STEFFIMYCIN B, A NEW MEMBER OF THE STEFFIMYCIN FAMILY : ISOLATION AND CHARACTERIZATION

T. F. BRODASKY and F. REUSSER

Research Laboratories, The Upjohn Company, Kalamazoo, Michigan 49001, U.S.A.

(Received for publication August 13, 1974)

Steffimycin B, an anthracycline, has been isolated from cultures of *Streptomyces elgreteus* by solvent extraction and purified by silica gel chromatography. NMR and mass spectroscopy indicate the presence of a methoxyl group in the sugar moiety of steffimycin B not found in steffimycin, a related antibiotic. Steffimycin B is not active against bacteria in experimentally infected animals, but has shown some potential antitumor activity in an *in vitro* screen. Further testing is in progress.

In 1967 BERGY & REUSSER[1] reported the discovery of a new antibacterial agent, steffimycin (U-20661), isolated from *Streptomyces steffisburgensis*. In the present paper we describe the isolation of a second member of this family, steffimycin B. Steffimycin B was isolated from a new species of streptomyces designated *Streptomyces elgreteus*,* which also produces steffimycin. In addition to fermentation, isolation and biological properties, we will report on physical chemical data which differentiates steffimycin B from steffimycin. Although steffimycin B has not demonstrated *in vivo* antibacterial activity in experimentally infected animals, it was active in inhibiting the growth of L-1210 mouse leukemia cells *in vitro*. Further antitumor testing is now in progress.

Experimental

Fermentation

All fermentations were conducted under submerged culture conditions in 500 ml Erlenmeyer flasks containing 100 ml of culture medium. Seed cultures were prepared in a medium containing 25 g/liter glucose monohydrate and 25 g/liter pharmamedia (Trader's Oil Mill Co., Fort Worth, Texas). The pH was adjusted to 7.2 prior to sterilization. The seed flasks were inoculated with a loop of soil stock prepared as described by REUSSER[2] and incubated at 28°C for 3 days on a rotary shaker (250 rpm, 6-cm stroke). The fermentation medium contained 25 g/liter Blackstrap molasses (Knappen Milling Co., Augusta, Michigan), 25 g/liter yellow corn meal (Wilson Corn Products, Inc., Rochester, Indiana) and 15 g/liter pharmamedia. The fermentation flasks were seeded with 5 ml aliquots of seed and were incubated at 28°C on a rotary shaker as described above. Peak antibiotic titers were usually obtained after 3~4 days of incubation.

Steffimycin B titers were determined by a microbiological disc-plate assay[2] with *Sarcina lutea* (UC 130) as the test organism. Samples for assay were diluted with 0.1 M phosphate buffer (pH 7.4).

Isolation and Purification

Five percent (w/v) of Dicalite was added to the fermentation broth which was then filtered. The clear filtrate was extracted twice with equal volumes of methylene chloride and the combined extracts were evaporated to dryness at 40°C under vacuum. The antibacterial potencies of the clear broth, spent broth and extracts were determined by disc-plate assay on *S. lutea*-

* The organism has been studied and classified by Ms. A. DIETZ of The Upjohn Company and has been designated UC 5453 in the Upjohn Culture Collection.

seeded agar. Extracts were examined by TLC on silica gel using the mobile phase, methylene chloride* 30 parts, acetone 10 parts, hexane 3 parts and methanol 2 parts. In this system steffimycin B has an R_f of 0.4~0.5.

Methylene chloride extracts of steffimycin B were purified by chromatography on silica gel H (0.2~0.5 mm Merck, Darmstadt) using the mobile phase described above. The column was packed with 50~100 g of silica gel H per gram of extract to be processed and charged with a solution of the extracted solids in a minimum volume of CH_2Cl_2. During elution, several colored bands developed. Fractions containing steffimycin B, a deep orange band, were collected so that none of the steffimycin, a red-orange band, was included. These fractions were pooled and analyzed for bioactivity and antibiotic composition by TLC.

The sodium salt of steffimycin B was prepared by titrating a methanolic solution of the compound with methanolic NaOH until no further color change was observed. On evaporation of the solvent a violet-colored solid was collected. NMR and IR spectra were consistant with spectra of steffimycin B.

