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Conformational analysis of 4-demethoxy-7-*O*-[2,6-dideoxy-4-*O*-(2,3,6-trideoxy-3-amino-α-L-*lyxo*-hexopyranosyl)-α-L-*lyxo*-hexopyranosyl]adriamicinone, the first doxorubicin disaccharide analogue to be reported

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Abstract

The solution conformation of 4-demethoxy-7-*O*-[2,6-dideoxy-4-*O*-(2,3,6-trideoxy-3-amino-α-L-lyxo-hexopyranosyl)-α-L-lyxo-hexopyranosyl]adriamicinone, the first doxorubicin disaccharide analogue to be reported, has been analysed using nuclear magnetic resonance data and molecular mechanics calculations. In order to consider the possibility of conformational averaging we have used the NAMFIS programme (NMR analysis of molecular flexibility in solution), that considers all reasonable structures and classifies them with regard to their relative capability of reproducing the experimental data. In this way we have determined the conformation of ring A of the aglycone of the sugar moieties and the preferred orientation around the glycosidic linkages. © 1997 Elsevier Science Ltd.

Keywords: Doxorubicin disaccharide; NMR; Conformational search; NAMFIS

1. Introduction

Anthracyclines are the class of anticancer agents with the widest spectrum of activity in human malignancies. However, considerable room for improvement exists because of the limitation of the present chemotherapy, due to the natural and acquired resistance of tumours. We have now found that 4-demethoxy-7-*O*-[2,6-dideoxy-4-*O*-(2,3,6-trideoxy-3-amino-α-L-*lyxo*-hexopyranosyl)-α-L-*lyxo*-hexopyranosyl]adriamicinone **1** (Fig. 1) shows, according to our experience, unprecedented superior activity when compared with doxorubicin in different human gynaecological and lung cancers implanted in immunodepressed mice [1]. This compound is the first example of a doxorubicin disaccharide analogue and belongs to the new series of synthetic anthracycline aminodisaccharides in which the aminosugar moiety appears as the second residue from the aglycone. As

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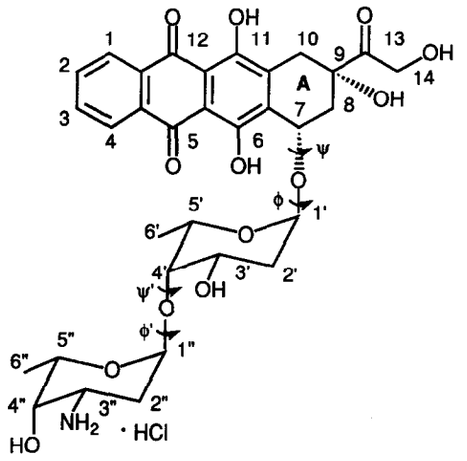

Fig. 1. Chemical structure and labelling of compound **1**.

it can be deduced from the observed remarkable difference in bioactivity between the 4′-axial and 4′-equatorial disaccharides of this type [2], the orientation of the aminosugar seems to be important for the molecular interaction [1] responsible for the antitumor effects of the compound [3].

With the aim of studying the conformational effect of this structural change, we have performed a conformational analysis of this new compound in Me_2SO-d_6 solution. In particular, the geometry around the glycosidic linkages was studied using NOE data and three bond ^1H–^{13}C coupling constants together with molecular mechanics calculations.

2. Experimental

Nomenclature.—The geometry at the glycosidic bonds is defined by the angles:

$\phi = C(7)–O(7)–C(1')–H(1')$,

$\psi = H(7)–C(7)–O(7)–C(1')$,

$\phi' = C(4')–O(4')–C(1'')–H(1'')$,

$\psi' = H(4')–C(4')–O(4')–C(1'')$.

