Complexation of antiretroviral nucleosides 2′,3′-dideoxyinosine, 2′,3′-dideoxyadenosine and 2′,3′-dideoxyguanosine with β-cyclodextrin. A ^{1}H NMR study

Bozenna Golankiewicz,[a] G. Perlovich,[bc] J. Poznański,[bd] J. Sitkowski,[ef] the late L. Stefaniak,[e] J. Zeidler[a] and Wojciech Zielenkiewicz*[b]

[a] *Institute of Bioorganic Chemistry, PAS, Noskowskiego 12/14 61-704 Poznań*
[b] *Institute of Physical Chemistry, PAS, Kasprzaka 44/52 01-224 Warszawa*
[c] *Permanent address: Institute of Solution Chemistry, Russian Academy of Sciences, 153045 Ivanovo, Russia*
[d] *Institute of Biochemistry and Biophysics, PAS, Pawinskiego 5a, 02-106 Warszawa*
[e] *Institute of Organic Chemistry, PAS, Kasprzaka 44/52 01-224 Warszawa*
[f] *Drug Institute, Chełmska 30/34, 00-725 Warszawa*

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A number of successful applications have been found that use cyclodextrin complexation ability. In the present work we analyse β-cyclodextrin (β-CD) complexes with 2′,3′-dideoxynucleosides (ddA, ddG, ddI) exhibiting high antiviral activity against HIV strains. The formation of the complexes has been analysed by ^{1}H NMR-monitored titration. On the basis of concentration dependencies of proton chemical shifts, the nucleosides in this study form complexes of 1:1 stoichiometry with β-CD. The bonding constants depend on ligand type and could be estimated as 35 ± 10 M^{-1} for ddA; 55 ± 10 M^{-1} for ddI and 85 ± 20 M^{-1} for ddG–β-CD complexes. ROESY spectra demonstrate that ligands penetrate the hydrophobic cavity of the β-CD. Finally, on the basis of ROESY data, the "low resolution" ddG–β-CD complex structure has been determined by multistep restrained molecular dynamics calculations. Calculated ddG–β-CD complex structure fully agrees with experimental data obtained for other β-CD complexes.

Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides which have six, seven and eight D-(+)-glucopyranose units for α-, β- and γ-CD respectively. The well-known advantage of using CDs comes mainly from their inclusion complex formation. This complexation can lead to alteration of the physical, chemical and biological properties of the guest molecule and may eventually have considerable pharmaceutical potential.[1]

From our preliminary thermodynamic analysis of cyclodextrins–antiviral nucleoside mixtures, including in particular the determination of transfer partial molar volumes, we obtained encouraging results in complexation of β-cyclodextrin (Fig. 1) with three antiretroviral dideoxy purine nucleosides (ddNs), 2′,3′-dideoxyinosine (didanosine, ddI, **1**), 2′,3′-dideoxyadenosine (ddA, **2**) and 2′,3′-dideoxyguanosine (ddG, **3**) (Fig. 2).[2]

Didanosine has been approved by the FDA for the treatment of HIV infections. Dideoxyadenosine has a degree of activity similar to that of ddI *in vitro*, but it is almost instantaneously deaminated to ddI *in vivo*. As upon oral administration ddA liberates a nephrotoxic metabolite, adenine, ddI is favoured in clinical use.[3] Dideoxyguanosine and its congeners have recently been intensely investigated as potent and selective antihepatitis B agents both *in vitro* and *in vivo*.[4–6] An important problem focusing a lot of attention in the case of ddN antiretrovirals is proper delivery of drugs to the targeting site *e.g.* central nervous system[7] or liver.[6] CDs, potent candidates for carrier materials, have not been used in this role with ddNs so far.

In the present paper we report a study into the ability of ddI, ddA and ddG to bind to β-CD and the structure of the complexes thus formed, aiming at future development of chemically modified CDs as a new way of delivery of ddNs to desired targeting sites.

