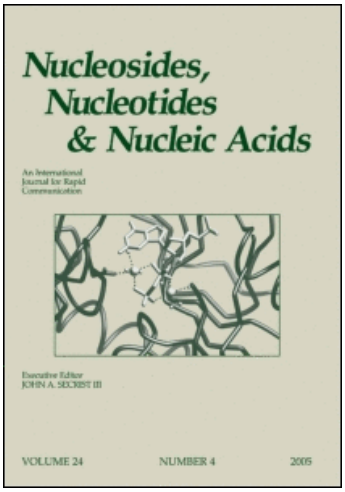

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Synthesis of An Acid-Stable 2,5'-Cyclo-2-oxo Analogue of Wyosine (Nucleosides and Nucleotides. Part 61[1])

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SYNTHESIS OF AN ACID-STABLE 2,5'-CYCLO-2-OXO ANALOGUE OF WYOSINE (NUCLEOSIDES AND NUCLEOTIDES. PART 61[1])

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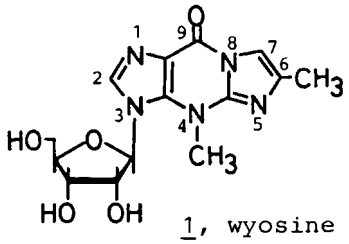
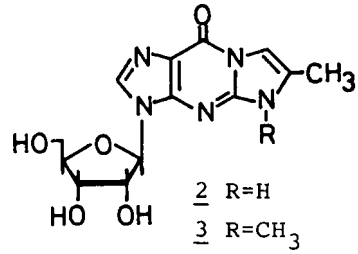
ABSTRACT: Methylation of a 4-desmethylwyosine derivative fixed in anti-conformation has afforded a higher yield of fluorescent N-4-methyl isomer, 2,5'-cyclo-2-oxo-2',3'-O-isopropylidenewyosine (7), which has been shown to be relatively stable in acidic media.

The Y nucleosides, hypermodified units from $tRNA^{Phe}_{s}$[2], deserve special attention due to their unusual physico-chemical properties, i.e. fluorescence and exceptional lability of glycosidic bond in acidic media. Location of Y nucleosides in the position adjacent to the 3'-end of anticodon also suggests their distinctive biological function in protein synthesis. Wyosine (1), the simplest representative of the Y-nucleoside family, has been isolated from Torulopsis utilis $tRNA^{Phe}$ [3] and already obtained by a total multistep synthesis via 3-methylguanosine[4,5].



1, wyosine



2 R=H
3 R=CH_3

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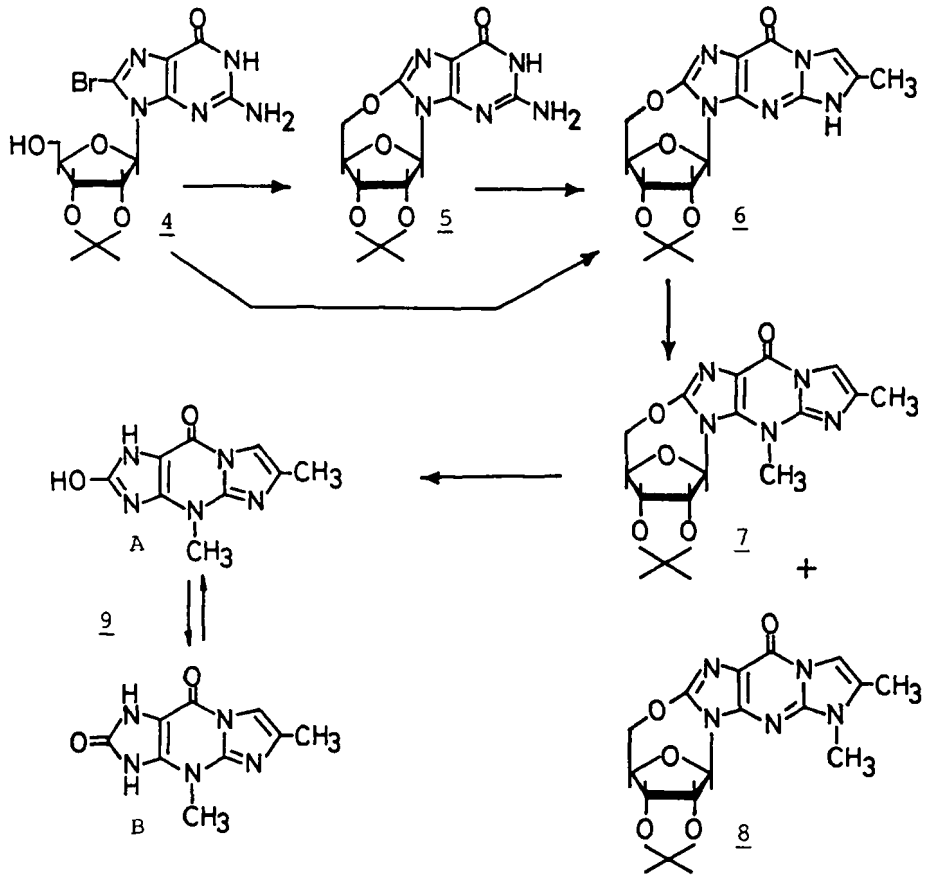
Recently it has been shown that methylation of 4-desmethylwyosine (2)[3,6] system with diazomethane[7,8] results in formation of wyosine,obtained as a minor product in addition to predominant amount of N-5-methyl isomer (3). Since the substrate 2 is easily accessible from guanosine[3], the approach via 4-desmethylwyosine seems to be a convenient synthetic route towards wyosine and its derivatives despite the low yield (1-3%)[7,8] of methylation at the N-4 position. In order to improve this yield, a mesoionic N-1-benzyl derivative of 2 was methylated with dimethyl sulfate, but it resulted in a quantitative substitution at the N-5[9].