NMR spectroscopy and molecular mechanics calculations.—The proton magnetic resonances of compound **1** were assigned by standard COSY, TOCSY and NOESY experiments on a Bruker AMX-500 MHz spectrometer. The spectral width was 4500 Hz in each dimension, and 4 K data points were recorded in 512 increments. The ^{13}C and HETCOR spectra were obtained at 75.45 MHz on a Varian Gemini 300 MHz. For the HETCOR 128 experiments of 480 scans each were obtained using a spectral width of 2700 Hz in F_1 and 18762 Hz in F_2, a relaxation delay of 3 s and a 4 K × 128 data set. The $^3J_{CH}$ coupling constants through the glycosidic bonds were measured by the application of pulse sequence *a* proposed by Poppe and van Halbeek [4] on a Bruker AMX-600 MHz spectrometer; 3200 scans were accumulated for each experiment with 5500 Hz of spectral width and the data points were fixed in order to have a final resolution of 0.5 Hz/point. All experiments were performed using a 7 mM solution of the compound in Me_2SO-d_6 at 25 °C. NOESY spectra were recorded at four mixing times (70, 100, 150 and 200 ms) to check the linearity of the cross-relaxation buildup. Interproton distances were calculated using the initial rate approximation according to a two spin model (calibration distance, H-3′, H-5′).

Molecular mechanics calculations were performed with the AMBER* force field [5] of DISCOVER (Biosym) augmented with parameters [2] in order to improve the description of the aglycone moiety and the ring A-fucose linkage. Atomic partial charges were obtained from a fitting of the electrostatic potential calculated at the semiempirical PM3 level. All non-bonded interactions were calculated without any cut-off using a distance dependent dielectric function $\varepsilon(r) = 4.0 \times r$ and a scale factor of 0.5 for the 1–4 van der Waals and electrostatic interactions.

3. Results and discussion

NMR data and conformational analysis.—The conformational study of **1** was performed in Me_2SO-d_6, as the extreme broadness of the NMR signals in water impaired any detailed analysis. In the former solvent our experimental data revealed the existence of an association process (negative NOE regime in the NOESY spectra, indicating a mobility typical of macromolecular species; changes in chemical shifts on increasing the temperature), but the broadness of the signals was significantly diminished with respect to that observed in water. Preceding conformational analyses of anthracyclines [6] were performed in

[1] The inhibition of cancer cells by anthracyclines is related to the apoptotic response to drug stimulation of topoisomerase II-mediated DNA cleavage consequent upon the formation of a drug-DNA-protein ternary complex.

[2] A. Madami, E. Monteagudo, F. Animati, P. Lombardi and F. Arcamone, unpublished results.

chloroform to avoid the formation of aggregates, but this solvent could not be used in this case because of the very low solubility of **1**. However, the conformation of **1** in the aggregates may represent a preferred physical state of the molecule reasonably similar to that adopted in the drug-DNA complex (a model for the aggregates has proposed the vertical stacking of the anthraquinone system [7]).

The ^{1}H spectrum of **1** in Me_2SO-d_6 is shown in Fig. 2. Both ^{1}H and ^{13}C spectra were assigned by means of COSY and ^{1}H–^{13}C correlation experiments. All the data are reported in Table 1. The NOE data and coupling constants employed in the conformational study are presented in Tables 2 and 3.

The molecular shape of this molecule is determined by the conformation of the sugar rings, of the saturated ring A and also, by the orientation of the fucose unit with respect to the aglycone and of the daunosamine unit with respect to the fucose ring. As in related analyses concerning anthracyclines [7–9], the preferred conformation of ring A was established as a $^{9}H_{8}$ from the coupling constants values for H-7, H-8eq and H-7, H-8ax of 2.5 Hz and 4.9 Hz respectively, together with the four bond coupling between H-8eq and H-10eq of 2 Hz. The fucose and daunosamine rings both present a $^{1}C_{4}$ chair conformation, as determined by the characteristic values of proton-proton coupling constants, in particular the values of the couplings between H-2′ax and H-3′ax and between H-2″ax and H-3″ax of 12.6 Hz and 12.5 Hz respectively. The NOE data also supported this result, as a significant dipolar interaction was observed between H-3′ and H-5′ and between H-3″ and H-5″, indicating a 1,3 diaxial relationship.

