figure 3 the effects of γ-CyD on the ^{1}H chemical shifts of Dx are presented. Unfortunately, some proton signals overlap with γ-CyD or are too weak to allow quantitative determination of the shifts of these signals under the present experimental conditions. In addition to changes of the proton signals in the D ring, shifts

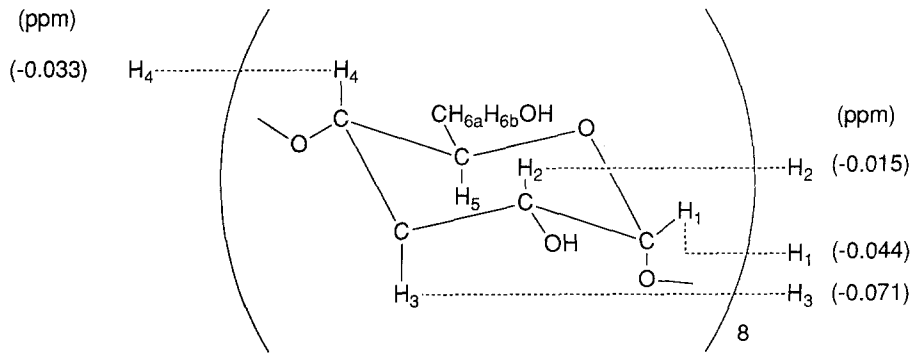

Fig. 2. Influence of doxorubicin on the ^1H chemical shifts of γ-CyD in D_2O. The values in parentheses are calculated from the expression $\Delta\delta = \delta_{complex} - \delta_{\gamma\text{-CyD}}$.

were also observed in the sggnals of the A ring and sugar moiety protons. Presumably, the D ring is included in the CyD cavity, whereas the A ring and the sugar part protrude from it. The sugar molecule may bend in the direction of the exterior of γ-CyD, and may interact with the free hydroxyl groups. The shifts on the outside of γ-CyD and in the A ring of Dx, probably caused by the interaction with sugar and the distortion of the A ring, by the interaction of the sugar with CyD, respectively, support this hypothesis.

The ^1H-NMR spectra of mixtures of Dr and γ-CyD show results similar to those obtained with Dx.

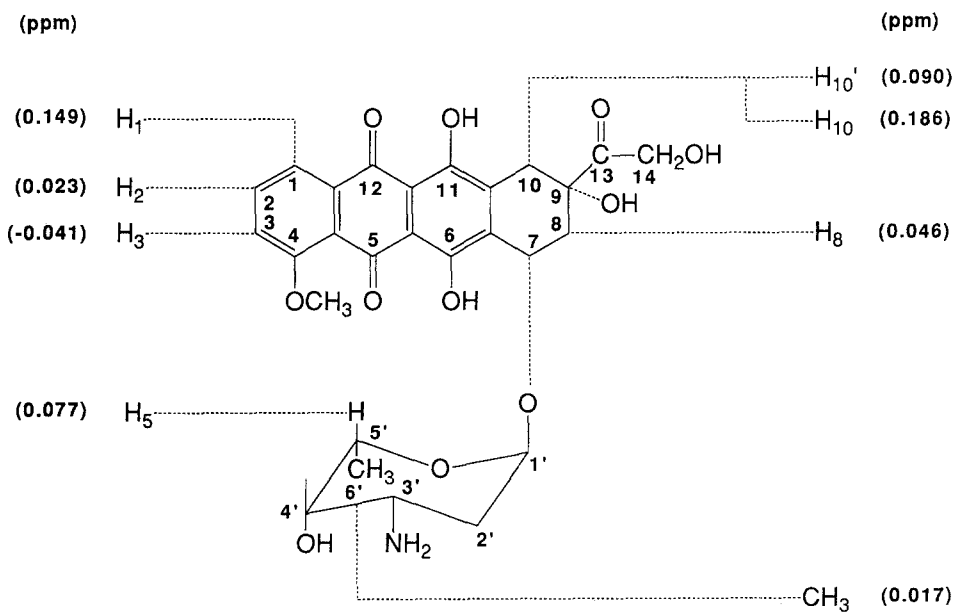

Fig. 3. Influence of γ-CyD on the chemical shifts of doxorubicin in D_2O. The values in parentheses are calculated from the expression $\Delta\delta = \delta_{complex} - \delta_{doxorubicin}$.

