Solution Conformation of the Antitumor Drug Streptonigrin

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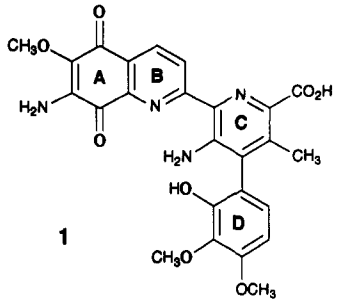
The solution conformation of the antitumor drug streptonigrin in THF-d_8 has been determined by dynamic ^{1}H NMR spectroscopy (400 MHz). The major solution conformation agrees with the structure observed in the solid state [Chiu, Y.-Y.; Lipscomb, W. N. *J. Am. Chem. Soc.* **1975**, *97*, 2525–30]. Rings A, B, and C are coplanar, with ring C held in place by a hydrogen bond from the amino group on ring C and the pyridyl nitrogen in ring B. This conformation is stable in the range pH 3.9–8.9. At lower pH, the hydrogen bond is disrupted due to protonation of the pyridyl nitrogen in ring B. The major species present at pH 3.9–8.9 and 180 K is the zwitterion **1b** (80%). Below 190 K, slow proton transfer between the free acid **1a** and the zwitterion **1b** is observed on the NMR time scale. Addition of a catalytic amount of base to the solution increases the rate of exchange **1a** ⇌ **1b**, and only one set of resonances is observed. In CD_2Cl_2 this proton transfer is not observed. Implications for the structure(s) of metal complexes formed by streptonigrin are discussed.

Streptonigrin (1), a highly functionalized 7-aminoquinoline-5,8-dione, has broad spectrum antitumor activity and is active against lymphoma, melanoma, and cancers of the breast, cervix, head, and neck.[1-6] However, severe toxic



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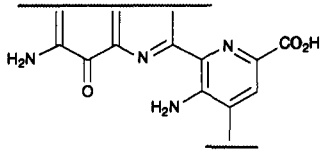
side-effects[2,3] have resulted in the drug being withdrawn from clinical use.[7] Studies *in vivo* and *in vitro* suggest that streptonigrin exerts its antitumor activity by degradation of DNA and by interfering with the cell respiratory mechanism.[8-11] Extensive studies on synthetic analogues have shown that the substructure **2** is the minimal unit required for biological activity.[1b,12] The role of ring D, although not clearly understood, does not appear to be crucial to the antitumor properties of the drug.



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Mechanistic studies by several groups have shown that streptonigrin-mediated DNA degradation requires reduction to the semiquinone and the presence of metal ions and that the process involves oxygen and radicals.[9-13] The exact role of the metal ions in this process has not been established.[15] Analysis of the structure of streptonigrin (1) shows that there are several metal binding sites. The substructure **2** contains all the potential coordination sites of streptonigrin, and the reduced clinical activity in derivatives not containing this subunit strongly suggests that metal-streptonigrin interactions play a pivotal role in determining the biological activity of streptonigrin.[15]

The active species responsible for DNA cleavage and the presumed mechanism of antitumor activity of streptonigrin has been suggested to be either the streptonigrin semiquinone radical which interacts directly with DNA[9] or the hydroxyl radical (OH·) that is generated in a series of redox reactions that involve several species including streptonigrin semiquinone and superoxide.[10,11,14] Metal ions have been suggested to play a role in these processes by direct interaction with streptonigrin, thus activating the system to reduction, and/or complexation with reduced streptonigrin.[10,13] Alternatively, metal ions have been implicated in the catalytic production of hydroxyl radical from hydrogen peroxide and superoxide.[10,11,13] Thus, while streptonigrin interacts with metal ions[15] and substantial enhancement of streptonigrin–DNA binding occurs in the presence of metal ions,[13a,16-18] the significance of these observations on the *in vivo* mechanism of action of streptonigrin remains to be elucidated.