The geometry at the glycosidic bonds, defined by the angles ϕ, ψ and ϕ', ψ' were studied with the assistance of NOE and $^{3}J_{CH}$ data (Tables 2 and 3) and conformational search. As a contrast with the moieties already described, for which a single conformer can be safely considered (extreme values of coupling constants ruled out the existence of more than one conformer in fast equilibrium), the glycosidic angles are intrinsically more flexible. Our experimental data were not a conclusive evidence by them-

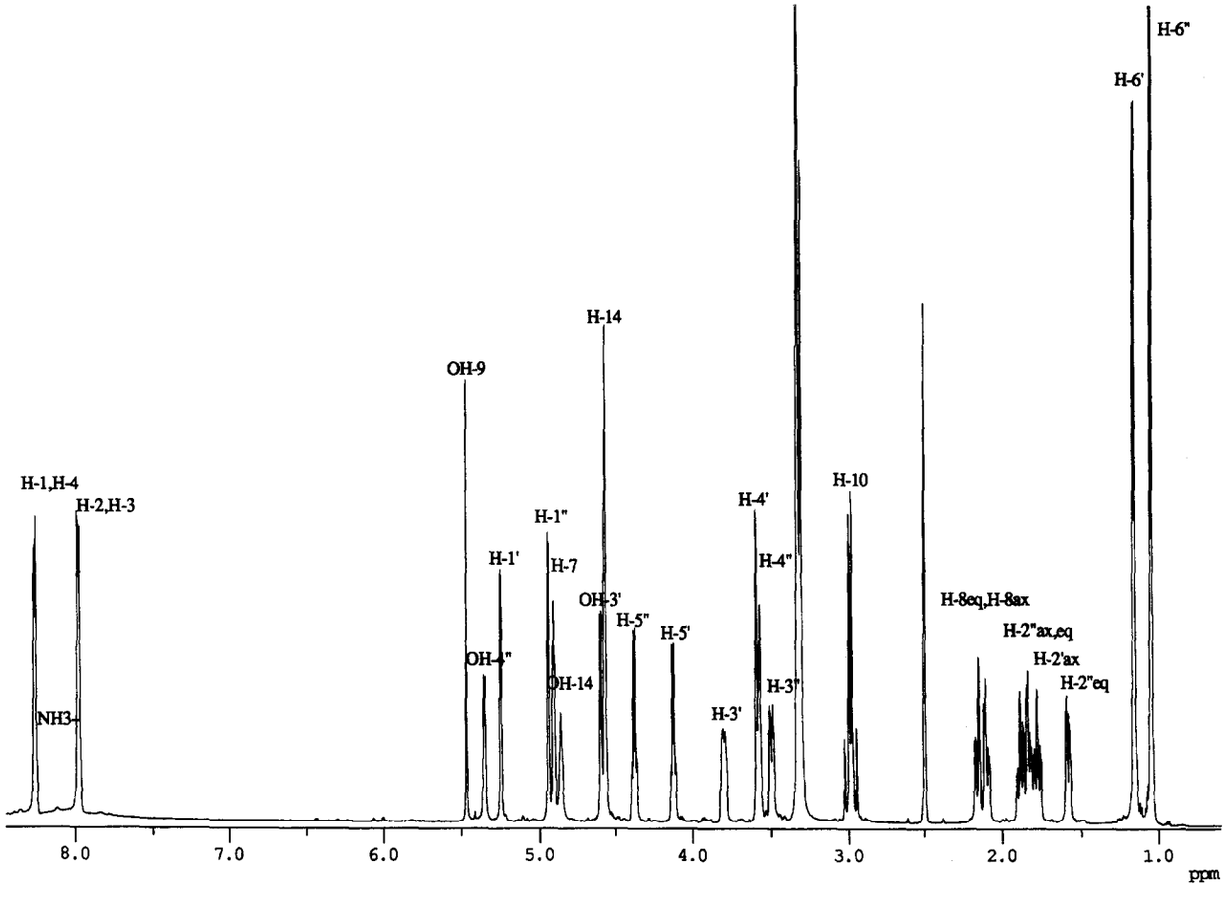

Fig. 2. ^{1}H spectrum of **1** in Me_2SO-d_6 at 298 K.

Table 1
Chemical shifts of the ^{1}H and ^{13}C resonances for **1** [a]

H-1	8.28	C-1	126.61
H-2	7.95	C-2	135.08
H-3	7.95	C-3	135.08
H-4	8.28	C-4	126.61
H-7	4.92	C-4a	132.70
H-8eq	2.17	C-5	186.36
H-8ax	2.10	C-5a	110.70 [b]
OH-9	5.52	C-6	156.48
H-10	2.99	C-6a	135.88
H-14	4.56	C-7	69.59
OH-14	4.85	C-8	36.50
H-1′	5.24	C-9	74.93
H-2′ax	1.77	C-10	32.29
H-2′eq	1.57	C-10a	135.08
H-3′	3.79	C-11	155.17
H-4′	3.58	C-11a	110.04 [b]
H-5′	4.11	C-12	186.19
H-6′	1.16	C-12a	132.70
H-1″	4.93	C-13	213.69
H-2″ax	1.88	C-14	63.73
H-2″eq	1.81	C-1′	100.81
H-3″	3.49	C-2′	33.43
H-4″	3.55	C-3′	64.10
H-5″	4.37	C-4′	77.70
H-6″	1.04	C-5′	66.84
OH-3′	4.60	C-6′	17.21
OH-4″	5.30	C-1″	96.83
$NH3^{+}$-3″	7.90	C-2″	28.18
OH-6	13.4	C-3″	46.74
OH-11	13.4	C-4″	66.22
		C-5″	65.74
		C-6″	16.70

[a] In Me_2SO-d_6 at 298 K.
[b] Values may be interchanged.

Table 2