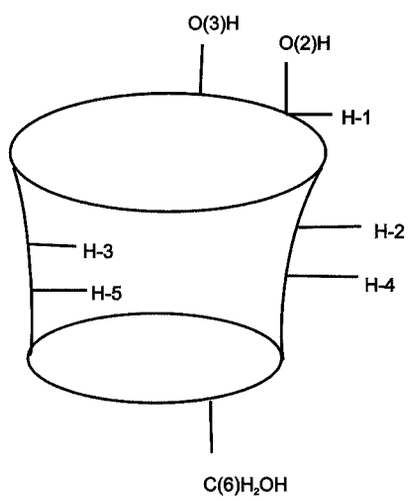

Fig. 1 Localisation of the "external" and "internal" protons in β-CD molecule.

Results and discussion

Variation of ^{1}H signals upon complexation

Heteronuclear GHMBC correlation experiments leading to unequivocal assignment of H-2 and H-8 protons of ddI and ddA, crucial for our study, showed that previous literature data[8–14] are not correct. The H-2 proton should be ascribed to upfield and the H-8 proton to downfield resonances respectively.

A comparison of the ^{1}H-NMR spectra of β-CD mixed with ddI, ddA or ddG dissolved in D_2O and that of free β-CD under the same conditions exhibits a number of systematic changes of

Table 1 Variation of the chemical shift of selected β-CD protons upon complexation with ddN

		[ddN] (%)								
ddN	$\Delta\delta_H$/Hz	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
ddI	H-1	0.4	0.9	1.4	1.7	2.2	2.6	3.0	3.2	3.6
	H-3	2.2	4.6	7.2	9.1	11.6	13.7	15.8	17.8	19.8
ddA	H-1	0.4	0.9	1.9	2.6	3.7	4.2	5.0	5.8	6.7
	H-3	2.0	5.2	8.5	11.2	14.6	17.0	19.9	22.3	25.0
ddG	H-3	1.7	3.2	4.9	6.7	8.6	10.2	11.8	13.2	15.7
	H-5	2.2	3.6	5.4	7.3	9.0	10.4	12.1	14.0	19.1

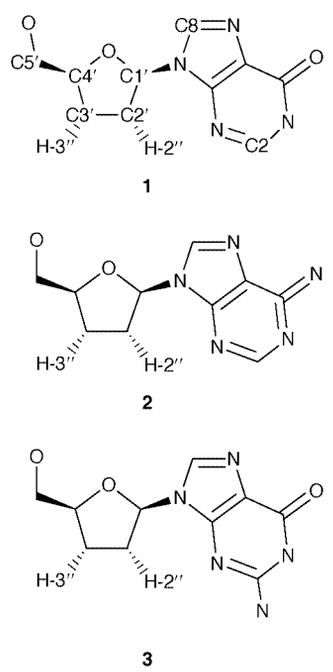

Fig. 2 Schematic view of ddI (**1**), ddA (**2**) and ddG (**3**).

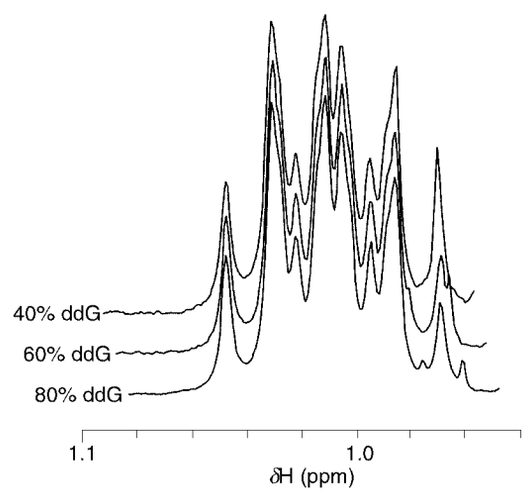

Fig. 3 Comparison of the ddG H-3″ resonance shape measured for different dideoxynucleoside : β-CD concentration ratios.

chemical shift values of β-CD protons (*cf.* Table 1). For each studied guest molecule the same tendencies in the chemical shift changes upon complexation were observed. Namely the β-CD H-3 proton, located inside the cavity, experiences a large upfield shift whereas the solvent-exposed H-1 undergoes a significantly smaller shift in the same direction. In contrast, chemical shift changes of H-2 and H-4 "external" protons are negligible. Unfortunately, known as diagnostic, the signal of β-CD H-5 proton could not be fully analysed due to partial overlapping with β-CD H-6 resonances, as well as H-5′ signals coming from nucleoside species. Despite that, it could be concluded that "internal" H-3 and H-5 protons are more sensitive to the complexation effect than H-1, H-2 and H-4 protons located on the outside of the host cavity. This indicates that guest molecules interact with the cavity interior of the β-cyclodextrin moiety. Estimated values of spin–spin coupling constants do not depend on the relative guest-to-host concentration ratio proving that the generated complexes are so weak that they do not change the average solution structure of the β-CD cycle upon the dideoxynucleoside binding. The estimated magnitude of the chemical shift drifts slightly depending on the type of ligand molecule. The effects diminish in the order ddA > ddI > ddG, but this observation cannot be directly attributed only to the stability of the formed complexes because small changes of the complex structure may play a role.

