1-METHYLPSEUDOURIDINE, A METABOLITE OF *STREPTOMYCES PLATENSIS*

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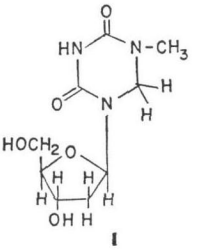
1-Methylpseudouridine is a new metabolite isolated from culture filtrates of *Streptomyces platensis*. The structure of this compound was determined from its physical and spectral properties.

Streptomyces platensis var. *clarensis* produces[1] antibiotic U-44590, an antibacterial and antiviral agent, the structure of which is represented by **I** (Fig. 1).[2]

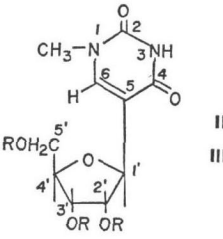
Fig. 1.



I

In the course of studies related to the production of nucleoside-like metabolites by *S. platensis* we isolated two carbon-carbon linked ribonucleosides identified as pseudouridine and 1-methylpseudouridine (**II**, Fig. 2). Pseudouridine has been isolated from several sources including culture filtrates of *Streptomyces* sp.[3,4,5]. 1-Methylpseudouridine has never been isolated and characterized chemically or biologically. The compound has been mentioned briefly in connection with work related to the structure of pseudouridine[6,7].

Fig. 2.



II: R = H
III: R = -COCH$_3$

The present paper discusses our studies which led to the isolation, characterization and determination of the structure of 1-methylpseudouridine.

Experimental

Spectroscopic Methods.

Proton magnetic resonance spectra were observed in D_2O (1-methylpseudouridine) or $CDCl_3$ (1-methylpseudouridine triacetate) on a Varian XL-100-15 spectrometer operating at 100 MHz. Carbon magnetic resonance spectra were observed in D_2O (1-methylpseudouridine) or $CDCl_3$ (1-methylpseudouridine triacetate) on a Varian XL-100-15 spectrometer operating in the C. W. mode at 25.2 MHz. Infrared spectra were obtained in mineral oil suspension.

Thin-layer Chromatographic Procedures.

Thin-layer chromatograms were run on silica gel G using ethyl acetate - methanol (6: 1, v/v) as the solvent when 1-methylpseudouridine, pseudouridine or antibiotic U-44590 were involved. The nucleosides were detected by the method described by D. R. BUHLER[8].

Preparations or fractions containing the triacetate of 1-methylpseudouridine and the diacetate of antibiotic U-44590 were analyzed by tlc on silica gel G using ethyl acetate - Skellysolve B - methanol (60: 30: 1, v/v) as the solvent. The compounds were detected by spraying with a "permanganate-carbonate" reagent prepared by dissolving 5 g of Na_2CO_3 and 1 g of $KMnO_4$ in 200 ml of water.

Fermentation and Assay Procedures.

The procedures described by DEBOER *et al.*[1] for the production of antibiotic U-44590 were used. The antibiotic titer of the fermentation was determined by an agar plate disc assay using *Klebsiella pneumoniae* as the assay organism. High titers of antibiotic U-44590 are indicative of high titers of 1-methylpseudouridine. Therefore the *K. pneumoniae* assay indirectly indicates production of 1-methylpseudouridine. Beers were harvested after a total fermentation time of 120~192 hours.

Isolation of 1-Methylpseudouridine and Pseudouridine.

Recovery from Fermentation Broth: Fermentation broth (harvest volume 4,800 liters) was adjusted to pH 3.0 with aqueous sulfuric acid solution and filtered with the aid of 240 kg of filter aid. The cake was washed with water and the aqueous wash was combined with the clear filtrate. The clear filtrate-wash was then passed over columns containing 76 kg of CAL-carbon packed in water. The spent beer was discarded. The columns were washed with water and the aqueous wash was also discarded. The columns were then eluted with acetone - water (4: 1, v/v). The eluates were tested for bioactivity against *K. pneumoniae* (indicative of the presence of antibiotic U-44590) and the active fractions were concentrated to a small volume. This solution was mixed with 4 volumes of acetone. The precipitated material was isolated by filtration, found to be bioinactive and was discarded. The filtrate was again concentrated to a small volume and the concentrate was mixed with 10 volumes of acetone. The precipitated material was also discarded. The new filtrate was concentrated to a solution containing *ca* 400 g/liter of solid material. Five hundred ml of this concentrate was freeze-dried to a preparation designated "Solid A", 200 g, which was subjected to the purification procedures described below.