One of the possible reasons for low yield of fluorescent N-4-methyl isomer in methylation of 2 with diazomethane may be a steric hindrance of ribofuranosyl moiety, being in a close neighbourhood of the N-4 center. Therefore, we have undertaken study on methylation of 2,5'-cyclo-2-oxo analogue of desmethylwyosine, a nucleoside fixed in anti-conformation, in which steric hindrance caused by 3-β-D-ribofuranosyl portion seems to be partially decreased.

Treatment of 8-bromo-2',3'-O-isopropylideneguanosine (4)[10] with sodium hydride in dimethylformamide according to Srivastava et al.[11] afforded 8,5'-cyclo-8-oxo-2',3'-O-isopropylideneguanosine (5). Application of silica gel chromatography for isolation of the product instead of precipitation in water[11] allowed to obtain 5 in much better yield of 96%. Reaction of N-1-sodium derivative of cyclonucleoside 5 with bromoacetone followed by alkaline hydrolysis gave 2,5'-cyclo-2-oxo-2',3'-O-isopropylidene-4-desmethylwyosine (6) in 89% yield. Transformation of 8-bromo derivative 4 to 6 may be also performed in a one-step procedure but in this case overall yield from 4 was somehow lower (59%).

Methylation of the cyclic analogue of desmethylwyosine 6 was carried out according to the procedure of Golankiewicz and Folkman[8] using diazomethane in dichloromethane at room temperature. Methylated products were successfully isolated from the reaction mixture by silica gel chromatography. The minor fluorescent product, 2,5'-cyclo-2-oxo-2',3'-O-isopropylidenewyosine (7) was obtained in 8.8% yield, when its non-fluorescent N-5-methyl isomer 8 was isolated in a yield of 60.6%. Both isomers were obtained in crystalline state



and their structures were confirmed on the basis of spectroscopic data. Their characteristic ultraviolet spectra were closely similar to those reported[3,8] for uncyclic wyosine (1) and N-5-methyl derivative 3. In ^{1}H NMR the newly introduced N-4-methyl group appeared at 4.12 ppm in compound 7, whereas N-5 methyl of product 8 was found at 3.62 ppm.

Even higher yield of methylation had been achieved when reaction was performed in benzene solution of diazomethane at 75°. In the latter case fluorescent 7 was obtained in 12.9% and its isomer 8 in 55.5%. Thus the ratio of N-4/N-5 methylation was like 1:4.3, being approximately as 1:30 for methylation of uncyclic 2',3',5'-tri-O-acetyl-4-desmethylwyosine[8].

It unquestionably shows that fixed anti-conformation of desmethyl substrate increases the yield of wyosine-type product, and therefore, the discussed steric hindrance of 3-β-D-ribofuranosyl moiety appears to be an obstacle, that

makes access of methylating reagent to nitrogen N-4 more difficult.