The chemical shift changes of ddI, ddA and ddG protons in the presence of β-CD are presented in Table 2. Detectable effects are observed for the majority of the dideoxynucleoside proton resonances. In contrast to the chemical shift changes observed for the β-CD resonances in the studied complexes, for ddN resonances there are no common rules for either the direction or magnitude of the resonance frequency drifts. For all compounds, a large downfield shift of H-5″ and H-3″ accompanied by an upfield shift of H-3′ is observed. The rest of the proton resonances exhibit various properties. Upon binding to β-CD H-1′ and H-2″ proton signals move downfield in ddA and ddG whereas no changes are observed for ddI ones. In the case of H-2′ and H-4′ resonances, downfield shifts in ddA, no changes in ddI and upfield shifts in ddG are observed. From the diagnostic point of view the most important is the different sensitivity observed for the base H-2 and H-8 resonances. This clearly indicates the topological differences in host–guest interaction between ddI, ddA and ddG emphasising the role played by the base moiety in the complex stabilisation. Analysis of the estimated sugar moiety NMR spectra parameters proved that upon addition of β-CD only small changes of the resonance shapes caused by individual protons' chemical shift drifts were observed. No significant changes of either geminal or vicinal coupling constants between furanose ring protons were found. It is shown by superimposition of H-3″ signals of ddG measured for 40, 60 and 80% ddG in ddG–β-CD mixtures (Fig. 3) that they have an identical resonance shape, clearly indicating that $^3J_{H\text{-}2',H\text{-}3''}$, $^3J_{H\text{-}2'',H\text{-}3''}$, $^3J_{H\text{-}4',H\text{-}3''}$, $^3J_{H\text{-}2',H\text{-}3'}$ as well as $^2J_{H\text{-}3',H\text{-}3'}$ coupling constants do not change upon complexation. In consequence it could be easily concluded that complexation does not have an effect on the conformational equilibrium of the furanose ring. Additional 1-D ^{1}H NOE difference spectroscopy experiments demonstrate that for ddA and ddG nucleosides the *syn–anti* equilibrium on the glycosylic bond does not change upon complexation whereas in the case of ddI the complexation process stabilises the *anti* conformer (*cf.* Table 3). The method used for the estimation of the *syn–anti* equilibrium is based on the analysis of the k_η values derived from the experimentally measured NOE enhancements N(H) upon irradiation of the H-8 proton of the base moiety, see eqn. (1) where the term

Table 2 Chemical shifts changes of ddI, ddA and ddG protons in the presence of β-CD