3.1.2. *Stoichiometric Ratio of the Complex*

Job plots [17] have been constructed to determine the molar ratios of the Dx–γ-CyD and Dr–γ-CyD complexes at pH 3. This pH value has been chosen since, at pH 3, Dx as well as Dr show maximum stability [8]. Both anthracyclines appear to yield 1 : 1 complexes with γ-CyD, this is illustrated for Dr in Figure 4.

3.1.3. *Effect of pH*

The influence of the pH on the proton chemical shifts has been studied for the γ-CyD complexes of both anthracyclines (molar ratio 1 : 1). The shifts at pH values 8.8 and 10 do not show significant differences when compared with the data obtained at pH 3. Exceptions are the C_{10} protons. At pH 10 the change in chemical shifts is about twice as large as the change at pH 3 and 8.8. This effect may be due to the instability of the anthracyclines in alkaline medium. On the other hand it might result from partial deprotonation of the OH group at C_{11} with concomittant disappearance of the hydrogen bond with the oxygen at C_{12} and distortion of ring A. The overall result suggests that the mode of complex formation for the anthracyclines is the same in acidic, neutral and alkaline media, indicating that the charge on the aminofunction of the sugar molecule (pK_a around 8 [18]) had no influence on the complexation mode. Since, generally, the charge of a molecule strongly influences the inclusion of a molecule in CyD [19], it is reasonable to assume that the sugar moiety does not penetrate into the CyD cavity.

3.1.4. *Determination of the Complex Constant*

The complex constants of Dx and Dr with γ-CyD have been determined previously by high performance liquid chromatography, circular dichroism and absorption

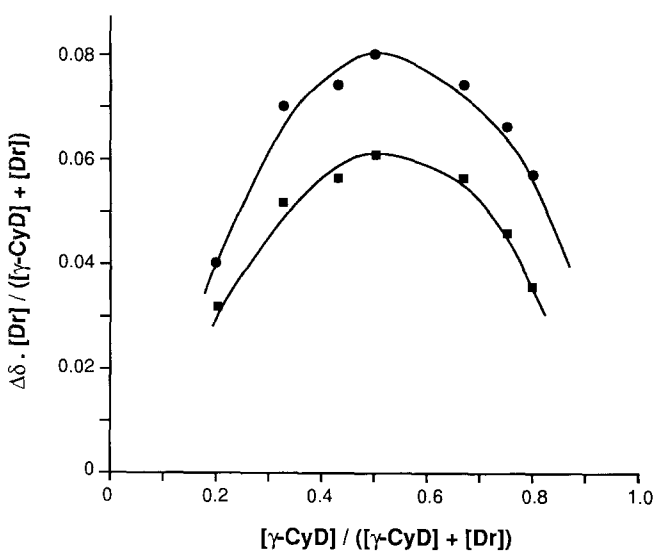

Fig. 4. Job plot for the complex formation of daunorubicin ((●) = H_3 and (■) = H_1) with γ-CyD.

spectroscopy [9, 20]. In the present study, the complex constants, K_s, of Dx–γ-CyD and Dr–γ-CyD were obtained by a modification of the Benesi–Hildebrand equation [21, 22], using Expression (1):

$$\Delta_{c.s.}/C_0 = -K_s \cdot \Delta_{c.s.} + K_s \cdot \Delta_0 \quad (1)$$

where $\Delta_{c.s.}$ represents the difference between the chemical shifts of the guest alone and the same guest in the presence of γ-CyD, C_0 the host concentration and Δ_0 the chemical shift of the guest in a complexed state with reference to an uncomplexed state. The complex constant can be calculated from the slope of the straight line obtained by plotting $\Delta_{c.s.}/C_0$ versus $\Delta_{c.s.}$. Only values for protons that show the largest chemical shift changes were used for the K_s calculation. The condition for using Equation 1 is that the host must be present in excess. During the present study, the CyD concentration with respect to the guest was in excess only up to a factor of almost four since otherwise the proton signals of the guest could no longer be measured. The K_s values found are $345 \pm 35\ M^{-1}$ ($n = 8$) and $323 \pm 58\ M^{-1}$ ($n = 7$) at pH 3 for Dx and Dr, respectively. These constants, considering the experimental conditions, are on the low side; however, they are of the same order of magnitude as the K_s values obtained earlier by HPLC, circular dichroism and absorption spectroscopy [9, 20]. This means that ^{1}H-NMR is a suitable technique for calculating complex constants of anthracyline–CyD complexes.

