STEFFIMYCIN C, A NEW MEMBER OF THE STEFFIMYCIN ANTHRACYCLINES

ISOLATION AND STRUCTURAL CHARACTERIZATION

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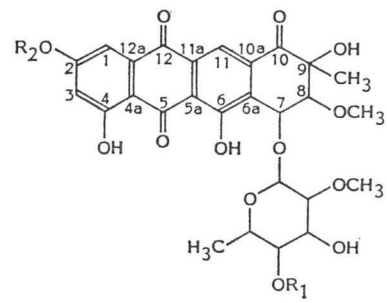
Mother liquors from steffimycin B crystallizations have been processed to yield steffimycin C, a new member of the steffimycin family of anthracyclines. It has been identified, using spectroscopic methods, as 10-deoxysteffimycin B. Steffimycin C has antibacterial activity only against *Streptococcus pneumoniae*. Whether steffimycin C is a precursor of steffimycin B or a metabolic reduction product is unknown at this time.

In 1967 BERGY and REUSSER[1] reported the isolation and chemical characterization of steffimycin (**I**), a novel anthracycline antibiotic. Aranciamycin (**II**), the second member of the family was reported in 1970[2]. In 1974, BRODASKY and REUSSER[3] described the isolation and characterization of the third member of this family, steffimycin B (**III**).

WILEY and KELLY *et al.* subsequently published the structures of the two steffimycin antibiotics and several hydrolysis products[4]. During a recent isolation of steffimycin B, it was discovered that the mother liquors from the crystallization of this antibiotic contained a component with the characteristic orange color and chromatographic behavior of the steffimycins but with an Rf value on silica gel TLC plates slightly lower than steffimycin B. The current paper describes the isolation, purification and structural characterization of this fourth member of the steffimycin family, steffimycin C. Its antibacterial properties are also described.



I (Steffimycin) $R_1=H$, $R_2=CH_3$
II (Aranciamycin) $R_1=H$, $R_2=H$
III (Steffimycin B) $R_1=CH_3$, $R_2=CH_3$

Experimental

Steffimycin B Mother Liquors

During the isolation of steffimycin B from a 250-liter fermentation, HPLC analysis of the mother liquors from the crystallization step indicated a new major component in a ratio with the remaining steffimycin B of 1:1. After concentrating the mother liquors to dryness, 32.4 g of solid containing steffimycin B, steffimycin C and other components was obtained.

Isolation of Crude Steffimycin C

The solid from the concentration of the mother liquors was processed to isolate the steffimycin C by preparative HPLC using a Waters Prep 500 (Waters Associates, Milford Mass.) system. The

stationary phase was silica gel and the mobile phase consisted of MeOH - CH_3CN - H_2O - $CHCl_3$ - $(C_2H_5)_3N$ (61: 26: 5: 7: 0.7). 2 g of solid was dissolved in a minimum volume of mobile phase for each run. The fractions containing steffimycin C (by TLC analysis) were pooled and concentrated to an aqueous phase. After adjusting the pH of the aqueous phase to 7 with HCl, the steffimycin C was extracted into EtOAc. Crude steffimycin C was obtained by precipitation from the EtOAc with excess cyclohexane. TLC analysis of the precipitate on Whatman (Whatman, Clifton, New Jersey) linear high performance silica gel plates using a mobile phase of CH_2Cl_2 - $(CH_3)_2CO$ - C_6H_{14} - MeOH (30: 10: 3: 2) revealed the presence of steffimycin C and 5~10% of steffimycin B.

