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Assignment of the ^1H NMR Spectrum and Solution Conformation of the Antitumour Antibiotic Ditrisarubicin B

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Abstract: Complete assignment of the ^1H NMR spectrum of ditrisarubicin B, a member of the anthracycline antitumour antibiotics, in acetonitrile is reported. The two trisaccharide chains are highly structured and their conformation supports their role as preorganised DNA minor groove binders which bind to DNA on intercalation of the tetracyclic chromophore.
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INTRODUCTION

Several classes of naturally occurring antitumour antibiotics, whose biological activity is related to their interaction with DNA, contain carbohydrate side chains.[1] Several recent studies have established that these side chains serve an important role in both DNA binding and recognition of specific DNA sequences.[2-5] NMR studies of chromomycin[2] and DNA footprinting studies of calicheamicin[3] specifically addressed the role the sugars play in DNA binding. In the case of calicheamicin, the sugar side chain was cleaved from the aglycone and shown to bind specifically to DNA, with different specificity to the parent drug.[3]

We have initiated studies aimed at understanding the role of the sugar side chains present in the anthracycline antitumour antibiotics.[6,7] This class of antitumour drugs contains a tetracyclic chromophore to which is attached a saccharide chain that generally is comprised of between one and three sugars (*e.g.*, Figure 1). A large number of studies have shown that these compounds interact with DNA by intercalation, and/or inhibition of topoisomerase II.[5] While daunomycin, which contains a single sugar residue, has been extensively studied both alone and complexed to DNA and oligonucleotides,[1,5,8] there is comparatively little data available on anthracyclines containing two or more sugars. Recent crystallographic[9] and NMR studies[10] have suggested that, in anthracyclines containing more than one sugar residue, the sugars are preorganised for interaction with the minor groove of DNA.

We have recently reported chemical and enzymatic DNA-footprinting studies of anthracyclines containing a monosaccharide, trisaccharide and two trisaccharide chains respectively.[7] These studies have confirmed the location of the sugars in the minor groove and some sequence selectivity and increased protection from enzymatic cleavage was observed with increasing length of the sugar chains. Based on these results, we are currently studying the conformation and DNA-binding properties of ditrisarubicin B[11] (Figure