Counter Double Current Distribution:

The solvent system used consisted of equal amounts of 1-butanol and water. The starting material, 50.0 g of "Solid A," obtained as described above, was dissolved in the solvent system. The two phases were separated and added in 4 tubes of the lower phase side of an all glass counter double current distribution machine (100 tubes, 25 ml/phase). Both the upper and lower phases coming out of the apparatus were collected in 25 ml fractions. A total of 172 transfers were run. Both the collected fractions and the tubes in the machine were analyzed by testing for bioactivity and by tlc. Fractions containing a mixture of pseudouridine, 1-methylpseudouridine and small amounts of antibiotic U-44590 were combined and the solution was concentrated to a dry material, 14.0 g, which was purified by silica gel chromatography as described below.

Silica Gel Chromatography. Isolation of Pseudouridine and 1-Methylpseudouridine:

A column was prepared from 1.8 kg of silica gel (Merck-Darmstadt, 7734) packed in the solvent system consisting of ethyl acetate - methanol (6: 1, v/v). The starting material, 13.5 g (obtained as described above), was dissolved in methanol. This solution was mixed with 100 g of silica gel and 500 ml of ethyl acetate and the mixture was concentrated to dryness. The resulting powder was added on the top of the column and the column was then eluted with the above-described solvent system. Fractions were analyzed by bioactivity determination, tlc, solids determination and U.V. spectra. Early fractions contained uridine (100mg) followed by antibiotic U-44590 (189mg), 1-methylpseudouridine (307 mg) and pseudouridine. Characterization of 1-methylpseudouridine follows in the characterization section.

Acetylation of Mixture of 1-Methylpseudouridine and Antibiotic U-44590:

A mixture of 1-methylpseudouridine and antibiotic U-44590, 43 g, obtained by the silica gel chromatography described above, was dissolved in 350 ml of acetic anhydride and 15 ml of pyridine. The solution was stirred and heated at 75°C for 72 hours. The reaction mixture was then concentrated to dryness *in vacuo*. The residue was dissolved in 200 ml of boiling ethyl acetate, and the solution, clarified by filtration, was allowed to stand at room temperature for 72 hours. Crystalline U-44590 diacetate, 31.3 g, was isolated by filtration. The mother liquors containing 1-methylpseudouridine triacetate was concentrated to dryness to give a viscous oil, 20.3 g, which was chromatographed as described below.

Silica Gel Chromatography. Isolation of Crystalline 1-Methylpseudouridine Triacetate:

A column was prepared from 1.8 kg of silica gel (Merck Darmstadt, 7734) packed in the solvent system consisting of ethyl acetate - Skellysolve B - methanol (60: 30: 1, v/v). The starting material, 20.3 g of crude 1-methylpseudouridine triacetate (obtained as described above), was dissolved in ethyl acetate - methanol (60: 1, v/v). The solution was mixed with 100 g of silica gel and 30 ml of Skellysolve B and the mixture was concentrated to dryness. The resulting powder was added to the top of the column and the column was eluted with the described solvent system. Fractions containing 1-methylpseudouridine triacetate (by tlc) were combined and the solution was concentrated to dryness to yield 6.2 g of crystalline (colorless needles) of 1-methylpseudouridine triacetate which is described in the characterization section.