ddN	[ddN] (%)	$\Delta\delta_H$/Hz H-8	H-2	H-1′	H-2″	H-2′	H-3″	H-3′	H-4′	H-5′	H-5″
ddI	0.1	20.4	−5.2				−20.3	22.1			−21.4
	0.2	17.2	−5.1				−17.8	19.7			−19.3
	0.3	15.1	−4.1				−15.5	17.6			−17.0
	0.4	13.1	−3.6				−13.7	14.7			−14.8
	0.5	11.0	−2.6				−11.3	12.1			−12.2
	0.6	9.0	−1.6				−9.2	9.7			−10.0
	0.7	7.0	−0.6				−6.9	7.1			−7.4
	0.8	5.0	−0.4				−4.7	4.3			−5.0
	0.9	3.2	−1.9				−2.4	1.9			−2.6
ddA	0.1	6.7	−33.0	−18.0	−17.5	−27.2		28.8	−9.5		−26.9
	0.2	5.4	−31.7	−17.1	−15.8	−25.3		25.5	−8.4		−24.1
	0.3	4.2	−28.6	−15.8	−13.9	−26.9		22.3	−7.3		−21.0
	0.4	2.8	−26.1	−14.6	−12.3	−24.7	−18.2	18.6	−6.4		−18.1
	0.5	1.9	−22.5	−12.6	−10.0	−21.4	−14.1	15.2	−5.1		−14.6
	0.6	0.8	−19.1	−10.9	−8.3	−14.8	−11.5	11.6	−4.3		−11.8
	0.7	0.3	−14.5	−8.3	−6.1	−11.2	−8.3	8.2	−3.1		−8.5
	0.8	0.1	−9.9	−5.7	−4.0	−7.6	−5.4	5.3	−2.0		−5.4
	0.9	0.1	−4.1	−2.3	−1.4	−3.3	−2.3	2.9	−0.6		−2.3
ddG	0.1	17.2		−16.6	−10.4	9.2	−13.7	10.6	5.2	7.1	−20.8
	0.2	14.8		−15.5	−9.7	7.7	−12.6	9.0	4.3	6.6	−19.0
	0.3	13.2		−14.0	−8.5	6.6	−11.1	8.0	3.9	6.2	−16.8
	0.4	11.1		−12.3	−7.3	5.4	−9.5	6.8	3.4	5.4	−14.4
	0.5	9.1		−10.4	−5.9	4.3	−7.9	5.7	2.9	4.6	−11.8
	0.6	7.0		−8.6	−4.9	3.1	−6.4	4.3	2.2	3.6	−9.5
	0.7	5.4		−6.6	−3.7	2.4	−4.8	3.4	1.8	2.9	−7.1
	0.8	3.2		−4.6	−2.6	1.2	−3.4	2.0	0.9	1.7	−4.1
	0.9	1.9		−1.9	−1.7	0.6	−1.4	1.2	0.4	0.7	−2.3

Table 3 *Syn–anti* equilibrium on the glycosylic bond analysed by 1-D ^{1}H NOE difference spectroscopy

Object	NOE enhancement (%) H-1′	H-2′	H-3′	k_η	*Anti* (%)	*Syn* (%)
ddI	1.9	2.4	2.3	2.4	72	28
ddI + β-CD	1.3	2.3	2.5	3.6	82	18
ddA	2.5	2.3	2.1	1.8	65	35
ddA + β-CD	1.9	1.6	1.7	1.8	65	35
ddG	2.2	3.0	2.7	2.5	74	26
ddG + β-CD	1.7	2.3	2.3	2.8	76	24

$$k_\eta = \frac{\mathrm{N(H\text{-}2')} + \mathrm{N(H\text{-}3')}}{\mathrm{N(H\text{-}1')}} \quad (1)$$

N(H-2′) + N(H-3′) is a measure of the *anti* conformer population whereas N(H-1′) corresponds to the *syn* conformation. Values of k_η correspond to the *syn–anti* equilibrium on the glycosylic bond.[15] In the method used the ratio of NOE enhancements does not depend on the correlation time, as directly measured NOE values do, so this enables estimation of the *syn–anti* equilibrium for dideoxynucleoside both free and partially bonded to β-CD without any additional correction or molecular ruler.

Complex stoichiometry

Chemical shifts of the appropriate signals of the β-CD, ddI, ddA and ddG as a function of relative concentration of the host and guest molecules were analysed in terms of the standard "Job plots".[16] The parabola-like curves presented in Fig. 4 exhibit maxima for the 1:1 concentrations of the host and the guest species, clearly indicating 1:1 stoichiometry for all complexes studied.

Estimation of the complex stability

The complexation process is so weak that the observed changes of $\Delta\delta_H$ are almost a linear function of the ligand concentration.

