THE STRUCTURE OF SHOWDOMYCIN, A NOVEL CARBON-LINKED NUCLEOSIDE ANTIBIOTIC RELATED TO URIDINE*

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Communicated by Lyman C. Craig, January 5, 1966

Showdomycin was first isolated from *Streptomyces showdoensis* by Nishimura and co-workers.[1] It is a white crystalline antibotic of empirical formula $C_9H_{11}NO_6$ which is active against both gram-positive and gram-negative bacteria and is especially active against *Streptococcus hemolyticus*[1] and *Streptococcus pyogenes*.[2] Showdomycin possesses significant antitumor activity against Ehrlich mouse ascites tumor *in vivo* and is active against cultured HeLa cells.[3] It has a melting point of 153–154°C, a specific rotation of $[\alpha]_D^{22.5}$ + 49.9° (C = 1, H_2O), and a major ultraviolet absorption maximum of 220 mμ in aqueous solution.[1] Antibiotic MSD-125A was obtained[4] from Merck, Sharp and Dohme Research Laboratories, and a rigorous comparison of physical data indicated MSD-125A to be identical to showdomycin.

The ultraviolet absorption spectrum of showdomycin[1] was suggestive of a maleimide-type structure. Treatment of showdomycin with dilute aqueous ammonia produced an absorption maximum at 328 mμ which rapidly disappeared upon further exposure to base. Similar behavior was exhibited with maleimide and is interpreted as a removal of the —NH proton by the base followed by basic hydrolysis to give ring opening. Table 1 illustrates the effect of dilute aqueous ammonia on showdomycin and certain maleimides. Inspection of Table 1 indicates the similarity of maleimide and showdomycin and strongly suggests the presence of hydrogen on the nitrogen atom.

Determination of the pK_a by the potentiometric method gave a value of 9.29 ± 0.04 for showdomycin. Similarly, determination of the pK_a of maleimide by the same procedure[5] gave a value of 9.46 ± 0.03. These data are again strong support for the imide-type structure and the presence of an acidic "NH"-type proton.

The infrared spectrum of showdomycin[1] exhibits a very strong carbonyl band at 1704 cm^{-1} which is very similar to that exhibited by maleimide at 1704 cm^{-1}. Showdomycin was hydrogenated using palladium on carbon catalyst and was found to consume 1.1 moles of hydrogen in ten minutes with loss of ultraviolet absorption. Maleimide under similar conditions absorbed 1.0 mole of hydrogen in six minutes with similar loss of ultraviolet absorption.

Examination of the proton magnetic resonance spectra of showdomycin revealed a number of interesting features.

(1) In dry deuterated dimethylsulfoxide-d_6 a definite single absorption peak (one proton) was noted at 10.78δ, typical of the "NH" proton of a cyclic amide. A sharp doublet (one proton) was observed at 6.74δ, typical of an aromatic or vinylic proton. The remaining nine protons were observed as multiplets in the 3.2–5.34δ region.

(2) When showdomycin was examined in deuterium oxide in the presence of deuterated acetic acid-d_4 (Fig. 1), the "NH" proton at 10.78δ was absent due to deuterium exchange. Similarly, three protons previously found in the 3.2–5.3δ

TABLE 1

EFFECT OF AQUEOUS AMMONIA ON ULTRAVIOLET ABSORPTION SPECTRA OF SHOWDOMYCIN AND CERTAIN MALEIMIDES

Compound	$\lambda_{max}^{H_2O}$ ($n \rightarrow \pi^*$)	λ_{max}^{base} ($n \rightarrow \pi^*$)	Time required for λ_{max} to disappear (min)
Showdomycin	275	328	5
Maleimide	275	326	5
N-Ethylmaleimide	300	300	<1

A small sample of each of the compounds was dissolved in water, and the absorption maximum due to the $n \rightarrow \pi^*$ transition in the 300-mμ region was noted. Several drops of conc. aqueous ammonia were added, the λ_{max} was measured immediately, and the approximate time required for its disappearance observed.

region had also exchanged with deuterium. Thus showdomycin has four exchangeable protons.