Experimental interproton distances of **1** and associated errors [a]

NOE type	Distance (Å) [b]	Δd (Å)
H-3′, H-5′	2.50 [c]	
H-1′, H-7	2.23	0.11
H-5′, H-8eq	2.54	0.25
H-1″, H-4′	2.60	0.25
H-1″, H-6′	2.90	0.30

[a] List of ADCs employed: H-7, H-2′eq; H-7, H-3′; H-8eq, H-1′; H-1′, H-3″; H-2′ax, H-3″; H-4′, H-2″ce; H-4′, H-3″; H-6′ce, H-3″; H-6′ce, H-5″; H-2′ax, H-2″ce; (ce = centroid).
[b] 'Virtual distances' determined by the averaging of the corresponding NOEs that are produced by the different conformers in rapid exchange equilibrium.
[c] Calibration distance.

Table 3
Experimental three bond coupling constants (Hz) [a] of **1** used for the analysis

Heteronuclear [b]		Homonuclear	
$^{3}J_{C-1',H-7}$	5.7	$^{3}J_{H-7,H-8eq}$	2.5
$^{3}J_{C-7,H-1'}$	4.9	$^{3}J_{H-7,H-8ax}$	4.9
$^{3}J_{C-1'',H-4'}$	5.9	$^{4}J_{H-8eq,H-10eq}$	2.0
$^{3}J_{C-4',H-1''}$	3.3	$^{3}J_{H-2'ax,H-3'ax}$	12.6
		$^{3}J_{H-2''ax,H-3''ax}$	12.5

[a] Heteronuclear coupling constants are given at ±0.5 Hz; homonuclear coupling constants are given at ±0.1 Hz.
[b] These values were obtained using pulse sequence *a* of ref. [4].

selves of a single conformation, and so we decided to analyse them taking into account the possibility of conformational averaging. For this purpose we used a modified version of the NAMFIS programme [10]. This programme intentionally provides an experimental picture, where all reasonable structures are considered and classified with regard to their relative capability of reproducing the experimental data.