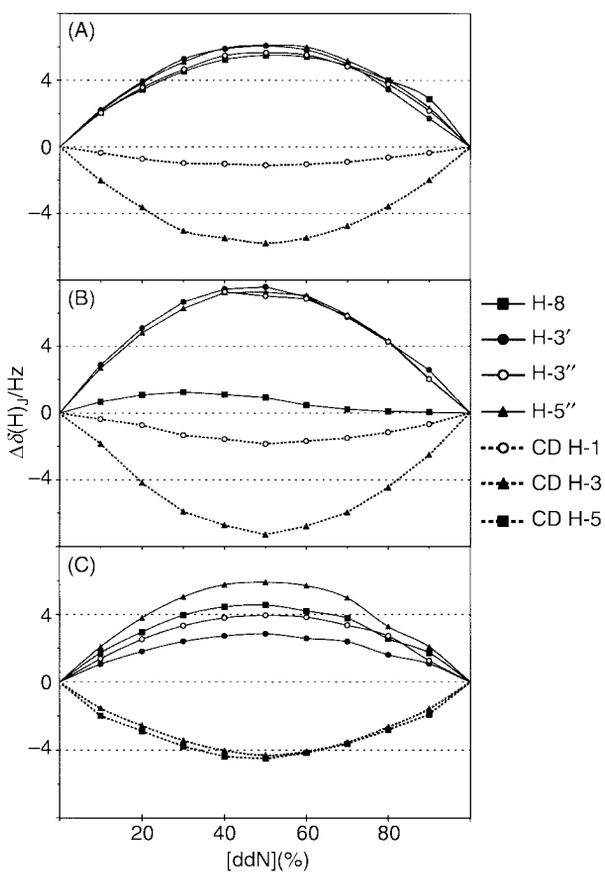

Fig. 4 Job plots obtained for ddI–β-CD (A), ddA–β-CD (B) and ddG–β-CD (C). The negative values correspond to the β-CD resonances. The maxima obtained for 50:50 concentration ratio prove 1:1 complexation stoichiometry.

In addition, as strong coupling of 2′2″ 3′3″ 5′5″ spin systems of dideoxynucleosides is observed, the analysis has to be limited to cyclodextrin protons only. Besides, due to signal overlapping,

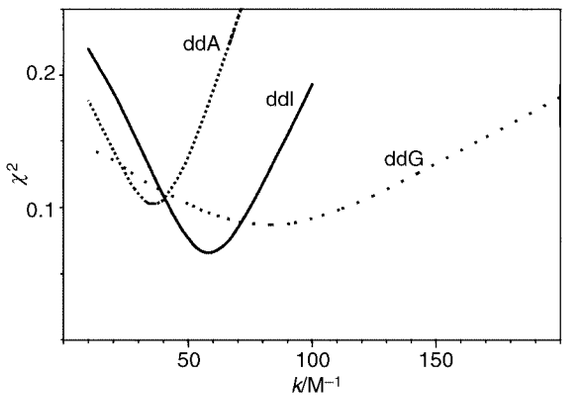

Fig. 5 Estimation of the complex association constant k. The χ^2 value is a measure of the fit of the complexation model [*cf.* equation (3)] to the experimental δ(CD H-3) values. The k values at the minima are expected to be close to the experimental bonding constants k_b.

the analysis for the whole range of concentrations may only be carried out for the H-3 proton. Assuming the following complexation model: L + N↔X, the experimentally measured value of the chemical shift of any proton under the conditions of fast exchange may be determined by eqn. (2), where $\delta(H_o)$,

$$\delta^{calc}(.....) = \frac{\delta(H_o)[L] + \delta(H_x)[X]}{[L] + [X]} \quad (2)$$

$\delta(H_x)$ are the chemical shifts corresponding to the free L and bonded X forms of the β-cyclodextrin, concentrations of which are equal to [L] and [X] respectively. [L] and [X] values are related to the complex dissociation constant k and the total concentrations of the host $[L_o]$ and guest $[N_o]$ by eqn. (3):

$$\frac{([L_o] - [X])([N_o] - [X])}{[X]} = k \quad (3)$$

The solution to eqns. (2) and (3) allows us to determine the theoretical dependence of the chemical shifts upon the concentration of both molecules. The value of $\delta(H_o)$ was determined independently whereas values of k and $\delta(H_x)$ were determined using a simplified analysis based on the relation $\chi^2(k, \delta(H_x))$ (4), being a measure of the deviation between the experimental data and the assumed theoretical relation. For each specified value of k, the value of $\delta(H_x)$ was optimised (eqn. (4)).

$$\chi^2(k,\delta(H_x)) = \Sigma_i\{\delta_i^{exp} - \delta_i^{calc}(k,\delta(H_x),[N_o^i],[L_o^i])\}^2 \quad (4)$$

The obtained patterns show a regular area of minimal values $\chi^2(k)$ for each of the three ddN (Fig. 5), thus allowing us to estimate relevant values of complex formation constants for ddI (55 ± 10) M^{-1}, ddA (35 ± 10) M^{-1} and ddG (85 ± 20) M^{-1}. Due to strong correlation between the adjusted values of k and $\delta(H_x)$ in the case under study, it was not possible to achieve convergence of the standard minimisation procedure either using the simplex method or the methods of coupled gradients. Uncertainty of the obtained values is relatively high as the calculations were based on the chemical shifts change analysis of only one β-cyclodextrin proton H-3.