Purification of Crude Steffimycin C

Crude steffimycin C was further purified by column chromatography on a Merck (E. Merck Darmstardt, Germany) prepacked silica gel column (bed volume 473 ml) using a mobile phase composed of CH_2Cl_2 - $(CH_3)_2CO$ - C_6H_{14} - MeOH (30: 10: 3: 2). For a typical run, 0.7 g of crude steffimycin C was dissolved in a minimum volume of mobile phase and the solution was charged onto the column which was subsequently eluted with the above mobile phase at a linear velocity of 1 cm/minute. Eleven fractions (10 ml each) were collected on the basis of color banding. Using TLC analysis, those fractions containing steffimycin C with a minimum of steffimycin B contaminant were pooled. Evaporation of the solvent from the pool resulted in the isolation of 0.3 g of steffimycin C whose purity, by TLC, was estimated as >98%. This material was suitable for spectroscopic characterization but to verify the antibacterial behavior of steffimycin C, a small amount of the purified material was chromatographed on a Whatman high performance TLC plate using the above mobile phase. After drying the plate, the silica gel containing only the steffimycin C component was removed from the plate and extracted with MeOH. Approximately 5 mg of steffimycin C containing no steffimycin B was obtained upon removal of the MeOH from the eluate.

Spectroscopy

UV spectra were recorded using a Hewlett-Packard 8450A spectrophotometer. IR spectra were obtained using a Perkin Elmer 298 spectrophotometer. ^1H NMR spectra were obtained in DMSO referenced to TMS using a Varian XL 200 instrument while ^{13}C NMR were run in DMSO or $CDCl_3$ referenced to TMS using a Varian CFT 20 spectrometer.

Results and Discussion

For some time purified samples of steffimycin B have contained trace quantities of an impurity which from its color and Rf on TLC plates indicated a probable relationship to the steffimycin family. Normally this contaminant did not present problems in various biological testing programs but when it was discovered that mother liquors from steffimycin B crystallizations contained significant amounts of this unknown component, it was decided to attempt its isolation, structural characterization and biological evaluation.

The Nujol mull IR spectrum of steffimycin C is given in Fig. 1 and it is characterized by the absence of the strong 1750 cm^{-1}/carbonyl band found in the spectrum of steffimycin B. A significant change in the 200~300 nm region of the UV spectrum of steffimycin C compared with steffimycin B, Fig. 2, was also observed. The absorption maxima and log ε values for steffimycin C appear in Table 1. These spectral data are consistent with the alteration or loss of a carbonyl group in the steffimycin B structure resulting in steffimycin C.

The ^{13}C NMR spectrum of steffimycin C in DMSO referenced to TMS is given in Fig. 3. Off resonance experiments allowed determination of line multiplicities and led to the correlation diagram shown in Fig. 4. Line positions and multiplicities for both compounds are given in Table 2.

These data indicate a 1 to 1 correspondence of the 28 carbons in steffimycin C with similar carbons in steffimycin B and support the absence or alteration of the C-10 carbonyl in steffimycin C. The

Fig. 1. IR spectrum (Nujol mull) of steffimycin C.