We have performed a systematic search for ϕ and ψ (in steps of 12°) for a simplified analogue of **1**, in which the daunosamine moiety is replaced by a methyl group, obtaining eight minima. Each of them was employed as a starting point to perform for **1** a systematic search for ϕ' and ψ' in steps of 18°. All generated conformers were minimized and clustered using an rms criterion for the deviations of the angles ϕ, ψ, ϕ', ψ' with a threshold of 5°. After this procedure we obtained a set of 54 minima. As no experimental data correlate the aglycone with the daunosamine unit, we decided to perform two independent analyses for the two glycosidic linkages. The list of ϕ, ψ and, ϕ', ψ' values obtained from our molecular mechanics studies was:

$(\phi, \psi) = (-173°, -24°); (-29°, -34°);$
$(9°, -37°); (37°, 131°);$
$(42°, -15°); (43°, 108°);$
$(50°, -160°); (63°, 51°),$
$(\phi', \psi') = (-55°, -29°); (-51°, 139°);$
$(-45°, 11°); (-28°, 72°);$
$(-25°, 180°); (49°, 6°);$
$(52°, -146°); (89°, 24°);$
$(173°, 0°).$

Our objective was to examine, with the help of NAMFIS, the occurrence and relevance of these dihe-

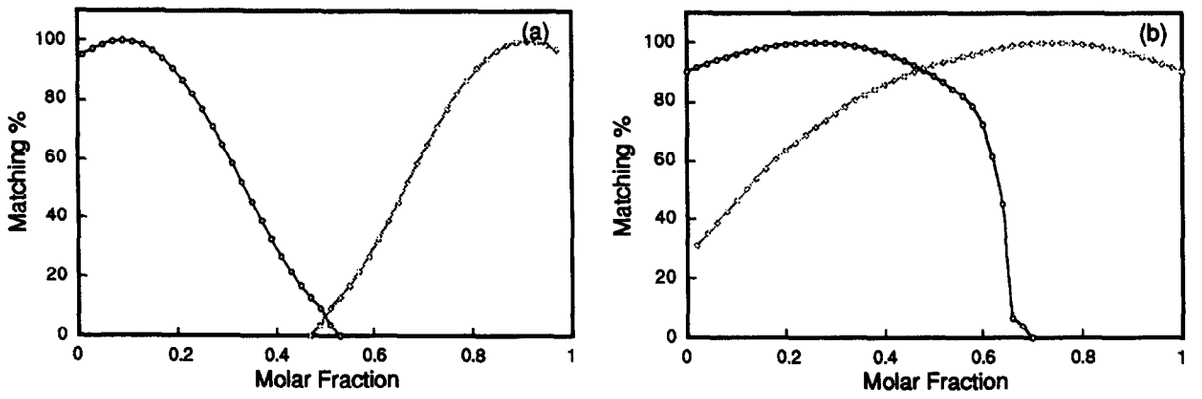

Fig. 3. Matching curves for conformers with: (a) $\phi = 42°$, $\psi = -15°$ (grey); $\phi = 37°$, $\psi = 131°$ (black); (b) $\phi' = 49°$, $\psi' = 6°$ (grey); $\phi' = 89°$, $\psi' = 24°$ (black).

dral angles as described by our experimental data. The programme first filtered all the conformers that violated at least one of the experimental absent distance constraints (ADCs) [11]; then for the sum of the conformers with a common geometry around the glycosidic linkage calculated the feasible domain and the corresponding matching functions (Fig. 3) of the sum of their molar fractions. All the distance constraints listed in Table 2 and $^3J_{CH}$ in Table 3 were used as experimental input. As theoretical input were employed the distances and the $^3J_{CH}$ for all the set of conformers. The $^3J_{CH}$ were calculated using the following Karplus-type equation: [12]

$$^3J_{CH} = 5.7\cos^2\Phi - 0.6\cos\Phi + 0.5$$

The values of ϕ and ϕ' obtained from our analyses satisfied the 'exo-anomeric effect' [13].