Results and Discussion

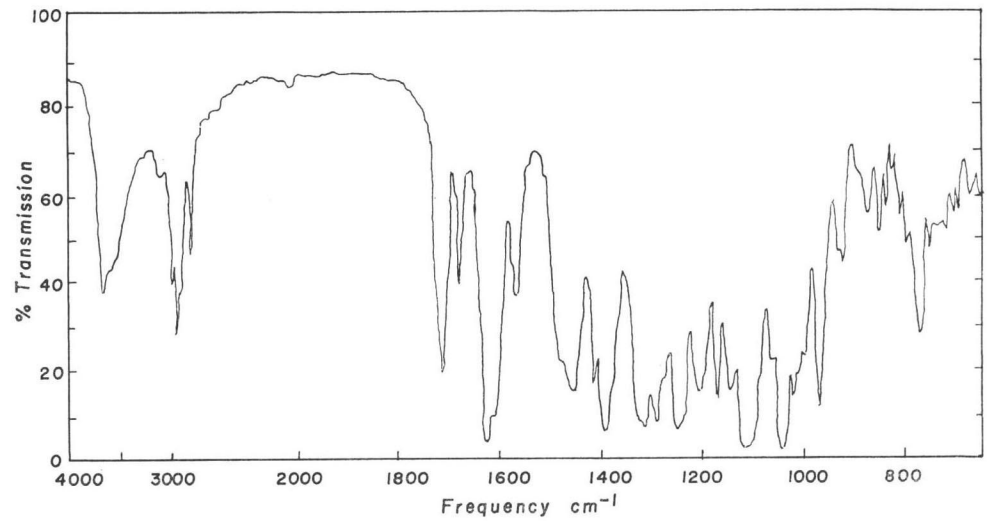
Chemical Characterization

Crystalline steffimycin B, $C_{29}H_{32}O_{13}$ (Calc. element percents C, 59.18; H, 5.48; O, 35.34; found, C, 58.68; H, 5.58; O, 35.74, by diff.), is orange and has a molecular weight of 588 by mass spectroscopy (MS). Its specific rotation is $[\alpha]_D^{25}$ +94 (*c* 1, $CHCl_3$) and it melts at 240~246°C. Acidic methanol solutions of steffimycin B are intensely orange but change abruptly to violet on addition of base. The UV maxima and log ε values are given in Table 1, while the IR and paper chromatographic pattern in seven mobile phases are

Table 1. UV Spectrum of stiffimycin B

	Wavelength	Log ε
In ethanol	λ max. at 234 nm	4.44
	sh at 254 nm	4.34
	sh at 274 nm	4.29
In 0.1 N ethanolic KOH	λ max. at 233 nm	4.50
	sh at 259 nm	4.42
In 0.1 N ethanolic HCl	λ max. at 234 nm	4.44
	sh at 254 nm	4.33
	sh at 274 nm	4.29

Fig. 1. IR (KBr pellet) of steffimycin B



* All solvents were Burdick & Jackson distilled in glass (Burdick & Jackson, Muskegon, Michigan).

given in Figs. 1 and 2, respectively. All of these data indicate that this compound is related to steffimycin (**I**, Fig. 3) the structure of which was proposed by Dr. R. C. KELLY of The Upjohn Company based on UV, IR and NMR data as well as degradation studies.* Steffimycin B has a higher R_f value than steffimycin in chromatographic system 7 (Fig. 4), indicating the less polar nature of steffimycin B. In addition to paper chromatography the two substances were easily separated using TLC or GLC (of TMS derivatives), Figs. 5 and 6.

The proton NMR spectra, Fig. 7, of the two materials were indistinguishable except for the broadening of the aliphatic (-OMe) at 3.6 ppm. C^{13} NMR spectra of steffimycin B reveal the presence of four (—OMe) groups (one aromatic and three aliphatic tentatively assigned at δ 56.1, 58.7, 60.0 and 60.7 ppm relative to TMS).

The mass spectra of steffimycin and steffimycin B show a 14 AMU difference in the molecular ion (574 and 588, respectively). This was consistent with the additional (—OMe)

Fig. 2. Paper chromatographic pattern of steffimycin B

1. 1-Butanol - water (84:16) developed for 16 hours
2. 1-Butanol - water (84:16) plus 0.25% *p*-toluene-sulfonic acid, developed for 16 hours
3. 1-Butanol - acetic acid - water (2:1:1), developed for 16 hours
4. 1-Butanol - water (84:16) plus 2% piperidine, developed for 16 hours
5. 1-Butanol - water (4:96), developed for 5 hours
6. 1-Butanol - water (4:96), plus 0.25% *p*-toluene-sulfonic acid, developed for 5 hours
7. Benzene - methanol - water (1:1:2), paper strip was equilibrated at 25°C in vapor from the mixed solvent and developed for 5 hours with upper phase