We have initiated a program aimed at characterization of the solution structure(s) of the metal complexes of streptonigrin and the interaction(s) of streptonigrin and metal complexes of streptonigrin with DNA using NMR spectroscopy. X-ray crystallography has shown that the A, B, and C rings of streptonigrin are in a nearly coplanar arrangement with ring D almost perpendicular to the plane defined by the other three rings.[19] While the proton (270 MHz) and carbon-13 (67.88 MHz) spectra of streptonigrin in dimethyl sulfoxide have been reported,[12] it has not been established whether the solution conformation of the drug is the same as the structure observed in the solid state.[15a] This paper reports a 400 MHz ^{1}H NMR study of the solution conformation of streptonigrin. The exact conformation(s) of the drug, and full assignment of the ^{1}H NMR spectrum, is an essential prerequisite to interpretation of NMR data obtained on streptonigrin metal complexes and in the NMR analysis of streptonigrin–DNA interactions.

Results

Streptonigrin is slightly soluble in water, the lower alcohols, ethyl acetate, dichloromethane, and chloroform and is readily soluble in dioxane, pyridine, dimethylform-

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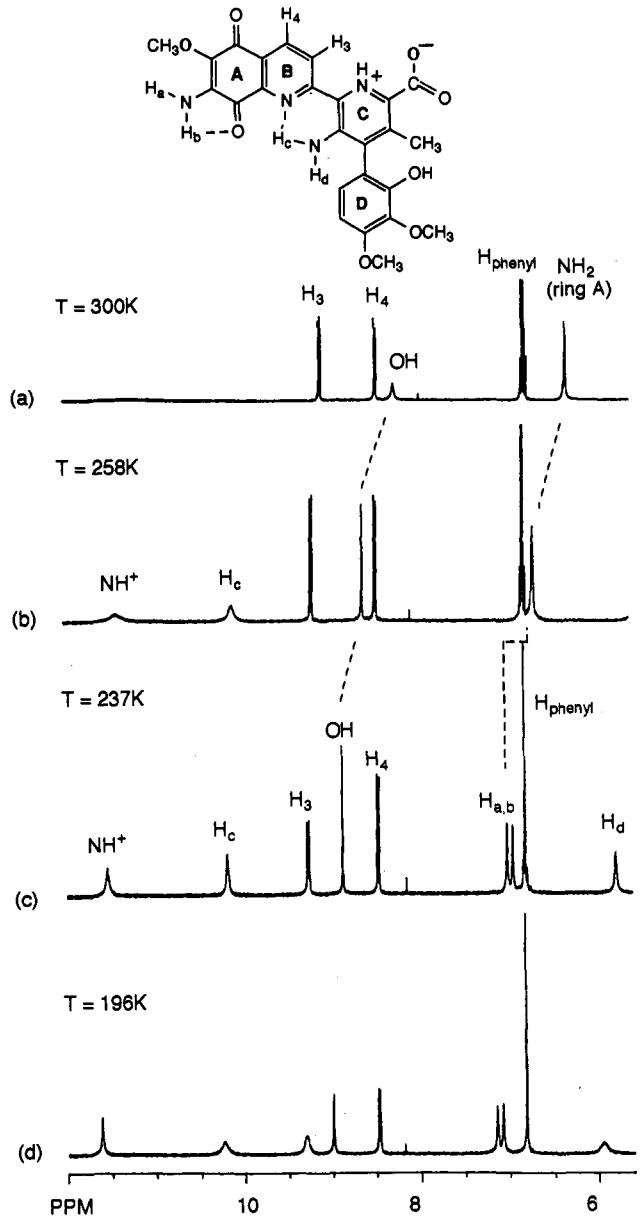
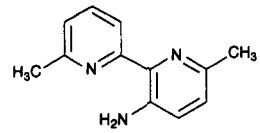


Figure 1. Section of the 400-MHz ^{1}H NMR variable temperature spectra of streptonigrin 1 in THF-d_8 (pH 8.9) at the temperatures indicated.

amide, and tetrahydrofuran.[12] Most studies were carried out in THF-d_8, due to the temperature range accessible in this solvent and because the exchangeable protons are observable in THF-d_8.