3.1.5 *Nuclear Overhauser Effect*

The two dimensional ^{1}H-NMR NOESY spectrum of the Dx–γ-CyD complex (molar ratio 1 : 1) is given in Figure 5. The penetration of the D ring of Dx into the CyD cavity is confirmed by the presence of cross peaks of H_1 (D ring) with H_5 (γ-CyD) and H_3 (D ring) with H_3 (γ-CyD).

The interaction of the sugar molecule with the exterior of the CyD molecule is indicated by cross peaks of H_4, (amino sugar) with H_2 (γ-CyD). This interaction was deduced form a comparison of the NOESY spectrum of γ-CyD itself with that of the complex. Unequivocal assignment of cross peaks is prevented by extensive overlap of signals and by the shift of the CyD and Dx signals upon complex formation.

3.2 MOLECULAR MODELLING

Although for Dx the configuration at C_7 is known to be (*S*) [14], grid searches were carried out for both the (*R*) and the (*S*) isomer. These grid searches yielded two minimum energy conformations for each isomer. It appears that, after docking of these structures into α-, β- as well as γ-CyD, only γ-CyD may form inclusion complexes. After full minimization, several complexes with low (negative) energies of interaction were obtained. However, introduction of a distance constraint of 3 Å between H_2 of the γ-CyD and H_4, of the amino sugar (as a result of the NOESY experiment; 3.1.5) and subsequent minimization, resulted in only two complexes with low interaction energies. Neither of the complexes obtained fulfil the distance constraint; however, these complexes possess the lowest energy in comparison with a reasonable distance. The energies of these complexes are shown in Table I, together with the distance between H_2 (γ-CyD) and H_4, (amino sugar).