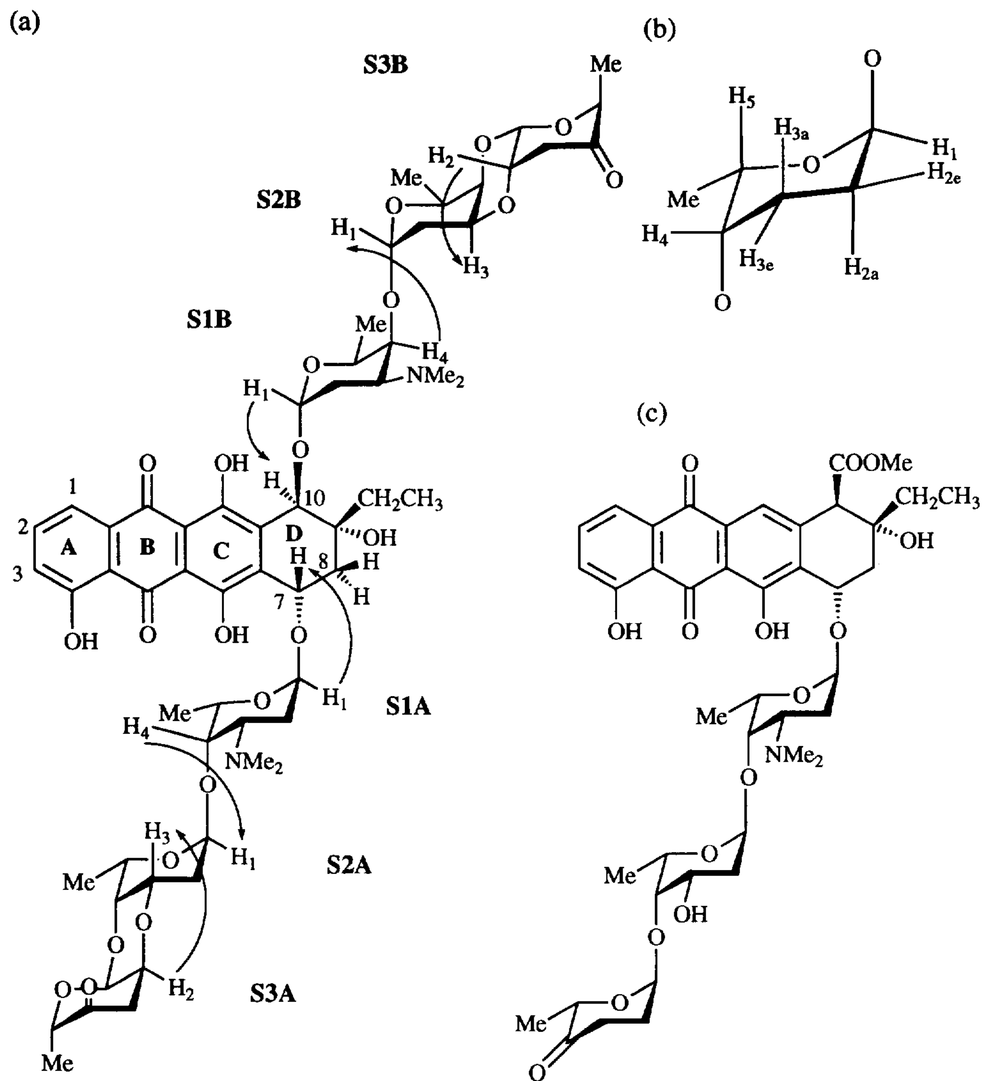

Figure 1 : (a) Structure and numbering scheme of ditrisarubicin B ; arrows indicate key inter sugar residue nOe contacts used for assignment of the A and B trisaccharide chains
(b) Numbering scheme of sugar residues
(c) Structure of aclacinomycin A

1a). Ditrisarubicin B is unusual compared with other anthracyclines in that it contains two trisaccharide chains. Furthermore there is an unusual connection between the terminal two sugars, S2 and S3, which constrains the conformation considerably in comparison to the corresponding trisaccharide in aclacinomycin A[12] (Figure 1c). This paper reports the complete ^1H NMR assignment of ditrisarubicin B and details of the solution conformation of the two trisaccharide chains in the drug. These data are an essential prerequisite for detailed ^1H NMR studies with oligonucleotide sequences. The structure is compared with that of aclacinomycin A, whose NMR spectrum[13] and complexes with oligonucleotides[10] were recently reported.

RESULTS AND DISCUSSION

Ditrisarubicin B has good solubility in chloroform, dichloromethane, methanol, and acetonitrile but has very limited solubility in water. All studies in this work were carried out in acetonitrile at 310 K as this gave a sharp, well-resolved spectrum. At lower temperatures, the aromatic resonances were broadened, suggesting intermolecular stacking of the aromatic rings. Similar broadening of the aromatic resonances was observed in methanol (273-323 K). In contrast, in less polar solvents such as chloroform, where stacking interactions are weak, sharp spectra were obtained at all temperatures. As a major application of this work was to allow ^1H NMR characterisation of the interactions of ditrisarubicin B with oligonucleotides to be carried out, acetonitrile was used as this is a water-misicible solvent suitable for the preparation of aqueous drug-oligonucleotide complexes.

Assignment of Spin Systems

The ^1H NMR spectrum of ditrisarubicin B was assigned from analysis of 600 MHz DQFCOSY, TOCSY, and ROESY spectra which were recorded under standard conditions. The chromophore protons on rings A and D were initially assigned. Ring A protons were identified by comparison with the reported data for daunomycin,[8] which contains a very similar ring system. The exchangeable -OH protons were not observed in the spectra due to exchange with solvent water, although they were noted in the range 12–14 ppm in spectra recorded in $CDCl_3$. Proton H_7 in ring D resonated downfield at 5.1 ppm and was coupled to both H_{8a} and H_{8b}, while H_{10} appeared as a singlet at 4.91 ppm. Similarly, the ethyl side chain at C_9 was readily identified in the DQFCOSY spectrum.