Variable-Temperature NMR Spectroscopy. The ^{1}H NMR spectrum of streptonigrin in THF-d_8 at 300 K is shown in Figure 1a. As the temperature is lowered, numerous new resonances appear in the spectrum (Figure 1). In neutral streptonigrin 1, there are six exchangeable protons, NH_2 (ring A), NH_2 (ring C), COOH (ring C), and OH (ring D). At 300 K only the OH and NH_2 (ring A) are observed in the spectrum. The amino protons on ring A appear as a broad singlet at 300 K (δ 6.1 ppm), but at lower temperatures they split into two singlets due to formation of hydrogen bonds to the adjacent oxygens. The amino protons on ring C are exchange broadened at 300 K but appear at 237 K as the two resonances, H_c and H_d, at δ 10.2 and δ 5.8 ppm, respectively. The large separation in chemical shift of H_c and H_d was attributed to the presence of a hydrogen bond between H_c and the pyridyl nitrogen in ring B. A similar difference in chemical shift has been observed in a study of the model system, 3-amino-6,6'-dimethyl-2,2'-bipyridine (3).[20] The resonances due to H_c



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and H_d broaden again at 196 K. The peak assignments of all resonances (Figure 1) were confirmed by a NOESY experiment at 190 K which showed the expected connectivities.

At 196 K, some of the resonances, notably H_c, H_d, and H_3, broadened further, and in the range 190–170 K two sets of signals were observed for some of the resonances Figure 2. The signals most affected are H_3, NH^+, OH, H_c, H_d, and CH_3, each of which splits into two signals in a ratio ~4:1. The presence of two species was attributed to slow proton transfer between the carboxyl proton and the adjacent pyridyl nitrogen in ring C, i.e., **1a** ⇌ **1b**. The broadening of H_3 (ring B) and not H_4 may be rationalized by steric interactions associated with the proton transfer; rings A–C are coplanar at this temperature, and as a result, H_3 is very close to the site of proton transfer.

Streptonigrin dissolved in THF-d_8 gives a solution with pH 8.9 and exists predominantly as the zwitterion **1b**. The pH of the solution was adjusted to 7.4, 5.6, 4.2, and 3.9, and spectra were recorded at 180 K in order to analyze the effect of pH on the proton transfer **1a** ⇌ **1b** (Figure 2) and the solution conformation of the drug. The chemical shifts of the resonances due to H_c and H_d did not change in this range indicating that the hydrogen bond between rings B and C is stable under these conditions. At pH < 3.9, however, the intensity of the signals due to H_c and H_d decreased, presumably due to protonation of the pyridyl nitrogen in ring B which prevents formation of the hydrogen bond. Increasing the pH of the solution from 8.9 to 9.7 by titration of base (NEt_3, 1 μL) at 180 K into the sample resulted in the disappearance of the minor resonances (Figure 2c) to give an averaged spectrum consistent with rapid exchange **1a** ⇌ **1b** as a result of a base-catalyzed proton transfer reaction.

Variable-temperature ^{1}H NMR spectra were also recorded in CD_2Cl_2 (300–190 K). In this solvent, sharp spectra, similar to the spectrum of 1 in THF-d_8 at 220 K (see Figure 1c), were obtained. No evidence for proton transfer between the carboxylic acid and the pyridyl nitrogen in ring C, such as the presence of minor peaks or further broadening of resonances at low temperatures, was detected in the temperature range studied.

Solution Conformation. There are five rotational barriers that may contribute to the line shape of the signals in the NMR spectra of streptonigrin Figure 3. Of particular relevance to interpretation of the variable temperature NMR spectra are barriers β, γ, δ, and ϵ. The relative magnitudes of barriers β, γ, and δ can be estimated from our previous work on the model system 3[20] and the work of Bott *et al.* [21] who reported a general method for calculation of rotational barriers in biaryl systems.