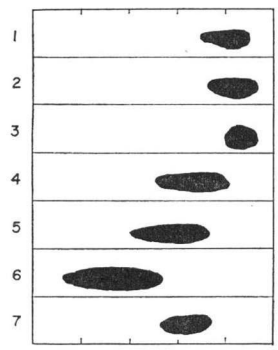
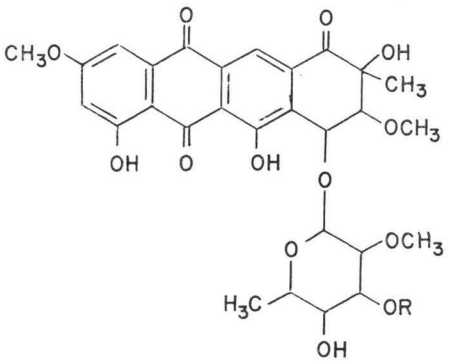


Fig. 3



I R=H (Steffimycin)
II R=CH_3 (Steffimycin B)

Fig. 4. Paper chromatogram of a mixture of steffimycin and steffimycin B in mobile phase 7 (see Fig. 2)

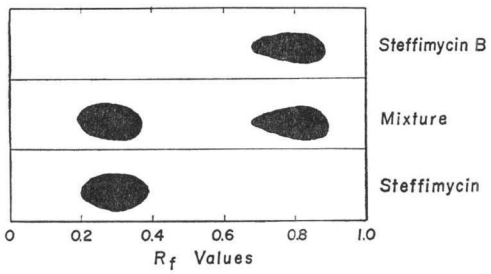
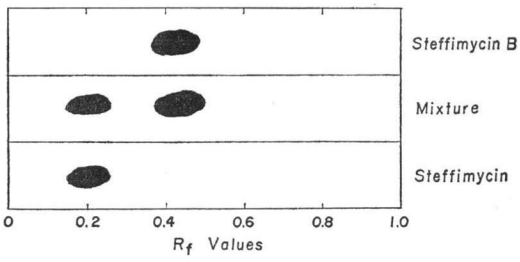


Fig. 5. TLC of a mixture of steffimycin and steffimycin B: Mobile phase methylene chloride 30 parts, acetone 10 parts, hexane 3 parts and methanol 2 parts



* Personal communication.

group indicated by NMR. Although low resolution mass spectra of underivatized steffimycin B gave low intensity peaks, it was possible to conclude that the aglycone of both steffimycin B and steffimycin were identical (*m/e* 414) and that the difference between the two molecules must therefore reside in the sugar moiety. An analysis of mass spectra indicated that the sugar in steffimycin gave a 161 *m/e* peak but the sugar in steffimycin B gave a 175 *m/e* peak or one CH_2 group higher in mass. It remained then to determine the site of this group.

High resolution mass spectroscopy verified that the aglycone of steffimycin B had a molecular ion of 414 and in addition a fragmentation pattern for the sugar moiety in which the ion **I** (Fig. 8) was observed. This, together with the other data presented, lead us to propose structure **II** (Fig. 3) for steffimycin B.

Fig. 6. GLC of a mixture of TMS ethers of (A) steffimycin B, (B) steffimycin; column-4′ glass, 3.8% UCW 98 on diatoport S at 242°C isothermal monitored with FID (Flame Ionization Detector)

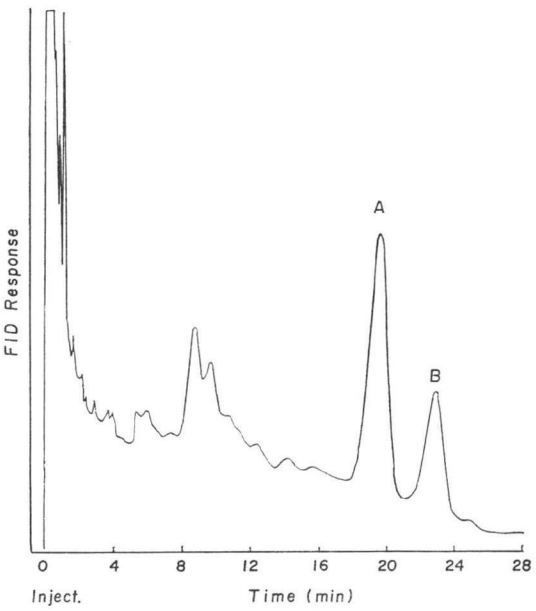
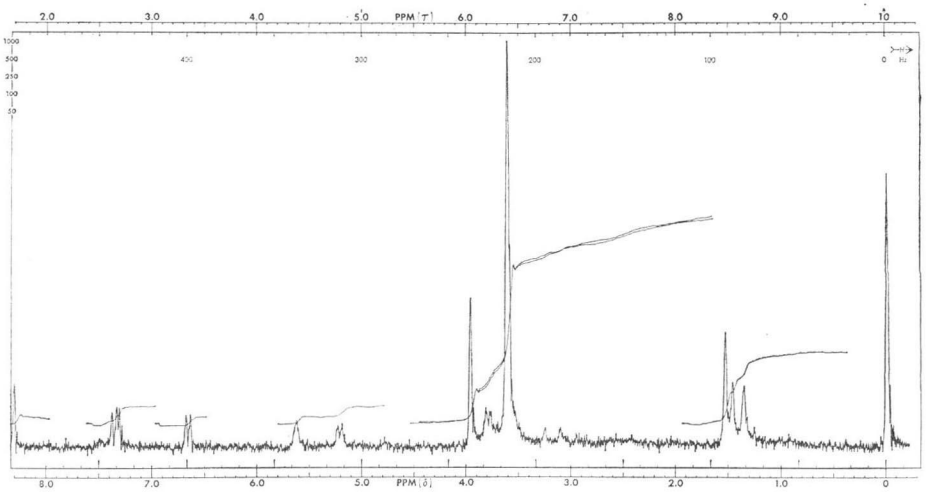