Intermolecular NOE determination. Structure of complex by computer modelling

All three ddN–β-CD complexes exhibit similar cross-peak patterns to the 2-D ROESY[17] spectra (Fig. 6). In all cases the unequivocal assignment of the (2, 8, 1′, 2′, 2″, 3′, 3″) ddN⇔H-3 β-CD cross-peaks could be made. Stereospecific assignment of the 2′,3′-dideoxyribose H-2′, H-2″ and H-3′, H-3″ protons was made using ROESY spectra. Due to conformational flexibility of the furanose ring the protons H-2″ and H-3″ are closer to H-4′ than their stereopartners, so in the ROESY spectra they exhibited stronger cross-peaks with the H-4′ proton. In the case of ddG also (8, 1′, 2′, 2″, 3′)⇔H-5 β-CD cross-peaks were assigned. Analogous cross-peaks were observed for the ddA and ddI, but there were alternative assignments to the intramolecular magnetisation transfer from the ddN 5′ proton. In all cases no interactions between ddN base protons and β-CD H-1 were found.

Due to signal overlapping, structural calculations were performed only for the ddG–β-CD complex. Eight cross-peaks (1′, 2′, 2″)⇔(H-3, H-5) and (3′, 3″)⇔H-5 were converted into 5 Å upper limit constraints whereas the weaker 3′⇔H-3 was converted into a 6 Å one. In order to make a correction for the experimentally confirmed *syn–anti* equilibrium on the glycosylic bond 8⇔(H-3, H-5) cross-peaks were converted into 6 Å constraints. In total, 11 constraints were used in the calculations.

The β-CD moiety is a cycle formed by 7 identical structural units. "Human driven" structure optimisations were performed. Standard methods of the pseudoatom correction[18] could not have been applied because all pseudoatoms were placed on the symmetry axis of the molecule.

After interactive docking of the ddG sugar moiety inside the β-CD cavity, manual assignment of the restraints to the particular proton pairs was made. The upper limits of the restraints were set as previously explained. 1000 ps molecular dynamics in the presence of restraints was performed with the frozen structure of β-CD and the flexible structure of ddG. The last step of structure determination, 1000 ps molecular dynamics simulation was performed, allowing conformational changes of both molecules.

The obtained ddG–β-CD complex (Fig. 7) generally agrees with various published analogous complexes in terms of stability data.[19–21] The sugar moiety deeply penetrates the β-CD cavity whereas the base is solvent exposed, so the *syn–anti* equilibrium on the glycosylic bond does not change. No specific interactions between molecules were found.

The structure of the β-CD–ddG complex explains the difference of the bonding constant among presently studied molecules. The nucleic base modifications change the complex stability by modulation of the base to a β-CD edge interaction. The change of 3′-OH in dN into H-3″ in ddN stabilises the complex by the decrease in unfavourable interactions of 3′-OH inside the hydrophobic cavity, so estimated values of bonding constants are larger than those obtained for deoxynucleosides.[21] An analogous effect had been observed for a series of modified pentoses, where methylation increased the complex stability.[19]

Experimental

Materials

β-Cyclodextrin produced by Sigma was dried in dry nitrogen atmosphere at 420 K. The content of water in the β-cyclodextrin crystals was monitored using a DuPont 951 Thermogravimetric Analyzer. ddI and ddG were synthesised and purified by the methods described previously.[14] ddA was a product of Sigma.