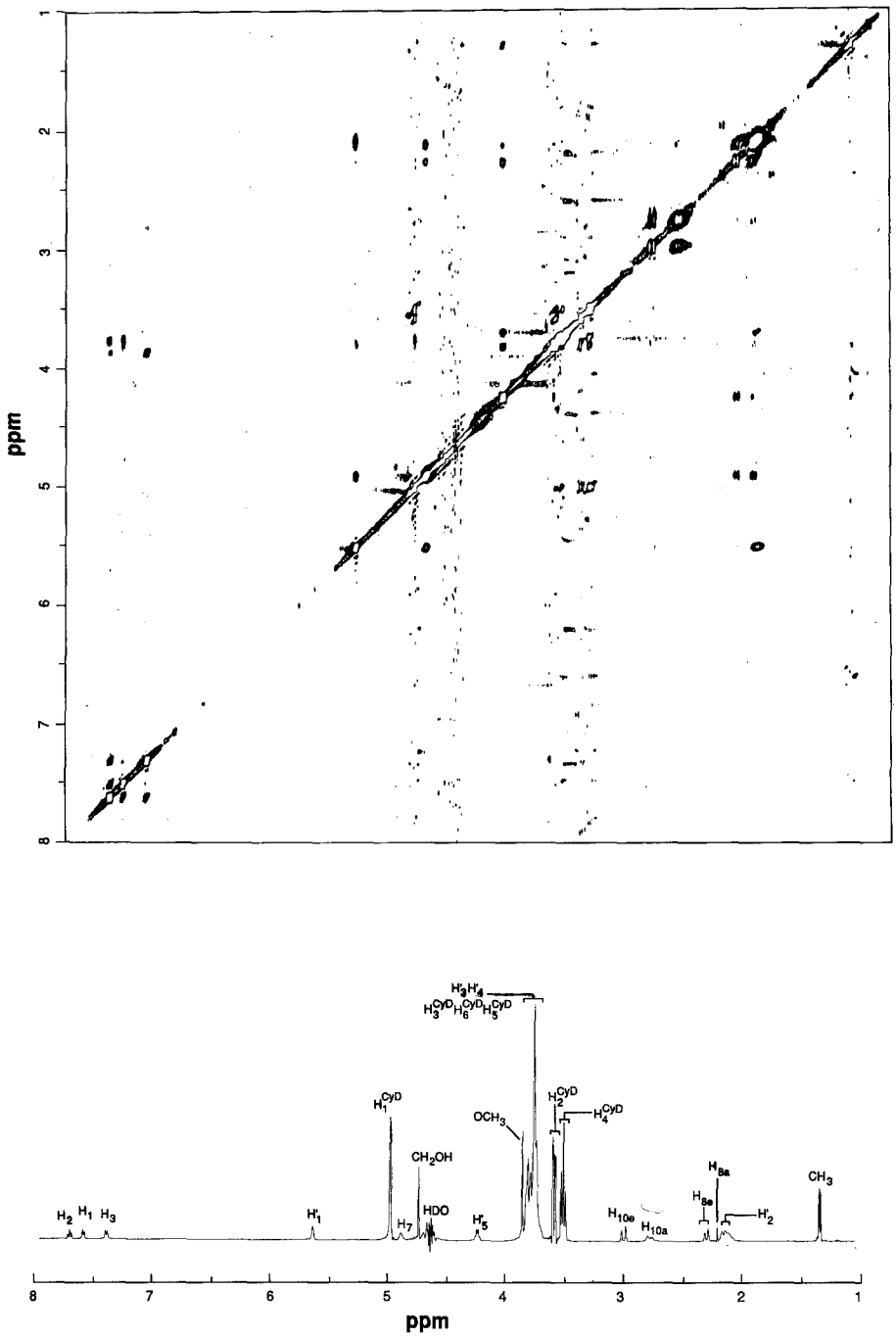

Fig. 5. ^{1}H-NMR and NOESY spectrum of the doxorubicin–γ-CyD complex in D_2O. The γ-CyD signals are denoted as H^{CyD}.

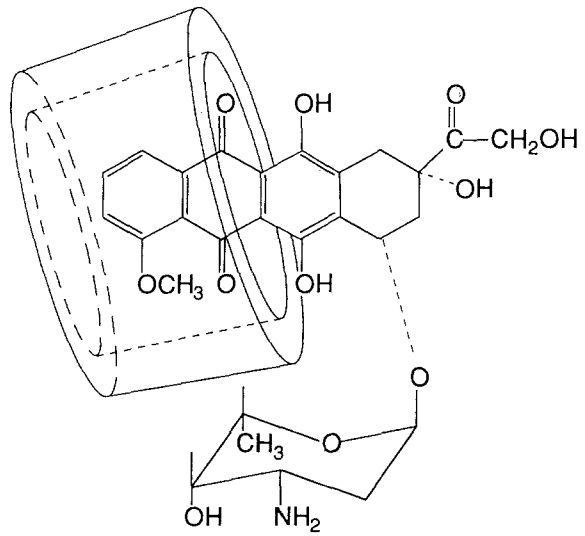

Fig. 6. Model of the proposed structure of the doxorubicin–γ-CyD complex.

Table I. Energies of some doxorubicin (Dx) C_7 configurations complexed with γ-CyD.

configuration Dx	$E^{a}_{(inter)}$ (kJ)	$E_{(complex)}$ (kJ)	$E_{(\gamma\text{-CyD})}$ (kJ)	$E_{(Dx)}$ (kJ)	distance[b] (Å)
S	−214	51.7	51.9	214	5.7
R	−291	−79.0	102	110	3.5

[a] $E_{(inter)} = E_{(complex)} - E_{(\gamma\text{-CyD})} - E_{(Dx)}$.
[b] Distance between H_4, of the Dx sugar part and H_2 of γ-CyD.

From the modelling study, it is obvious that the aglycone penetrates into the cavity, whereas the amino sugar points outwards and is in the neighbourhood of the hydroxyl groups of γ-CyD (Figure 6). This result is in agreement with the ^{1}H-NMR study. It is remarkable, however, that the $C_7(R)$ isomer of Dx seems to fit better into γ-Cyd than the (*S*) isomer, and that the distance between the H_2 of the γ-CyD and the H_4 of the amino sugar is larger in comparison with the observations from the NOESY experiment. This may possibly be explained by the flexibility of the complex structure. It seems that, after complex formation, the guest molecule can move in and out of the cavity [23], to some extent, and also that it may rotate within the CyD [24].

4. Conclusions

The present study shows that the anthracycline antibiotics Dx and Dr form inclusion complexes with γ-CyD. The ^{1}H-NMR study demonstrates that in aqueous media the aglyconic part of the anthracycline molecule is included in the cavity,

whereas the aminosugar moiety points outwards and may interact with the hydroxyl groups on the outside of the CyD. Molecular modelling confirms the existence of this complex structure; however, the modelling experiments were performed with crystal structures, so the solvent effect has not been taken into account. Furthermore it appears that the stoichiometry of the anthracycline–γ-CyD complex is 1 : 1 and the complex constants for Dx–γ-CyD and Dr–γ-CyD are 345 M^{-1} and 323 M^{-1}, respectively.

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