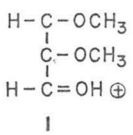
Fig. 7. Proton NMR spectrum of steffimycin B 60 MHz in $CDCl_3$ relative to TMS



Biological Characterization

Steffimycin B inhibits only gram-positive bacteria when assayed in a two-fold broth dilution test (Table 2). Mice experimentally infected with *Diplococcus pneumoniae* were not protected when steffimycin B was ad-

Fig. 8.



I

ministered subcutaneously at a dose of 400 mg/kg. No toxicity was noted at this level. The *in vitro* and *in vivo* antibacterial activities were determined by the methods described by LEWIS *et al.*[4]

Steffimycin B significantly inhibits the growth of L-1210 mouse leukemia cells in culture, giving an ID_{50} of 0.6 mcg/ml as determined by the method of BUSKIRK[5]. None of fourteen fungi pathogenic for humans was inhibited by steffimycin B. Antifungal tests were carried out on agar plates. The antibiotic was dissolved in nutrient agar to give concentrations of 1,000, 100, 10 or 1 mcg/ml.

Table 2. *In vitro* antibacterial activity of steffimycin B

Test organism	Minimal inhibitory concentration* (mcg/ml)
Staphylococcus aureus UC-80	4
Streptococcus hemolyticus UC-152	8
Streptococcus faecalis UC-3235	8
Escherichia coli UC-51	>500
Proteus vulgaris UC-93	>500
Klebsiella pneumoniae UC-57	>500
Pseudomonas aeruginosa UC-95	>500
Diplococcus pneumoniae UC-41	1

* Twofold dilution endpoints in brain heart infusion broth. Read after 20 hours of incubation.

Steffimycin B and steffimycin are very similar in both chemical and biological properties. Neither compound has shown any *in vivo* activity in tests run to date and it was postulated that the lack of *in vivo* activity might be in part due to their low solubility in protophilic solvents, particularly aqueous systems. The sodium salt of steffimycin B was prepared in an attempt to overcome this problem. Although the salt was very water soluble, it displayed less *in vitro* activity than did the parent compound and no *in vivo* activity in mice infected with gram-positive organisms.

Acknowledgements

The authors acknowledge gratefully Dr. L. BACZYNSKYJ and Mr. R. WNUK for assistance with the mass spectra, Dr. G. SLOMP and Mr. S. MIZSAK for assistance with NMR spectra, Mr. C. LEWIS for *in vivo* studies, Mr. H. BUSKIRK for performing the cell culture assays, and Ms. A. DIETZ for antifungal tests. We also thank Messrs. B. CZUK and J. KAY for their technical assistance with the fermentation, isolation and purification of steffimycin B.

References

1) BERGY, M. E. & REUSSER, F.: A new antibacterial agent (UC-20,661) isolated from a streptomycete strain. Experientia 23: 254~255, 1967.
2) REUSSER, F.: Stability and degeneration of microbial cultures on repeated transfer. Advances in Applied Microbiology 5: 189~215, 1963
3) HANKA, L. J.; M. R. BURCH & W. T. SOKOLSKI: Psicofuranine. IV. Microbiological assay. Antibiot. & Chemoth. 9: 932~935, 1959.
4) LEWIS, C.; H. W. CLAPP & J. E. GRADY: *In vitro* and *in vivo* evaluation of lincomycin, a new antibiotic. Antimicr. Agents & Chemoth. -1962: 570~582, 1963.
5) BUSKIRK, H. H.: Assay of cytotoxic agents with L-1210 cells. Proc. Tissue Cult. Assoc. 20: 23, 1969.