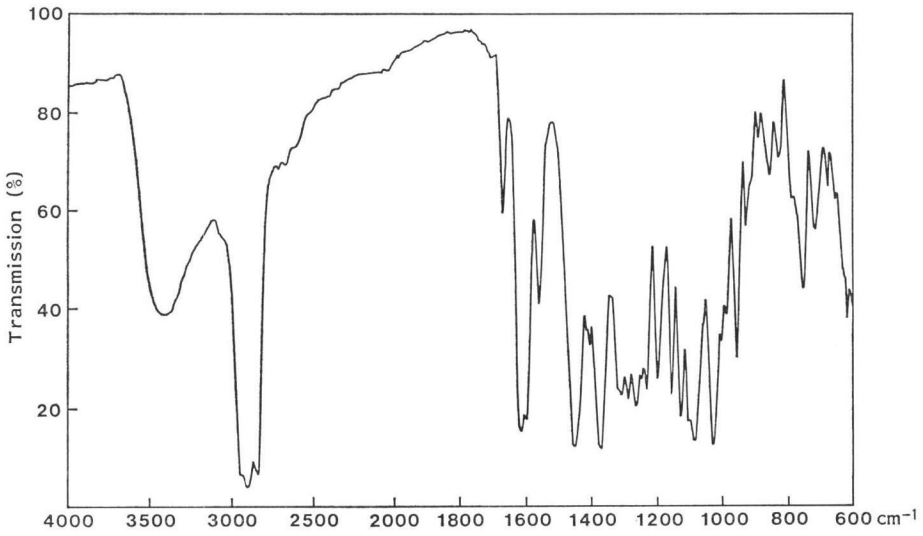
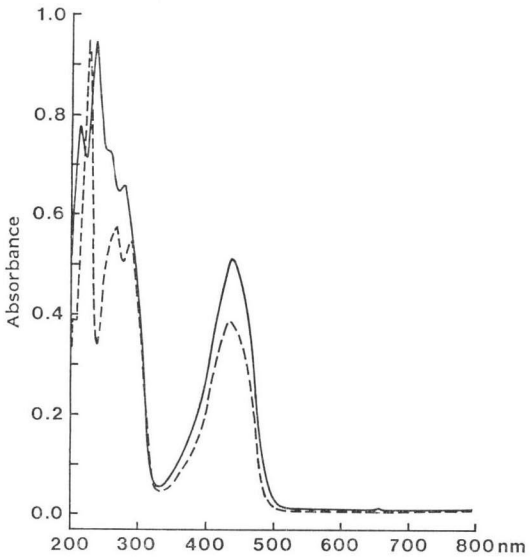
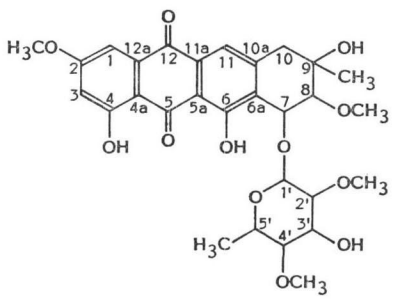


Fig. 2. UV spectrum of steffimycin C (---) and steffimycin B (—).

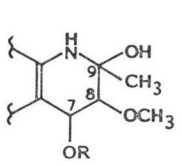




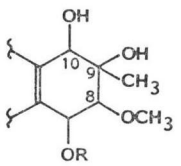
VI

Table 1. UV spectrum of steffimycin C.

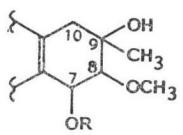
λ_{max}^{MeOH} (nm)	log ε
225	4.56
266	4.30
287	4.25
434	4.05



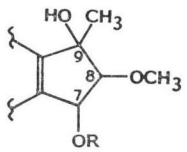
IV



V



VI



VII

absence of extra lines in appropriate regions of the ^{13}C NMR spectrum of steffimycin C preclude structures **IV** and **V**. These consideration left structures **VI** and **VII** as the only reasonable possibilities.

To aid in the resolution of the structure, a 200 MHz ^{1}H NMR spectrum of steffimycin C in

Fig. 3. ^{13}C NMR spectrum of steffimycin C (DMSO ref TMS).