The six anomeric protons (H_1), which resonated downfield in the region 5.0-5.5 ppm (along with H_7 from ring D) were readily identified in the DQFCOSY spectrum due to their coupling to the H_2 proton(s), and served as entry points for the assignment of the oligosaccharides (Figure 2). These and all other saccharide protons were easily distinguished from intercalator resonances in the two-dimensional spectra; the sugar connectivities invariably occurred in pairs, shifted in both dimensions by up to *ca* 0.1 ppm, indicating that the two trisaccharide chains exist in similar, but not identical, environments (Figure 2). For both the S2 and S3 sugars, only one of the two possible H_1-H_2 connectivities was observed in the DQFCOSY. The S3 spins systems, however, could still be distinguished from S1 and S2 on the basis of both the relatively downfield chemical shift of the $S3A_2$ (*i.e.*, H_2 of S3A) and $S3B_2$ protons (S3, in contrast to both S1 and S2, carries an oxygen at C_2), and the lack of a strong geminal coupling to the H_2 proton. Confirmation of this assignment was

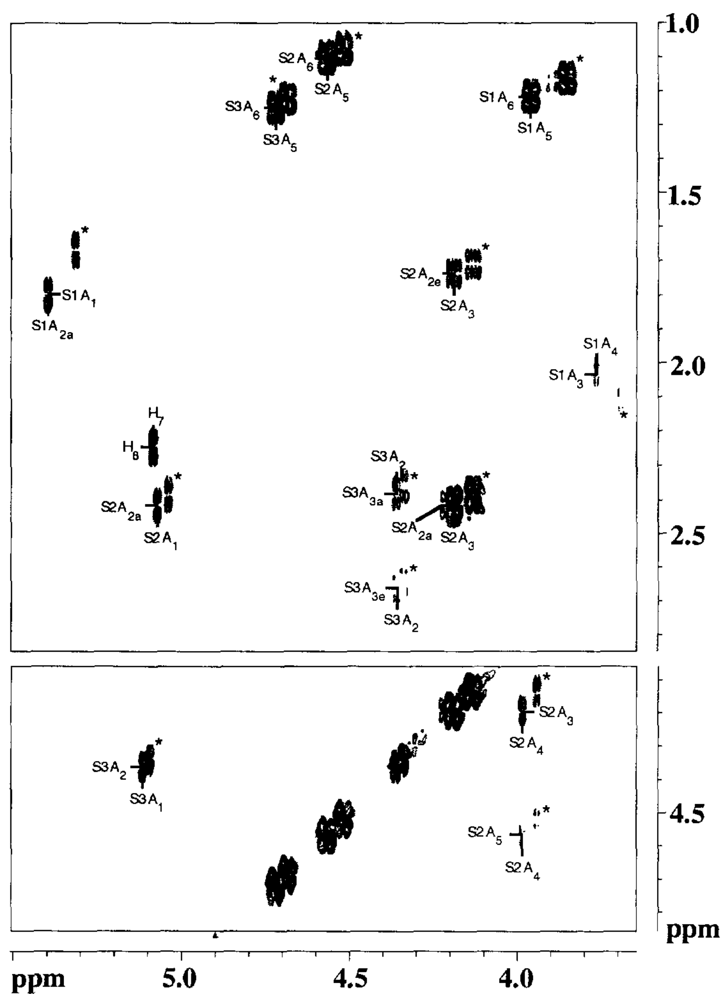

Figure 2: Section of the DQFCOSY of ditrisarubicin B, illustrating many of the connectivities which were used in the assignment of the ^1H NMR spectrum. Crosspeaks connecting protons in the A trisaccharide chain are labelled, and the corresponding crosspeaks from the B saccharide are indicated with an asterisk.

obtained from the TOCSY spectrum, where the missing H_1-H_2 connectivities for S1 and S2 were observed. From this assignment of H_1 in S3A and S3B, the connectivity H_1-H_2-$H_{3a,b}$ was established and defined this spin system in these two sugars.