The results of our study could be resumed as follow: the geometry around the ϕ and ψ linkage is well defined with values for these angles of circa 42° and −15°, in accordance with previous conformational analysis of anthracyclines [6]. These studies proposed a certain rigidity for ϕ and ψ, interpreting the NOE data as that of a single conformer about this point, rather than a fast equilibrium of different conformers. Nevertheless our analysis showed that with the data available also a significant population of conformers with ϕ and ψ of around 37° and 131° could not be ruled out. An additional experimental data such as the virtual distance between H-1′ and OH-6 might further clarify this matter. In our case, this information was missing because of the exchange rate of this hydroxyl group under the acidic conditions imposed by the preparation procedure of **1**.

For the orientation of the daunosamine moiety with respect to the fucose, we found that our data could be satisfactorily explained considering only conformers with $\phi' = 49°$ and $\psi' = 6°$, but conform-

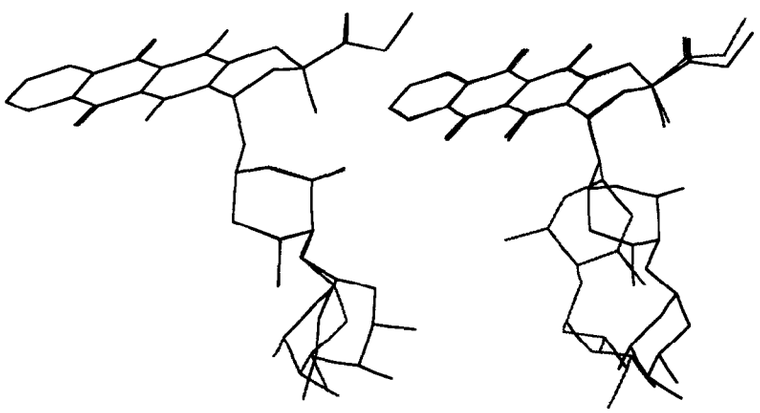

Fig. 4. Superimposition of conformers with: at left $\phi = 42°$, $\psi = -15°$, $\phi' = 49°$, $\psi' = 6°$ (grey); $\phi = 42°$, $\psi = -15°$, $\phi' = 89°$, $\psi' = 24°$ (black), and at right $\phi = 42°$, $\psi = -15°$, $\phi' = 49°$, $\psi' = 6°$ (black); $\phi = 37°$, $\psi = 131°$, $\phi' = 49°$, $\psi' = 6°$ (grey).

ers with $\phi' = 89°$ and $\psi' = 24°$ could be present as a molar fraction up to 0.6 with good degree of matching. This result can be interpreted as a two minima area, according to the force field calculations or as a broad area that exhibits quite a large libration motion (Fig. 4).

In conclusion we have now determined that the shape of the molecule up to the second sugar ring is similar to that of doxorubicin, so that the absence of the positive charge at the first sugar moiety seems not to affect the conformational preference of this portion of the molecule. We have also described the preferred spatial arrangement of the aminosugar, which as it has been mentioned in the introduction, is deemed to participate in relevant interactions with the topoisomerase II.

Our future aim is to apply this methodology to other disaccharide analogues with the daunosamine attached to the aglycone and a non aminated sugar moiety at the second position [14] since, in our opinion, the knowledge of the factors that influence the molecular conformation of the drug in solution can provide some insight into the molecular mechanism of drug action.

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