These data, the optical rotation, and empirical formula $C_9H_{11}NO_6$ suggested the presence of a carbohydrate moiety. The presence of a carbohydrate attached to maleimide could account for the slight increased acidity due to the electronegative effect of the sugar. Attempts to establish the presence of a sugar via acid hydrolysis were unsuccessful. Thus a possible carbon-carbon glycosidic bond was suggested since the nitrogen had been shown to be unsubstituted. Showdomycin was then treated with aqueous hydrazine at 100°. This procedure has been employed by Davis and Allen[6] for the detection of D-ribose in pseudouridine. Following this treatment of showdomycin with hydrazine, D-ribose was detected on paper chromatograms by the use of aniline phthalate spray (see *Experimental*). The establishment of the carbohydrate moiety as D-ribose was of considerable assistance in further structure elucidation. Periodate titration indicated the consumption of 1 mole of periodate which established the furanose configuration. Examination of the pmr spectra of showdomycin in deuterium oxide in the presence of deuteroacetic acid-d_4 revealed the anomeric proton centered at 4.82δ (Fig. 1). This proton (Hb) is split into a doublet by the proton at the 2′ carbon (Hc) which is further split into two doublets by the vinylic proton Ha. The coupling constant between Ha and Hb is 1.5 cps. The position of this anomeric proton is deciding proof of the attachment of the D-ribose moiety on carbon. Pseudouridine under similar conditions (Fig. 2) exhibits the anomeric proton at 4.72δ as compared to 6.0δ for the anomeric proton of uridine in the same solvent. Other naturally occurring carbon-substituted ribonucleoside antibiotics are formycin[7] and laurusin.[7] Formycin (7-amino-3-β-D-ribofuranosylpyrazolo[4,3-d]pyrimidine) exhibits the anomeric proton (Hb) at 5.14δ in deuterated dimethyl sulfoxide-d_6[7] and at 5.37δ in deuterium oxide and deuteroacetic acid-d_4 (Fig. 3).

The structure proposed for showdomycin based on present studies is 3-β-D-ribofuranosylmaleimide (I). Hydrogenation of showdomycin resulted in the loss of

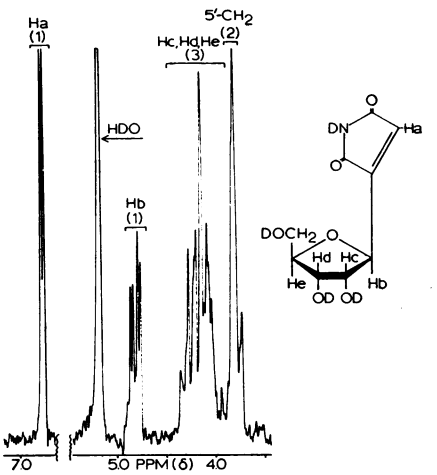

FIG. 1.—Showdomycin. Solvent: D_2O-D_3CCOOD. Int. std.: DSS.

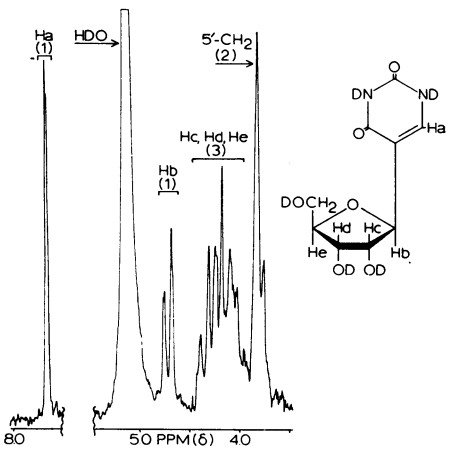

FIG. 2.—Pseudouridine. Solvent: D_2O-D_3CCOOD. Int. std.: DSS.

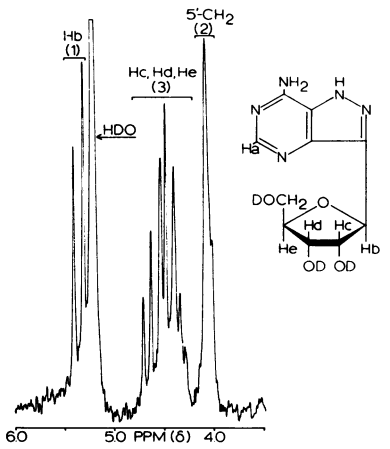

FIG. 3.—Formycin. Solvent: D_2O-D_3CCOOD. Int. std.: DSS.

ultraviolet absorption and loss of the vinylic proton at 6.8δ. The anomeric proton shifted 0.32δ upfield with a change in coupling constant from J = 1.5 to J = 2.5 cps. The hydrogenated product also showed the appearance of new multiplets (three protons) in the region 2.7–3.5δ.