Further analysis of the DQFCOSY, starting from H_1 for each of S1A, S2A, S1B, and S2B, allowed almost complete identification of the spin systems these four sugar residues. Due to the small coupling between H_4 and H_5 (< 1 Hz), this crosspeak was weak and not always possible to assign. In addition, spectral overlap in the DQFCOSY, particularly in the region 2.0-3.5 ppm, precluded the unambiguous assignment of all protons. The TOCSY spectrum, however, alleviated these problems, allowing all spin systems to be identified. In some cases (*e.g.*, S1B), TOCSY "skewers" at the H_1 frequency even revealed correlations to the distant H_5 and H_6 protons.

Assignment of Trisaccharides

The assigned spin systems were matched to individual saccharide residues using dipolar connectivities derived from a ROESY spectum. Initially, NOESY spectra were recorded, but the absence of a significant number of crosspeaks, even at longer mixing times (up to 400 ms), suggested the tumbling rate of ditrisarubicin B was not in the correct regime to give nOe enhancements. Similar observations have been made by other groups working with related compounds.[13] Use of the ROESY experiment alleviated this problem, and the key inter-sugar residue contacts obtained from this spectrum are shown schematically in Figure 1. Sugar S1A was identified by a strong nOe from H_7 (ring D) to H_1 of S1A. Examination of molecular models generated by MacroModel[14] indicated the internuclear distance between these protons is ~ 2.3 Å in an energy-minimised conformation. Similarly, S1B was identified from the nOe from H_{10} (ring D) to $S1B_1$, a distance of ~ 3.1 Å. The second sugar residue in chain A and chain B was identified in each case by the nOe from H_4 of S1 to H_1 of S2. Assignment of S2A and S2B allowed S3A and S3B to be identified by an nOe from H_3 of S2 to H_2 of S3.

Conformation of Sugar Residues

Table 1 summarizes the chemical shifts and coupling constants for ditrisarubicin B. The coupling constants, which may be measured either directly from the 1D spectrum or from the antiphase splittings in the DQFCOSY, can be used to infer the conformation(s) of the six sugar residues in the drug. Firstly, the medium sized coupling constants between $S1A_1$ and both $S1A_{2a}$ and $S1A_{2e}$ imply that $S1A_1$ is equatorial, and therefore that the glycosidic link to ring D of the chromophore is axial. Similarly, the $S2A_1$–$S2A_{2a/2e}$ ^3J values suggest that $S2A_1$ lies equatorial. The $^1C_4$ chair conformation for S2A is further confirmed by the large $S2A_{2a}$–$S2A_3$ coupling (12.6 Hz) and the medium sized couplings further around the ring towards $S2A_5$. It is also clear that S3A lies in a chair-like conformation, as evidenced by the two medium-sized $S3A_2$–$S3A_{3a/3e}$ couplings. All of the couplings constants measured for the B-chain sugars mirrored those of the A-chain within experimental error, so that the above conclusions may also be drawn for the B-chain.

TABLE 1 ^{1}H NMR Data for Ditrisarubicin B

Epitope	Proton	δ (ppm) A-Chain	J_{HH} (Hz)[b] A-Chain	δ (ppm)[c] B-Chain
S1	H_1	5.40	3.0	5.32
	H_{2a}	1.80	6.4*, 16.6*	1.73
	H_{2e}	1.80	16.6*	1.50
	H_3	2.06	6.5*	2.14
	H_4	3.78	6.5*, 5.3*	3.71
	H_5	3.87	5.3*, 6.5*	3.97
	H_6	1.22	6.6	1.24
	$N(Me)_2$	2.11	–	2.11
S2	H_1	5.09	3.6	5.05
	H_{2a}	2.43	4.0, 12.6	2.40
	H_{2e}	1.77	14.6*, 6.1*	1.74
	H_3	4.20	12.5, 3.5	4.14
	H_4	4.00	2.5	3.95
	H_5	4.58	6.9	4.53
	H_6	1.11	6.6	1.09
S3	H_1	5.13	3.3	5.11
	H_2	4.37	5.6*, 5.5*	4.35
	H_{3a}	2.39	11.5, 2.7	2.37
	H_{3e}	2.67	12.5*	2.65
	H_5	4.73	6.3	4.69
	H_6	1.26	6.3	1.23
Aglycone	H_1	7.82	7.3	
	H_2	7.75	7.6, 8.3	
	H_3	7.30	8.6	
	H_7	5.13	4.0	
	H_{8a}	2.26	5.0, 16.6	
	H_{8b}	2.16	15.3*	
	H_{10}	4.91	–	
	H_{13}	1.75	7.0*	
	H_{14}	1.03	7.3	