Deacetylation of 1-Methylpseudouridine Triacetate to 1-Methylpseudouridine:

Three g of crystalline 1-methylpseudouridine triacetate was dissolved in 40 ml of absolute methanol. Concentrated ammonium hydroxide (0.22 ml) was added to the methanolic solution and the mixture was allowed to stand at room temperature for 48 hours; it was then concentrated to dryness to give 3.0 g of crude 1-methylpseudouridine. This material was chromatographed over 450 g of silica gel (Merck-Darmstadt, 7734) using ethyl acetate - methanol (6: 1, v/v) as the solvent system. Fractions containing (by tlc) 1-methylpseudouridine were combined and concentrated to a volume of 100 ml. Crystalline 1-methylpseudouridine precipitated and was isolated by filtration; yield 900 mg.

Results and Discussion

Isolation of 1-Methylpseudouridine:

Two procedures have been used for the isolation and separation of 1-methylpseudouridine from antibiotic U-44590. The starting material for both procedures was obtained by absorption of both compounds on activated carbon followed by elution with acetone - water (4: 1).

In the first procedure the material obtained from the carbon eluate was purified further by counter double current distribution using 1-butanol - water (1: 1) as the solvent system. This procedure removes most of antibiotic U-44590 from a mixture of nucleosides including 1-methylpseudouridine, pseudouridine and uridine. Silica gel chromatography of the mixture of nucleosides separated 1-methylpseudouridine from pseudouridine and other metabolites.

In the second procedure the material obtained from the carbon eluate was chromatographed on silica gel to yield a mixture of 1-methylpseudouridine and antibiotic U-44590. Acetylation of the mixture afforded crystalline diacetate of U-44590 which was separated from crude 1-methylpseudouridine triacetate. Silica gel chromatography of the latter yielded crystalline 1-methylpseudouridine triacetate which by deacetylation gave crystalline 1-methylpseudouridine. The second procedure gives high yield of 1-methylpseudouridine or 1-methylpseudouridine triacetate of high purity.

Characterization and Structural Studies:

Pseudouridine was isolated as colorless crystalline material. It was identified by comparison to authentic sample and by high resolution mass spectroscopy.

1-Methylpseudouridine (**II**) was isolated as a colorless crystalline (needles) material, m.p. 181~184° C (unc.), $[\alpha]_D$ −25° (*c* 1, 50% aqueous ethanol); it is soluble in water and lower alcohols and insoluble in ketones, halogenated or saturated hydrocarbon solvents, ether, ethyl acetate or other ester type solvents.

Compound **II** forms a tetramethylsilyl-derivative having a molecular composition of $C_{10}H_{10}N_2O_6 \cdot [Si(CH_3)_3]_4$, (Calcd M^+, 546.2433; found, 546.2406). The molecular formula of 1-methylpseudouridine, therefore, is $C_{10}H_{14}N_2O_6$, molecular weight 258, identical to that of thymidine.

Fig. 3. Infrared spectrum of 1-methylpseudouridine (Nujol mull)

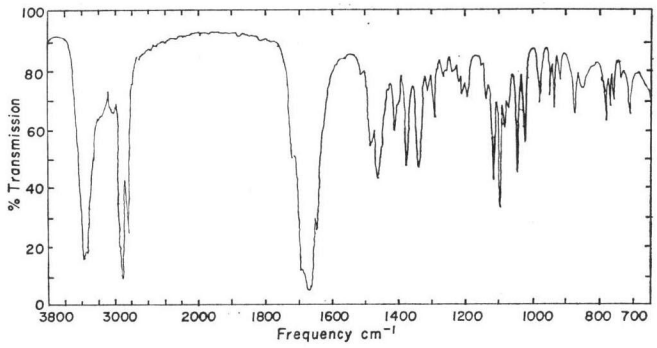
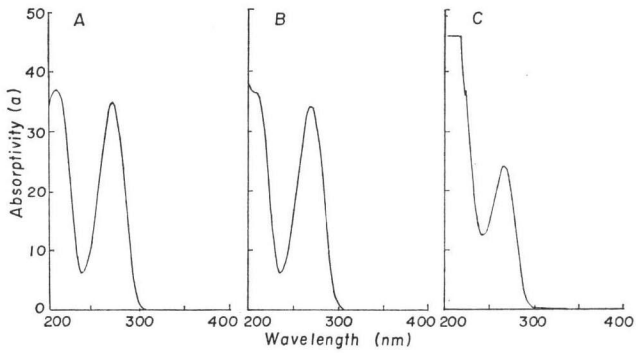


Fig. 4. UV spectra of 1-methylpseudouridine*



* A: Water; B: Water pH 1.0; C: Water pH 11.0.