Assignment of the configuration as β is made since a study of the pmr spectrum of the $C_{3'}$, $C_{4'}$, and $C_{5'}$ proton region, 3.7–4.8δ, reveals that this region shows an absorption pattern virtually identical to that for pseudouridine (Fig. 2) and formycin (Fig. 3). The pmr spectrum of the α-anomer of pseudouridine[8] (pseudouridine B) shows the anomeric proton 0.33 ppm δ downfield from that of the β-isomer. The $C_{3'}$, $C_{4'}$, $C_{5'}$ region, 3.7–4.8δ, of the pmr spectrum of the α-anomer of pseudouridine is distinctly different from that of the β-derivative, showing a large doublet at 4.38δ with very little absorption in the area of 4.2δ. These data provide strong support for the assignment of the β-configuration.

Showdomycin has recently been converted to the mono-, di-, and triphosphates by enzymatic means, utilizing a high-speed supernatant fraction from Ehrlich ascites cells.[9]

Although pseudouridine gives a red-brown color with the orcinol reagent,[8] both showdomycin and formycin give negative tests with this reagent. Chambers[10] suggests that pseudouridine possesses considerable carbonium ion character which is probably responsible for the positive orcinol reaction and for the isomerization of pseudouridine in acid solution. Such isomerization has not been observed for formycin or showdomycin and suggests greater stability of the C-glycosidic bond.

Comparison of the structure of showdomycin with that of uridine (Fig. 4) reveals considerable similarity. Indeed, Stuart models show a similar stereochemical relationship of the base to the sugar in each case, since maleimide is a planar molecule. Showdomycin bears a similar structural relationship to pseudouridine, and can be viewed as pseudouridine which has lost an —NH group in the contraction to a five-membered ring. Further similarity is noted by a comparison of pK_a values. The pK_a of showdomycin is 9.29 compared with 9.17 for uridine[11] and 9.1 for pseudouridine.[10]

The biosynthesis and function of pseudouridine remains obscure. The direct incorporation of pseudouridine into RNA apparently does not occur *in vivo*,[12] but pseudouridine would appear to arise by an intramolecular rearrangement of uridine at the polynucleotide level.

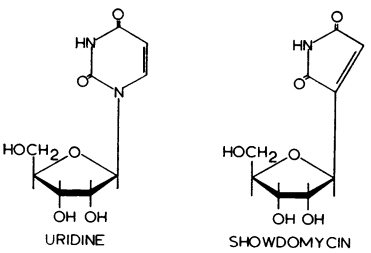

FIG. 4.

Of no small interest is the fact that showdomycin is a nucleoside derivative of maleimide. Maleimide derivatives have been rather extensively used as alkylating agents for the sulfhydryl group[13–16] or the amino group[17] in various proteins. Thus the presence of the β-D-ribosyl moiety might well convey desirable specificity to the maleimide molecule in these reactions. Indeed, showdomycin may well prove to be an exciting tool in the study of various enzymes concerned with nucleoside and nucleotide biochemistry.

Experimental.—All melting points were determined with a Thomas Hoover melting-point apparatus and are uncorrected. Pmr spectra were determined on a Varian A-60 spectrometer and all samples run in DMSO-d_6, D_2O, or a mixture of D_2O and acetic acid-d_4 which contained 1% sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard. Ultraviolet spectra were obtained on a Beckman DK-2 spectrophotometer. The infrared spectra were determined on a Beckman IR-5A spectrophotometer using pressed KBr pellets. Plates with an adsorbent layer of 0.35 mm thickness were used for thin-layer chromatography. Showdomycin (MSD-125A) was purified by recrystallization from anhydrous acetone.

Reaction of showdomycin with periodate: To a 250-ml erlenmeyer flask was added a 45-mg sample of showdomycin. The solid was dissolved in 5 ml of water. A 5-ml aliquot of 0.05 *M* periodic acid (prepared by dissolving 10.7 gm of $NaIO_4$ in water, adding 0.2 ml of conc. H_2SO_4, and diluting the solution to 100 ml) was added. The solution was stoppered, and after 1 hr, 20 ml of water and 1–2 drops of methyl red were added and the solution was neutralized with 0.05 *N* NaOH. One ml of saturated potassium iodide solution and 2.5 ml of 15% H_2SO_4 were added and the solution was titrated immediately with 0.0943 *N* $Na_2S_2O_3$ solution using Paragon as an indicator.