[a]Chemical shifts are measured from ^{1}H NMR data recorded at 600 MHz, 37°C, d_3-acetonitrile, and were calibrated against the residual acetonitrile solvent resonance (1.93 ppm).
[b]Coupling constants were measured from a Lorentz-Gauss resolution-enhanced 1D spectrum where possible; otherwise measurements were made from the antiphase splitting of the relevant crosspeak in the DQFCOSY. Measurements made using the latter method are marked with an asterisk, and and in some cases may overestimate the true value of the coupling constant by 1-2 Hz.
[c]The $^{3}J_{HH}$ coupling constants for the B-chain were in all cases within ±0.5 Hz of the corresponding values for the A-chain.

Comparison with Aclacinomycin A

The solution conformation of aclacinomycin A in chloroform was recently reported.[13] While our study has been carried out in a more polar solvent, comparison of the conformation of the trisaccharides in the two antibiotics is informative. The sugar residues of aclacinomycin A (Figure 1c) were reported to lie in essentially chair conformations with the glycosidic links diaxial and with considerable restriction in the rotation about the glycosidic bonds. Compared with aclacinomycin A, the trisaccharides in ditrisarubicin B (Figure 1a) are considerably more constrained due to the fused junction between rings S2 and S3. From the dihedral angles present in S2 and S3, estimated from the ^{3}J couplings in these rings (Table 1), S2 and S3 have a fixed conformation, which results in a distinct curved shape due to the chair conformations of the sugars. Sugars S1 and the glycoside connection to sugars S2 are identical to aclacinomycin A and our data support an almost identical conformation in this portion of the trisaccharide chain in both antibiotics.

The association constants of betaclamycin A (which carries the same trisaccharide as aclacinomycin A) and ditrisarubicin B for DNA (Kunimoto et al., 1988) have been reported to be 5.92×10^{6} M^{-1} and 2.36×10^{8} M^{-1} respectively.[15] It is well-known that preorganisation is often a pre-requisite to obtain tight binding. Recent studies on several DNA-binding compounds have noted the rigid, extended saccharide conformations present in these compounds.[2,3] Thus oligosaccharides appear to be subtantially preorganized for DNA-binding. As many of these sugars, including the anthracyclines, contain 6-deoxy sugars, hydrophobic interactions are also assumed to be important in minor groove binding with DNA. The major contribution to the higher binding constant of ditrisarubicin B for DNA compared with betaclamycin A is obviously the second trisaccharide chain. However, it is likely that the highly structured conformation of the two trisaccharide chains is also important in the overall binding process.

EXPERIMENTAL

Ditrisarubicin B was a gift from Professor Uchida, Pharmaceutical Research Laboratory, Kirin Brewery, Japan, and was used as provided. NMR spectra were recorded on a Bruker AMX600 spectrometer on a 5 mM sample in CD_3CN (99.9%, Aldrich) solution at 313 K. Spectra were recorded with quadrature detection employed throughout. Two dimensional spectra were acquired in the phase sensitive mode using time-proportional phase incrementation (TPPI).[16] Datasets comprised 2048 (t_2) x 512 (t_1) data points, and were acquired with spectral widths of 5400 Hz in both dimensions. Typically 16–32 transients were recorded for each increment of t_1 with a recycle delay of 1.0 seconds. Double quantum filtered COSY (DQFCOSY)[17] spectra were acquired using the standard pulse sequence. TOCSY[18] spectra were acquired using a 10.0 kHz spin locking field with an MLEV-17 sequence of 70 ms. ROESY[19] spectra were recorded using a 2.0 kHz spin locking field and mixing times of 200 and 400 ms. Data were zero-filled once in each dimension and subjected to shifted sine-bell weighting functions in t_2 and t_1 prior to 2D Fourier transformation. Polynomial baseline corrections were applied in f_2. All data processing was carried out using Bruker UXNMR software on a Silicon Graphics workstation. Molecular models for the estimation of distances and dihedral angles were generated using MacroModel[14] (version 4.1) on a Silicon Graphics Indigo.