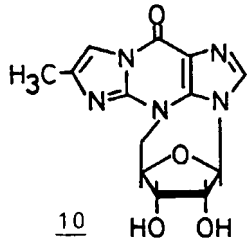
In the case of uncyclic analogues, methylation in hot solution of diazomethane in benzene was also superior to previously described conditions[8] in respect of formation of wyosine derivatives. In our introductory experiment, methylation of desmethylwyosine triacetate yielded respective fluorescent product, wyosine triacetate, in 4.2%.

The 2,5'-cyclo-2-oxo derivative 7 was surprisingly resistant to acidic hydrolysis.It remained virtually unchanged at pH 2.9, 37° [12] and at pH 2.1, 25° for 24 hours, but underwent very slow decomposition in 0.1 M hydrochloric acid at 25°; when half-times for hydrolysis of the glycosidic bond of wyosine under above listed conditions were 41 min, 19 min and 95 sec[5,13], respectively.

In 0.1 M hydrochloric acid at 50°, however, compound 7 was quantitatively hydrolyzed to a new fluorescent product after 20 h. Structure of this product was assigned as 2-hydroxywye base (9) on the spectroscopic basis. Its ultraviolet spectrum showed three maxima at 233, 279 and 298 nm, and was different from that reported for wye base[14]: 230, 265 and 300 nm. The difference can be explained in terms of possible tautomerism between form A and B in compound 9. In 1 M hydrochloric acid at 30° half-time of glycosidic linkage hydrolysis of 7 was found as $t_{1/2}$ 30 min, k_{obs} 2.31 x 10^{-2} min^{-1}.

These results indicate that 2,5'-cyclo-2-oxo analogue of wyosine (7) is more stable to acidic hydrolysis than wyosine by a factor of three orders of magnitude. However, selective deacetonation could not be achieved without cleavage of glycosidic bond. It is worthy to note that even in case of 8,5'-cyclo-8-oxo-2',3'-O-isopropylideneguanosine (5) attempted deacetonation resulted in formation of 8-hydroxyguanine[11].

Considering the reason for this unusual stability under acidic conditions, we must note that electronic effect of oxygen bridge attached to C-2 in compound 7 is not neglectable, since the most probable site of protonation of Y nucleosides is nitrogen



10

N-1. On the second hand, another cyclic analogue of wyosine, N-3,5'-cyclowyosine (10)[3,15] had been also shown to be relatively stable to acidic hydrolysis. The latter, however, does not posses an oxygen bridge and is fixed in syn-conformation. Therefore, a rigid skeleton of cyclonucleosides 7 and 10 must be responsible for the discussed stability, diminishing a dynamic interaction between N-4-methyl group and N-3-β-D-ribofuranosyl moiety.This interaction may be the main driving force for unusual susceptibility of Y nucleosides to acidic hydrolysis.

EXPERIMENTAL

Melting points were determined on a Yanagimoto MP-3 micromelting point apparatus and are uncorrected. UV spectra were measured on a Shimadzu UV-260 spectrophotometer. Mass spectra were taken on a JEOL JMS-D 300 mass spectrometer at 70 eV. ^{1}H NMR spectra were recorded on a JEOL JNM-FX 100 FT spectrometer with tetramethylsilane as an internal standard and are reported on δ scale in ppm. Thin-layer chromatography (TLC) was conducted on Merck precoated silica gel F_{254} Type 60 plates using following solvent systems (measured by volume): A, isopropanol - concd. ammonia - water (7:1:2); B, n-butanol - water (86:14); C, chloroform - methanol (9:1); D, ethyl acetate - isopropanol (9:1); E, n-butanol - glacial acetic acid - water (5:3:2). For a preparative short column chromatography Merck TLC gel HF_{254} Type 60 was used.

8-Bromo-2',3'-O-isopropylideneguanosine (4) was obtained from 8-bromoguanosine applying a protection procedure of Ikehara and Muneyama[10], and triacetate of 2 was synthesized according to Golankiewicz and Folkman[8].