Streptonigrin is optically active in solution,[22] which has been attributed to restricted rotation about the aryl bond connecting rings C and D as a result of severe steric hindrance between the amine and methyl group of ring C

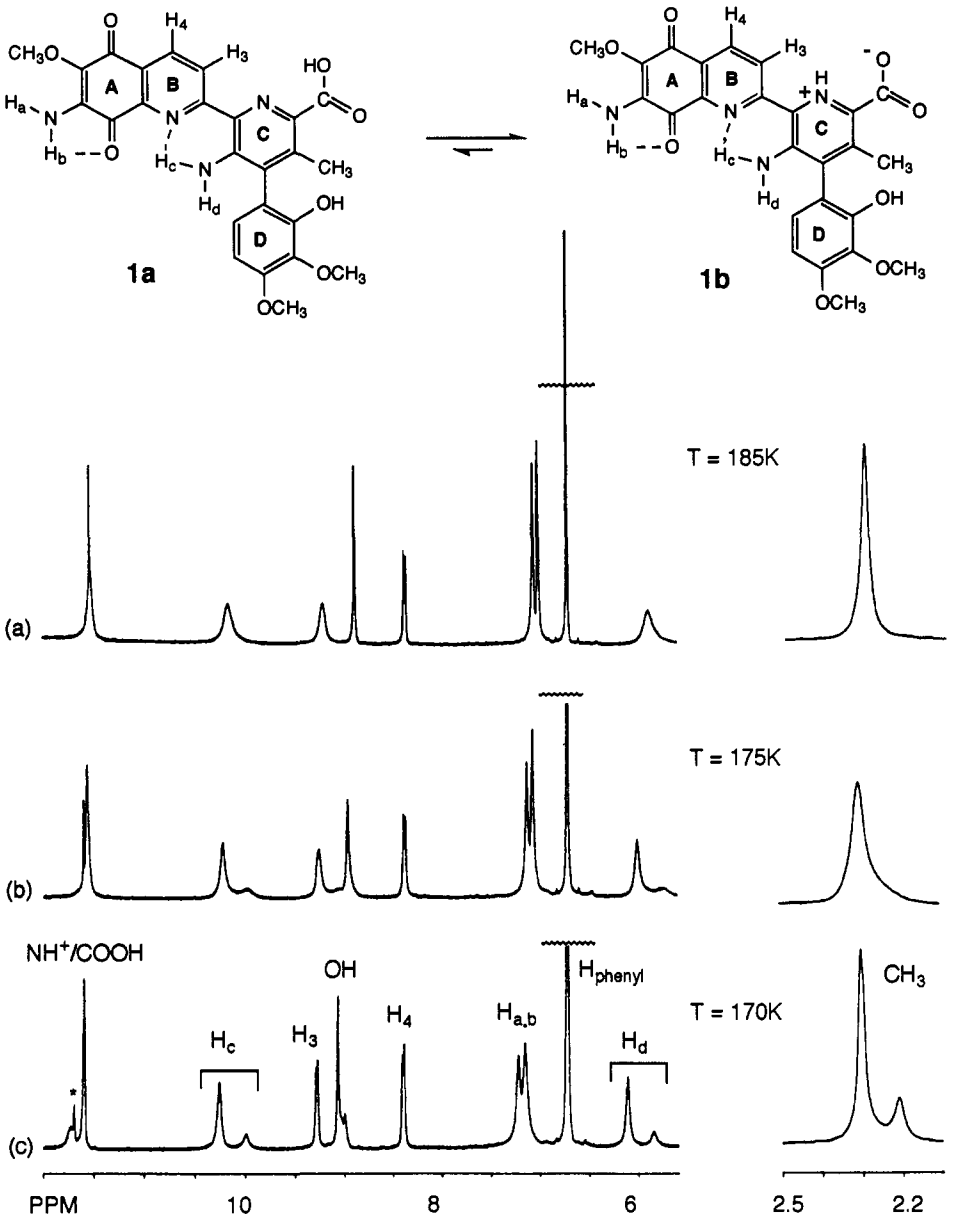


Figure 2. Section of the 400-MHz ^{1}H NMR variable temperature spectra of streptonigrin 1 in THF-d_8 (pH 8.9) at 170–185 K; *minor impurity.[30]