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REFERENCES

1. *Anthracycline and Anthracenedione-Based Anticancer Agents,* Ed. J. W. Lown, Elsevier, New York, 1988; *Chemistry of Antitumour Agents*, Ed. D. E. V. Wilman, Blackie, New York, 1990.
2. Keniry, M. A.; Banville, D. L.; Simmonds, P. M.; Shafer, R. *J. Mol. Biol.* **1993**, *231*, 753; Gao, X.; Mirau, P.; Patel, D. J. *J. Mol. Biol.* **1992**, *223*, 259; Silva, D. J.; Kahne, D. J. *J. Am. Chem. Soc.* **1993**, *115*, 7962; Silva D. J.; Kahne, D. E. *J. Am. Chem. Soc.* **1993**, *115*, 7962.
3. Nicolaou, K. C.; Tsay, S. -C.; Suzuki, T.; Joyce, G. F. *J. Am. Chem. Soc.* **1992**, *114*, 7555; Paloma, L. G.; Smith, J. A.; Chazin, W. J.; Nicolaou, K. C. *J. Am. Chem. Soc.* **1994**, *116*, 3697; Nicolau, K. C.; Aijoto, K.; Komatsu, H.; Smith, B. M.; Li, T.; Egan, M. G.; Gomex-Paloma, L. *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 576; Paloma, L. G.; Smith, J. A.; Chazin, W. J.; Nicolaou, K. C. *J. Am. Chem. Soc.* **1994**, *116*, 3697.
4. Ikemotos, N.; Kumar, R. A.; Dedon, P. C.; Danishefsky, S. J.; Patel, D. J. *J. Am. Chem. Soc.* **1994**, *116*, 9378.
5. Lown, J. W. *Chem. Soc. Revs.* **1993**, 165 and references cited therein.
6. Shelton, C. J.; Harding, M. M. *J. Chem. Res.* **1995**, 1219.
7. Shelton, C. J.; Harding, M. M.; Prakash, A. S. submitted for publication *Biochemistry.*
8. Tong, G. L.; Wu, H. Y.; Smith, T. H.; Henry, D. W. *J. Med. Chem.* **1979**, *22*, 912.
9. Gao, Y. -G.; Liaw, Y. -C.; Li, Y. -K.; van der Marel, G. A.; Boom, J. H.; Wang, A. H. -J. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 4845.
10. Yang, D.; Wang, A. H. -J. *Biochemistry* **1994**, *33*, 6595.
11. Uchida, T.; Imoto, M.; Masuda, T.; Imamura, K.; Hatori, Y.; Sawa, T.; Naganawa, H.; Hamada, M.; Takeichi, T.; Umezawa, H. *J. Antiobiot.* **1983**, *36*, 1080.
12. Oki, T.; Kitamura, I.; Yoshimoto, A.; Matasuzawa, Y.; Shibamoto, N.; Ogasawara, T.; Inui, T.; Takamatsu, A.; Takeuchi, T.; Masuda, T.; Hamada, M.; Suda, H.; Sawa, T.; Umezawa, H. *J. Antiobiot.* **1979**, *32*, 791.
13. Parkinson, J . A.; Sadler, I. A.; Pickup, M. N.; Tabor, A. B. *Tetrahedron* **1995**, *51*, 7215.
14. Mohamidi, F.; Richards, N. G. J.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrikson, T.; Still, W. C. *J. Computat. Chem.* **1990**, *11*, 440.
15. Kunimoto, S.; Takahoshi, Y.; Uchida, T.; Takeuchi, T.; Umezawa, H. *J. Antibiot.* **1988**, *41*, 655.
16. Marion, D.; Wütrich, K. *Biochim. Biophys. Res. Commun.* **1983**, *113*, 967.
17. Piantini, U.; Sorenson, O.; Ernst, R. R. *J. Am. Chem. Soc.* **1982**, *104*, 6800.
18. Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *65*, 355.
19. Bothner-By, A. A.; Stephens, R. L.; Lee, J. -M.; Warren, C. D.; Jeanloz, R. W. *J. Am. Chem. Soc.* **1984**, *106*, 811.

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