The IR spectrum (Fig. 3) shows absorptions at 3390, 3350 (OH, NH) and 3060, 3040 (C = CH) cm^{-1}. The most characteristic band at 1672 cm^{-1} is indicative of the presence of a pyrimidine system in the molecule of **II**. The UV spectra (Fig.4) showed absorptions at λ_{max} (ε), 209 nm (7546), 270 nm (9081) in water; 209 nm (sh) (10342), 270 nm (8923) in water, pH 1.0; 267 nm (6192) in water, pH 11.0. These UV maxima strongly suggest a 1,5-disubstituted uracil (thymidine-like) chromophoric system in II[7]. The PMR spectrum of II in D_2O showed absorptions at δ 3.42 (s, 3H) assigned to an NCH_3; δ 3.85 (2H) due to $-CH_2O-$; complex absorption at δ 3.95 to 4.4 (3 H) assigned to three -CHO-groups; a doublet at δ 4.71 (1 H, J=4.0 Hz) and a singlet at δ 7.81 (1 H). These data agree with the consideration of a pyrimidine-pentoside structure for **II**. Furthermore, the presence of the anomeric proton as a doublet at δ 4.71 indicated that **II** is a carbon-carbon linked nucleoside.* Since a methyl group is attached to the 1-position of the pyrimidine nucleus the pentose fragment must be linked to C-5 and consequently the singlet at δ 7.81 (1 H) is assigned to the hydrogen attached at C-6 of the pyrimidine moiety.

This conclusion is supported by ^{13}C-NMR studies (Table 1). In addition to an N-CH_3 (q, δ 36.92) and two carbonyls at C-2 and C-4 of **II** [δ 153.22 (s), δ 165.62 (s)] the CMR spectrum shows the presence of a singlet at δ 111.41 assigned to C-5 (point of attachment of the pentose moiety) and a doublet at δ 146.90 which is assigned to C-6 of **II**. Furthermore the chemical shifts and multiplicities of C-1′ to C-5′ indicate ribofuranosyl-structure for the pentose moiety[10]. Compound **II** was assigned the β-anomeric configuration since the absorption pattern of the C-3′, C-4′, C-5′ proton region in the PMR spectrum is identical to that of pseudouridine. In addition the PMR spectrum of the α-anomer of pseudouridine shows the anomeric proton 0.33 ppm downfield from that of the β-anomer[6].

Acetylation of **II** yielded, a crystalline (needles) triacetate (**III**), m.p. 117~120°C, $[\alpha]_D^{25}$, +9° (c 1, $CHCl_3$). **III** is soluble in most organic solvents and insoluble in water and saturated hydrocarbons. High resolution mass spectra indicated a molecular formula of $C_{10}H_{11}N_2O_6 \cdot (COCH_3)_3$. Other mass

* The proton spectrum of showdomycin a C-riboside shows anomeric proton as a doublet at δ 4.82; pseudouridine under similar conditions shows the anomeric proton at δ 4.71. This compares with δ 6.0 for the anomeric hydrogen of uridine[6].

ions observed were at *m/e*, 324, 281, 265, 264, 222, 205 resulting from loss of either CH_3COOH or CH_3CO- or both fragments. The most significant ion was observed at *m/e* 155 due to fragment IV.

The *m/e*, 155 ion is important since all carbon-carbon linked nucleosides like formycin[11], formycin B[11], showdomycin[11], and pseudouridine[12] are characterized by a fragmentation pattern containing base peak at B +30 (where B is the purine or pyrimidine moiety of the respective nucleosides). This indicates that the carbon-carbon bond of the glycosyl linkage is not cleaved in contrast to the C-N glycosyl linkage of regular nucleosides[13].