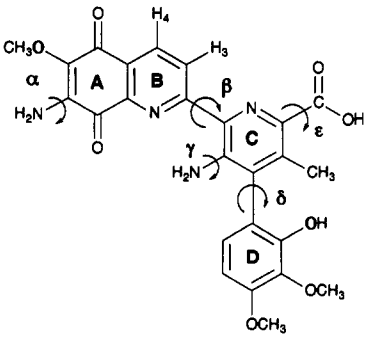


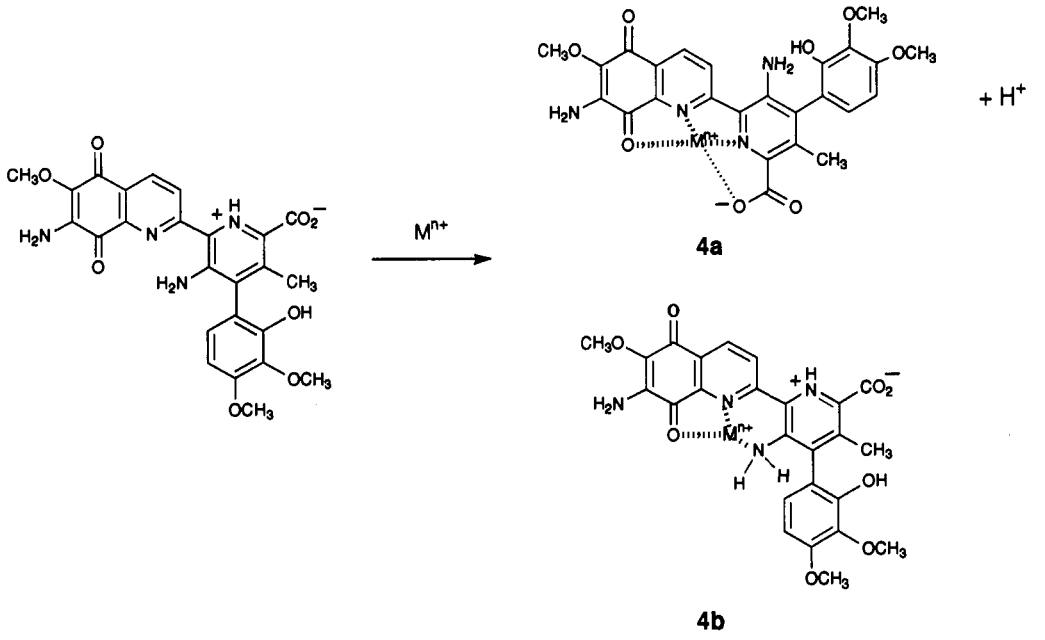
Figure 3. Definition of the rotational barriers in streptonigrin (1).

and the hydroxyl group of ring D;[21] i.e., process δ is very large. At 298 K, all processes, except δ, are fast on the NMR time scale. As the temperature is lowered, the appearance of H_c and H_d indicate that process β and/or γ are slow on the NMR time scale. At temperatures below 200 K, proton transfer between the carboxyl proton and the pyridyl nitrogen is detected and requires the carboxylic acid group to be oriented with the –OH placed near the pyridyl nitrogen of ring C; i.e., process ϵ is slow on the NMR time scale. In contrast to biaryl systems, where generally the most stable conformer has the two aryl rings perpendicular to each other,[20] the major solution conformation of streptonigrin **1b** contains rings B and C coplanar.

Discussion

Streptonigrin, dissolved in 0.15 M KCl solution, exhibits two titratable functions in the pH range 3–9.[23,24] The first function has a pK_a of 4.4 and has been assigned to the carboxylic acid in ring C,[23] while the second titratable function occurs at pK_a 6.6 and has not been assigned.[23] In neutral streptonigrin, there are acidic and basic sites that are relevant to the present study: COOH group on ring C (pK_a 4.4), pyridyl N (ring B), pyridyl N (ring C), NH_2 (ring C), and phenolic OH (ring D). From known compounds of related structure, the pK_a values of pyridyl nitrogens are generally in the range 5.0–6.0, and phenols with electron-donating groups substituted on the ring have pK_a values of typically 10–11.[25] In streptonigrin, it is likely that the pK_a of the two pyridyl nitrogens are significantly

Scheme I



different due to the different substitution patterns on the rings and the presence of the hydrogen bond between rings B and C.