NMR Spectroscopy

NMR Measurements were performed at 300 K in D_2O solutions, using Bruker AM 500 (heteronuclear experiments and 1-D spectroscopy) and Varian INOVA 500 MHz (2-D COSY, 2-D ROESY experiments) spectrometers. For the accurate determination of chemical shift changes upon complexation the external TMS (tetramethylsilane) in acetone-d_6 was used as reference. The investigated solutions were obtained by mixing 15 mM stock solutions of β-CD and dideoxynucleosides in a

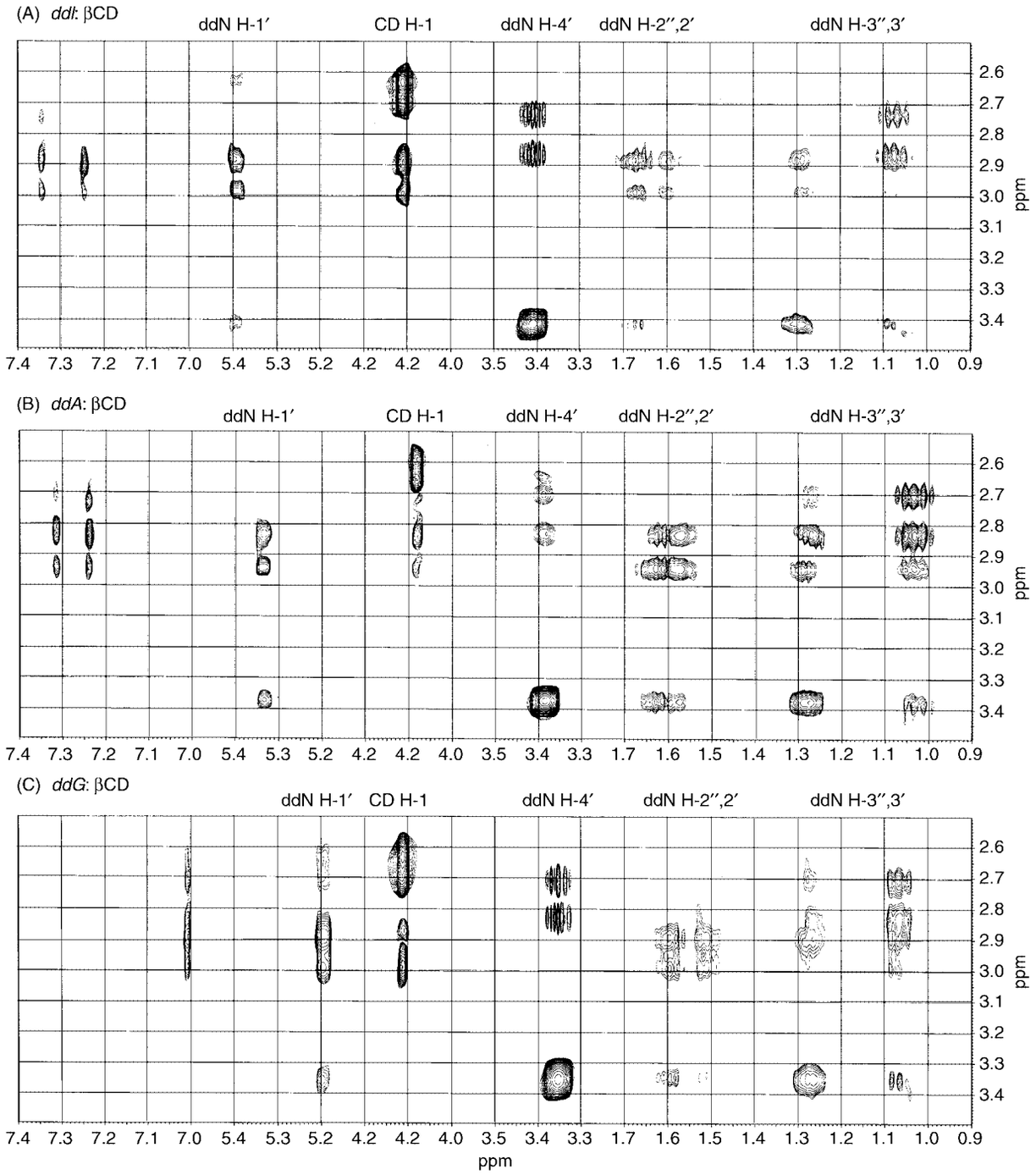

Fig. 6 Slices of the 2-D ROESY NMR spectra of β-CD–ddI (A), ddA–β-CD (B) and ddG–β-CD (C) complexes.