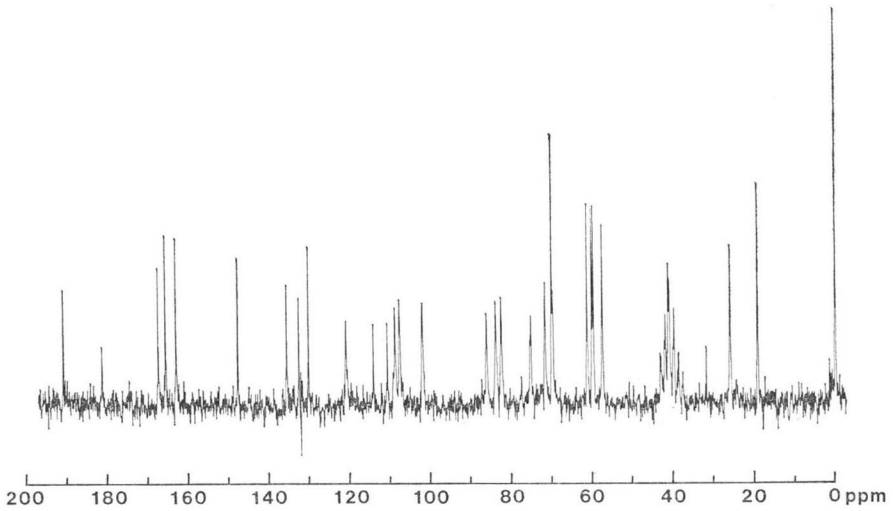
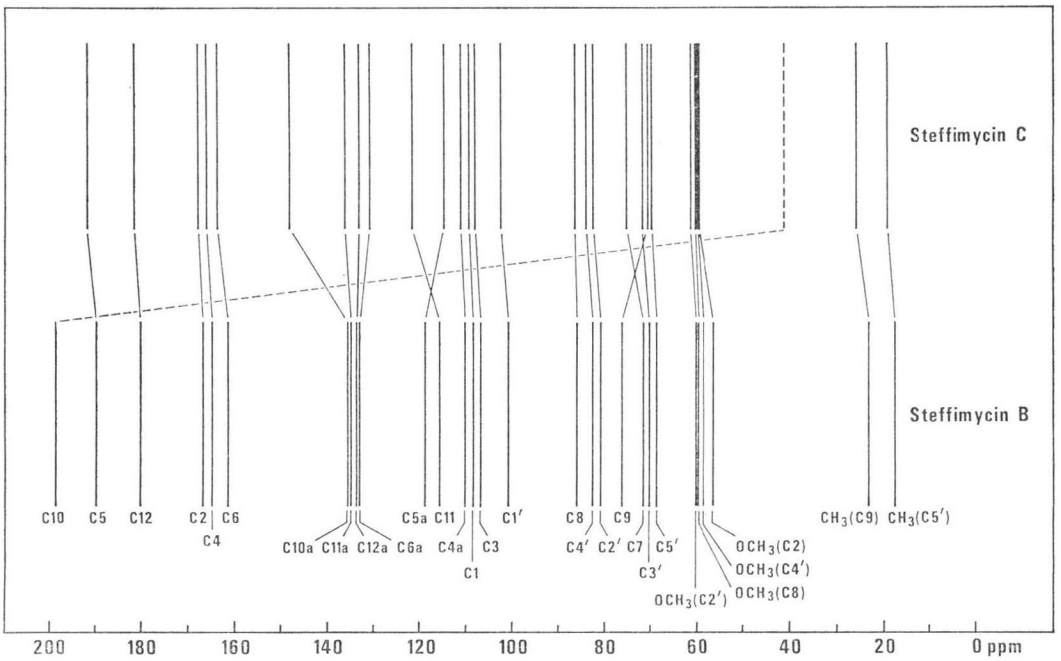


Fig. 4. ^{13}C NMR correlation diagram for steffimycin C and steffimycin B.



DMSO was obtained. A portion of the spectrum appears in Fig. 5. The salient feature of this partial spectrum is the presence of an AB pair of protons with a coupling $J_{AB}=17.5$ Hz. Analysis of this region of the ^{1}H NMR spectrum revealed that a 29th carbon atom in the steffimycin C structure exists as an isolated CH_2 group. Its chemical shift in the ^{13}C NMR spectrum would cause it to be masked by the central DMSO bands. A ^{13}C NMR spectrum of steffimycin C in $CDCl_3$ is shown in Fig. 6. The appearance of the strong line at 42.1 ppm supports structure **VI** for steffimycin C.

Table 2. ^{13}C NMR chemical shifts[a] for steffimycin C and steffimycin B.