In THF solution, and at low temperatures (170–195 K), proton transfer between the carboxylic acid and adjacent pyridyl nitrogen, **1a** ⇌ **1b**, is slow on the NMR time scale. Proton transfer has been observed in pyridinecarboxylic acids and is favored in polar solvents.[26] Thus, pyridine-2-carboxylic acid exists predominantly as the zwitterion in water but is in equilibrium with predominant amounts of the free acid in ethanol. In tetrahydrofuran, 80% of the zwitterionic form of streptonigrin is observed at 170 K (Figure 2). Addition of a catalytic amount of base to this solution led to an averaged spectrum consistent with a base-catalyzed proton transfer reaction to interconverts **1a** and **1b**. This assignment is supported by spectra recorded in the less polar solvent CD_2Cl_2; no signals due to minor species were detected in the range 190–300 K. In CD_2Cl_2, streptonigrin most probably exists as the free acid **1a** as stabilization of a charged species such as **1b** is much less favored.

There has been much speculation in the literature about the structure(s) of the metal complexes of streptonigrin.[13a,15,18,23] Two types of complexes have been proposed in which the metal ion coordinates to the bipyridyl nitrogens **4a** or the metal ion coordinates to one pyridyl nitrogen in ring B and the amino nitrogen of ring C **4b** (Scheme 1).[15] Hajdu and Armstrong have suggested that streptonigrin exists as a zwitterion in solution and reported that potentiometric titrations of streptonigrin in the presence of Cu(II) and Zn(II) are accompanied by the release of 1 mol of protons in 8% acetonitrile/0.1 M Tris buffer (pH 6.75).[15b]

The results of our work provide information about the proton transfer sites in streptonigrin. In aqueous solution, the relative population of the zwitterion 1b would be expected to increase compared to THF, due to the increase in solvent polarity. Under the conditions reported for the preparation of the metal complexes of streptonigrin (pH 6.5–70), the major species present is **1b**. Formation of a bipyridyl type complex **4a** (Scheme I) would require release of 1 mol of protons from the pyridyl nitrogen in ring C to form the complex. In contrast, formation of complex **4b**, in which the metal binds to the pyridyl nitrogen of ring B and the amino group of ring C, can be accommodated without proton transfer. The fact that interaction of both Cu(II) and Zn(II) with streptonigrin results in release of 1 mol of protons[14] strongly suggests that both metals form bipyridyl complexes **4a**.

Streptonigrin has been administered to patients[2,3] in both saline and glucose solutions.[2,3] Our results, obtained in THF-d_8 and CD_2Cl_2 solutions, suggest that the predominant species present in more polar aqueous solutions (pH 5–9) is the zwitterion **1b**. Characterization of the metal complexes of streptonigrin and their interaction with DNA using multinuclear high field NMR spectroscopy is currently underway.

Conclusions

Dynamic NMR (400 MHz) spectroscopy has been used to study the solution conformation of the antitumor drug streptonigrin in THF-d_8. Streptonigrin exists predominantly as a zwitterion in polar solvents. The major solution conformation has rings A–C in a planar arrangement with ring D perpendicular to the plane defined by the other three rings. A hydrogen bond between the pyridyl nitrogen in rings B and the amino group of ring C stabilizes the structure and is present in the range pH 3.9–8.9. This conformation agrees with the solid state structure of streptonigrin obtained from crystals grown from ethyl acetate. This study establishes the importance of both solvent and pH on the structure of streptonigrin, and these results need to be considered in the interpretation of data obtained on metal–streptonigrin complexes.

Experimental Section

NMR Spectroscopy. Streptonigrin was purchased from Sigma Chemical Company and was used as provided. NMR samples were prepared as 10 mM solutions. The pD of the solutions was measured and corrected to give the pH values by subtraction of 0.4.[27] For pH titration experiments CD_3COOD or NEt_3 was added in aliquots (1–5 μL) to give the required pH. NMR spectra were recorded on a Bruker AMX400 spectrometer over spectral widths of 5500 Hz with quadrature detection employed and referenced to the residual solvent proton reso-

nances. NOESY spectra were acquired in the phase sensitive mode using time-proportional phase incrementation (TPPI)[28] with a mixing time of 1 s. The spectrometer temperature was calibrated by comparison to the shift difference of the resonances in methanol.[29]

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