volumetric ratio 1 : 9, 2 : 8, 3 : 7, 4 : 6, 5 : 5, 6 : 4, 7 : 3, 8 : 2 and 9 : 1 respectively. In order to remove oxygen ions before NOE measurements the probes were frozen and then kept under the low pressure atmosphere. Signal assignments were made with the aid of the standard 2-D COSY[22] spectra. 2-D ROESY[17] measurements were made using the standard Varian software with the experimental conditions as follows: 256 increments of 2048 points each, 4 kHz of the spectral width, spin lock field in pulse mode centred at water resonance and 250 ms mixing time duration. All spectra were processed with $\pi/2$ shifted squared sinebell filter in both dimensions with the help of NMRPipe software.[23]

Chemical shifts and coupling constant values of furanose ring protons were estimated by use of the program based on the LAOCOON II algorithm.[24] Stereospecific assignments of ribose H2′ and H3′ protons were made by analysis of NOESY cross-peaks to H-4′ protons. Estimations of the *syn–anti* equilibrium on the glycosylic bond were made by 1-D NOE enhancement measurements upon irradiation of the nucleic base moiety H-8 resonance.[15]

Heteronuclear GHMBC correlation experiments with a partial low pass filter[25] allowed us to establish a whole pattern of direct and long-range coupling pathways, leading to unequivocal assignment of H-2 proton to upfield and that of H-8 to downfield resonances respectively. The 1-D steady state NOE difference spectroscopy[26] experiments with saturation of H-8 proton were performed to estimate the *syn–anti* equilibrium on the glycosylic bond of dideoxynucleosides.

Complex stoichiometries were obtained *via* the analysis of the standard Job Plots.[16] The bonding constants of complexes were estimated by the analysis of the chemical shift drift as a function of the host and guest concentrations. All calculations were done by the standard optimisation procedure of the Corel Quattro spreadsheet. Because of relatively low values of

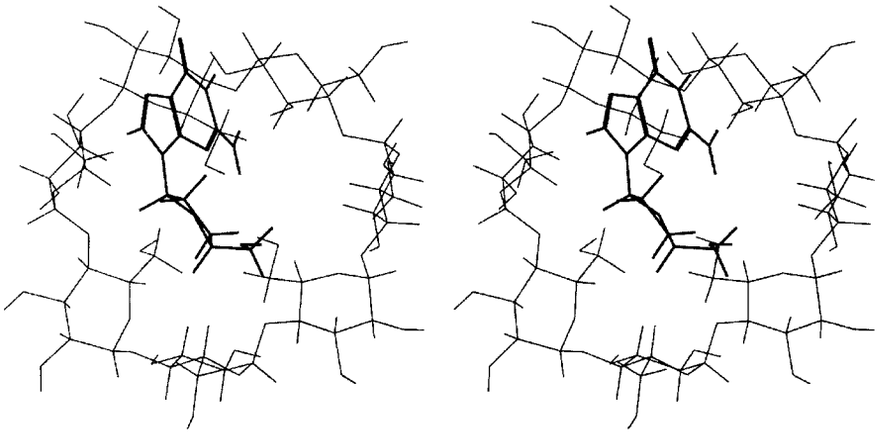

Fig. 7 Stereo view of the ddG–β-CD complex obtained on the basis of 2-D ROESY NMR experiment. The ddG molecule is marked by a thicker line.

bonding constants a simplified method for the analysis of the χ^2 value as a function of bonding constant was used.[27]

Computational methodology

The ddG–β-CD complex structure was obtained with the aid of the Sybyl package. All calculations were performed without explicit water molecules. The Tripos forcefield[28] and Amber charges[29] were used with the distance dependent relative permittivity ε set to 4.5r. The structure of β-CD was adopted from the crystallographic data whereas that of the ddG molecule was optimised by molecular mechanics calculations. Standard interactive Sybyl docking procedure was used to generate the initial complex structure. The complex structure was refined by restrained molecular dynamics (MD) calculations. For each intermolecular cross-peak found in a 2-D 250 ms mixing time ROESY spectrum a 5 Å upper distance constraint with 50 kcal mol^{-1} $Å^{-2}$ force constants was used. In order to improve calculations before final optimisation, the complex structure was tuned by 1000 ps restrained MD calculations at 400 K with the fixed conformation of β-CD. Finally, 1000 ps restrained MD calculations were performed at 300 K.

Acknowledgements

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