Three blank determinations were made using all the reagents as described above, giving an average value for v_{blank} of 20.98 ml. The sample size was selected such that it would consume 0.8 as much $Na_2S_2O_3$ as the blank. The number of moles periodate consumed was calculated from the following formula:

$$N_{Na_2S_2O_3}(v_{blank} - v_{sample}) = 2 \text{ (moles of sample) (moles } IO_4^-).$$

A summary of the results is given in Table 2.

Treatment of showdomycin with hydrazine: A mixture of 2 mg of showdomycin, 0.2 ml of water, and 0.5 ml of hydrazine (95%) was heated at 100° for 5 hr. After cooling the solution, benzaldehyde (2–3 ml) was carefully added and the mixture extracted three times with ether. The aqueous solution was then used for paper chromatography. Ten μl of the solution and 1 μl of 1% aqueous solutions of the sugars were used. The chromatograms were sprayed with aniline hydrogen phthalate[18] and then heated for 5 min at 100°. (See Table 3.)

Hydrogenation of showdomycin: A stirred suspension of 5% Pd/C in 5 ml of absolute ethanol was treated with hydrogen gas at 690.5 mm of Hg at room temperature (29°) until there was no further uptake. A solution of 52.2 mg of showdomycin in 5 ml absolute ethanol was added. The mixture was kept under hydrogen with stirring for 40 min although hydrogen uptake of 1.1 mole

TABLE 2

SUMMARY OF PERIODATE TITRATION DATA

Showdomycin sample (mg)	v_{sample} (ml)	No. moles IO_4^- consumed
45.8	16.60	1.04
45.3	16.80	1.00

TABLE 3

SUMMARY OF R_f VALUES OF VARIOUS SUGARS AND PRODUCT OBTAINED FROM SHOWDOMYCIN

	Solvent system I: butanol–acetic acid–water (5:1:4) R_f	Solvent system II: ethyl acetate-pyridine-water (2:1:2) R_f
Showdomycin solution after hydrazine treatment	0.56	0.53
Ribose	0.56	0.53
Xylose	0.48	0.49
Arabinose	0.43	0.42

was complete after 10 min. The catalyst was removed by filtration and the filtrate concentrated to dryness *in vacuo*. The residue was dissolved in D_2O and determination of the pmr spectrum showed complete loss of vinyl proton and the appearance of a three-proton multiplet between 3.60 and 2.54δ. The ratio of the number of protons in this region to that in the 4.5–3.6δ region was 0.54 (calculated for 3,4-dihydroshowdomycin 0.50). An ultraviolet spectrum showed complete loss of absorption. Thin-layer chromatography on SilicAR 7GF developed with EtOH-EtOAc(1:3) showed two spots, a major one at R_f 0.7 and a minor spot at R_f 0.8 (showdomycin, R_f 0.9). A second run using 50.7 mg of showdomycin also consumed 1.1 mole of H_2. For comparison a sample of maleimide treated under identical conditions consumed 1 mole of H_2 in 6 min.

Purification of pseudouridine: A commercial sample of pseudouridine[19] mp 204–210° (d) was estimated by pmr spectra to consist of approximately 75% of the β anomer (pseudouridine C) and 25% of the α anomer (pseudouridine B). A 300-mg sample was dissolved in 60 ml of boiling methanol, filtered, and the filtrate chilled overnight at −15°. A trace of dark solid was removed by filtration and the filtrate slowly evaporated to a volume of about 10 ml. The white solid (110 mg, mp 220–224.5°) was collected and recrystallized from 95% ethanol to yield 90 mg of the pure β-anomer of pseudouridine (pseudouridine C) mp 224.5–226°, (lit. mp 220–221°,[8] 223–224°[20]).

Summary.—Showdomycin has been shown to be 3-β-D-ribofuranosylmaleimide (I), a carbon-substituted nucleoside antibiotic structurally related to uridine and pseudouridine.

* Supported by research grant CA 08109 and CA 08109-02 from the National Cancer Institute of the National Institutes of Health, USPHS.

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[4] The authors wish to thank Dr. Frank J. Wolf of the Merck, Sharp and Dohme Research Laboratories for a generous sample of MSD-125A.

[5] The authors wish to thank Edward M. Eyring for the pK_a's of showdomycin and maleimide which were determined in his laboratory.

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