	Steffimycin C	Steffimycin B
C-10	(42.1)[b]	198.4 s
C-5	191.2 s	189.5 s
C-12	181.2 s	180.0 s
C-2	167.6 s	166.6 s
C-4	165.8 s	164.7 s
C-6	163.3 s	161.2 s
C-10a	147.9 s	135.3 s
C-11a	135.9 s	134.6 s
C-12a	132.8 s	133.1 s
C-6a	130.4 s	132.9 s
C-5a	114.4 s	118.4 s
C-11	121.2 d	115.3 d
C-4a	110.9 s	110.0 s
C-1	109.1 d	108.1 d
C-3	107.9 d	106.6 d
C-1′	102.1 d	100.7 d
C-8	86.3 d	85.9 d
C-4′	84.0 d	82.3 d
C-2′	82.7 d	80.9 d
C-7	75.3 d	71.6 d
C-3′	71.8 d	70.2 d
C-9	70.2 s	76.1 s
C-5′	70.0 d	68.7 d
CH_3O (C-2′)	61.4 q	59.9 q
CH_3O (C-8)	60.3 q	59.7 q
CH_3O (C-4′)	60.0 q	58.5 q
CH_3O (C-2)	59.9 q	56.4 q
CH_3 (C-9)	26.1 q	23.3 q
CH_3 (C-5′)	19.4 q	17.8 q

[a] ppm relative to TMS in DMSO.
[b] Observed only in $CDCl_3$: ppm relative to TMS.

Table 3. Specific activity[a] (biounits/mg) of steffimycins B and C.

Regression line statistics	B	C
Slope	3.5	3.3
Intercept	17.7	10.4
Syx	0.47	0.00
r^2	0.94	1.00
Biounits/mg	89	0.13

[a] *Micrococcus luteus* UC 130.

Table 4. Minimum inhibitory concentrations of steffimycins B and C.

Organism	MIC (μg/ml)	
	C	B
Staphylococcus aureus UC 76	>100	4
S. aureus UC 6685	>100	
S. aureus UC 6690	>100	
S. faecalis UC 694	>100	8
Streptococcus pyogenes UC 152	>100	
S. pneumoniae UC 41	1.56	1
Escherichia coli UC 45	>100	>500
Klebsiella pneumoniae UC 56	>100	>500
Salmonella schottmuelleri UC 126	>100	
Pseudomonas aeruginosa UC 95	>100	>500

Test method; agar dilution pH 7.4.

Steffimycin C gave an elemental analysis (found: C 59.95, H 6.12, O 33.93 by difference) suggesting the molecular formula $C_{29}H_{34}O_{12}$ (calcd: C 60.62, H 5.96, O 33.41) which has a ring/double bond number of 13. These data are consistent with structure VI for steffimycin C.

The difference in specific activity of the two antibiotics was measured using the biounit potency assay[5]. One mg/ml CH_2Cl_2 solutions of the antibiotics were serially diluted 1 to 2 down to a dilution of 1/128 and 80 μl of each solution was applied to 12.5 mm paper discs. After drying, the discs were applied to an agar tray seeded with *Micrococcus luteus* UC 130. The trays were incubated for 16 hours at 32°C and the resulting zones of antibiotics were read to the nearest mm. These data were subjected to regression analysis using a Hewlett Packard model 85A microcomputer. The dilutions necessary to give a 20-mm zone of inhibition were interpolated from the regression lines and converted to biounits, Table 3.

Examination of the *in vitro* antibacterial activity of steffimycin C reveals that it does not retain the activity of steffimycin B except for one organism *Streptococcus pneumoniae* UC 41, see Table 4.

Steffimycin C is the first member of the steffimycin family of antibiotics, isolated from fermentations, in which the C-10 carbon lacks a carbonyl oxygen. WILEY *et al.*[6] reported that in the biosynthesis of the steffimycins the aglycone is constructed from a polyketide intermediate derived from acetate. In this case the C-10 carbon in the steffimycins originates from the C-2 carbon of CH_3COONa

Fig. 5. ^{1}H NMR spectrum of steffimycin C (DMSO ref TMS).