The IR spectrum of **III** showed absorptions at 1745 cm^{-1} due to the ester carbonyls; the UV spectrum showed maximum at 269 nm (ε=8216). The PRM spectrum of **III** in $CDCl_3$ showed absorption at δ 2.1 (s, 3 $COCH_3$); δ 3.42 (s, N-CH_3); δ 4.25 (complex, 3H, 4′,5′α,5′β protons); δ 4.88 (d, C-1′ proton); δ 5.38 (complex, 2H, 2′, 3′ protons); δ 7.5 (s, C-6 proton) and δ 9.3 (s, -NH) all in agreement with the postulated structure. The chemical shifts observed in the CMR spectrum of III are presented in Table 2 and are also consistent with the proposed structure.

Biological Properties:

1-Methylpseudouridine and its triacetate were found inactive against a variety of Gram-positive and Gram-negative organisms. Both compounds showed marginal activity when tested for their ability to inhibit herpes virus (42D, type 1) replication *in vitro*. They did not show activity against L-1210 cells *in vitro*.

Table 1. Chemical shifts observed in the ^{13}C-NMR spectrum of 1-methylpseudouridine.

Chem. Shift (ppm)**	Number of carbons	Multiplicity	Assignment*
79.77	1	d	C-1′
74.17	1	d	C-2′
71.58	1	d	C-3′
84.14	1	d	C-4′
62.44	1	t	C-5′
153.22	1	s	C-2
165.62	1	s	C-4
111.41	1	s	C-5
146.90	1	d	C-6
36.92	1	q	N-CH_3

* See Structure **II** (Fig. 2).
** Relative to tetramethylsilane (TMS).

Fig. 5.

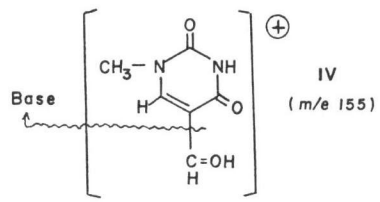


Table 2. Chemical shifts observed in the ^{13}C-NMR spectrum of 1-methylpseudouridine triacetate (U-50,227).

Chem. Shift (ppm)**	Number of carbons	Multiplicity	Assignment*
20.62	2	q	$2CH_3CO$
20.83	1	q	$\underline{CH_3}CO$
36.17	1	q	$\underline{CH_3}$-N
63.42	1	t	C-5′
71.33	1	d	C-3′
74.17	1	d	C-2′
77.47	1	d	C-1′
78.93	1	d	C-4′
111.28	1	s	C-5
143.28	1	d	C-6
150.92	1	s	C-2
162.53	1	s	C-4
169.78	2	s	$\underline{CO}$-CH_3
170.63	1	s	$\underline{CO}$-CH_3

* See Structure **III** (Fig. 2).
** Relative to tetramethylsilane (TMS).

1-Methylpseudouridine has not been isolated and characterized from any source prior to the present report. In the course of structural studies with pseudouridine W. E. COHN[6] reacted pseudouridine with diazomethane and the reaction mixture was characterized by paper chromatography. In addition to the starting material he observed three additional spots on the papergrams. On the basis of Rf values and UV spectra he considered the three reaction products as being 1,3-dimethylpseudouridine, 1-methylpseudouridine and 3- methylpseudouridine. Similarly SCANNELL *et al.*[7] methylated pseudouridine using dimethyl sulfate and analyzed their reaction mixture by paper chromatography. They did not isolated any of the methylation products.

As mentioned earlier *S. platensis* produces pseudouridine, 1-methylpseudouridine and a 5-aza-5,6-dihydropyrimidine nucleoside (I). SUHADOLNIK[14] has also isolated pseudouridine from culture filtrates of *Streptomyces ladakanus* which produces the antibiotic ladakamycin (5-azacytidine)[15].

The presence of pseudouridine and 1-methylpseudouridine in culture filtrates of streptomycetes which produce 5-azapyrimidine nucleosides presents interesting questions as to the biosynthesis of these C-nucleosides and their function as metabolic products.

Acknowledgment

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