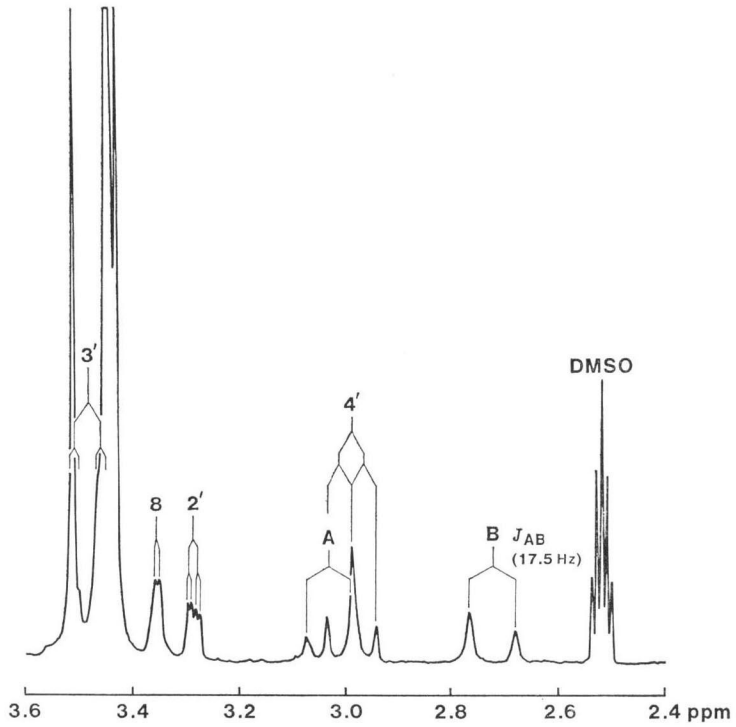
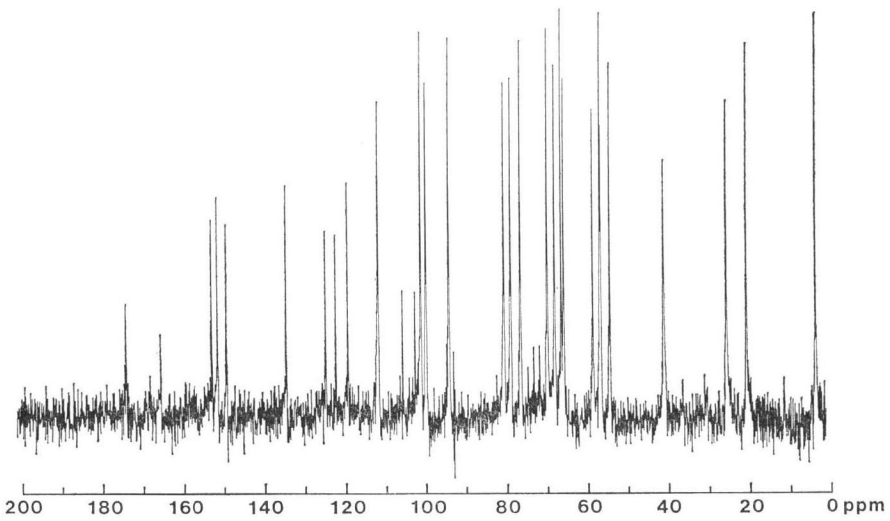


Fig. 6. ^{13}C NMR spectrum of steffimycin C ($CDCl_3$ ref TMS).



and it would seem that the C-10 carbon should be present as a methylene group. MARSHALL and WILEY[7] have demonstrated that *Chaetomium* and *Actinoplanes utahensis* both convert steffimycin B to 10-dihydrosteffimycin B. Although the reduction product 10-deoxysteffimycin B was not isolated, this study demonstrated that certain organisms have the ability to reduce the C-10 carbonyl. Whether 10-deoxysteffimycin B represents the precursor to the structure containing a C-10 carbonyl as might

be inferred from the biosynthetic studies or a metabolic reduction product as occurred in the bioconversion experiments is still in question.

Acknowledgments

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