8,5'-Cyclo-8-oxo-2',3'-O-isopropylideneguanosine (5)

To an anhydrous solution of 4 (1.0 g, 2.49 mmol) in DMF (10 mL) was added sodium hydride (0.239 g, 9.95 mmol) in 60% suspension in oil, and resulting suspension was stirred with exclusion of moisture for 18 h, according to method of Srivastava et al.[11]. DMF was then evaporated in vacuo, a residue was redissolved in water (20 mL) and adjusted to pH 5 with 10% acetic acid. Water was removed by evaporation and a dry residue was dissolved in solvent C (20 mL), then appl-

ied on a silica gel short column (4.5 x 7 cm). Product was eluted with solvent C, 30-mL fractions. Fractions containing the product were evaporated to give 770 mg (96%) of 5 as a white solid, homogenous by TLC. mp 281°. TLC R_F 0.54(B); 0.12(C). UV λ_{max} (H_2O): 251 (ε 13,100), 278 (8,000) nm. NMR (DMSO-d_6): 1.30, 1.45 (s each, 6, CMe_2), 3.97 (d, 1, H-5'b), 4.57 (dd, 1, H-5'a), 4.67 (bs, 1, H-4'), 4.85 (d, 1, H-2'), 5.06 (d, 1, H-3'), 5.80 (s, 1, H-1'), 6.48 (bs, 2, NH_2), 10.63 (bs, 1, NH).

2,5'-Cyclo-2-oxo-2',3'-O-isopropylidene-4-desmethylwyosine (6)

Method A. Sodium hydride (101 mg, 4.22 mmol) in 60% suspension in oil was added to a solution of 5 (1.13 g,3.52 mmol) in dry DMSO (15 mL) and this was stirred at room temperature for 2 h. Almost clear solution was treated with bromoacetone (578 mg, 4.22 mmol) for 1 h. After this time reaction mixture was alkalized by addition of 1 M KOH (25 mL) and incubated at 40° for 1 h. A red solution was then diluted with water (50 mL), neutralized with 10% acetic acid and extracted with chloroform (3 x 150 mL). Combined chloroform extracts were washed with water, dried over Na_2SO_4 and evaporated to dryness. The crude product was then purified by silica gel column (4 x 8 cm) chromatography in chloroform - methanol 95:5, collecting 15-mL fractions. Evaporations of fractions 11-24 yielded 6 (1.122 g 89%) as a pale-yellow solid. TLC R_F 0.80(A), 0.66(B), 0.39(C), 0.24(D). UV λ_{max} (MeOH): 229 nm (ε 32,700), 283 (11,600). NMR (DMSO-d_6): 1.31, 1.47 (s each, 6, CMe_2), 2.27 (d, 3, 6-CH_3), 4.04 (d, 1, H-5'b), 4.60 (dd, 1, H-5'a), 4.72 (bs, 1 H-4'), 4.89 (d, 1, H-2'), 5.10 (d, 1, H-3'), 5.94 (s, 1, H-1'), 7.34 (s, 1, H-7), 12.42 (bs, 1,NH).

Method B. To an anhydrous solution of 4 (3.33 g, 8.28 mmol) in DMSO (20 mL) was added sodium hydride (0.457 g, 19.0 mmol) and resulting suspension was stirred at room temperature for 2 h. After this time TLC in solvents B and C showed only the presence of 5 (R_F 0.54 and 0.12, respectively) and bromoacetone (1.47 g, 10.76 mmol) was added. The reaction mixture was maintained at room temp. for 45 min, then diluted with 1 M KOH (50 mL) and incubated at 40° for 1 h. The product was isolated and purified like in method A, what afforded 1.754 g of 6 (59%), identical in all respects with the material obtained in method A.

Methylation of 2,5'-cyclo-2-oxo-2',3'-O-isopropylidene-4-desmethylwyosine (6)

Method A. Saturated at 0° solution of diazomethane in dichloromethane (10 mL; ca 25 mmol) was poured to a flask containing dry, powdered 6 (180 mg, 0.5 mmol). After 10 min at room temp. diazomethane and solvent were evaporated in vacuo without heating. A resulting solid foam was redissolved in chloroform - methanol (98:2) and applied on a silica gel short column (3.7 x 11 cm). Products were eluted with chloroform - methanol (98:2), at a flow rate of 1.1 mL/min, 8-mL fractions. Fractions 41-47 contained fluorescent 2,5'-cyclo-2-oxo-2',3'-O-isopropylidenewyosine (7) homogenous in three solvent systems, 16.5 mg (8.8%) of a white solid after evaporation. This product was crystallized from isopropanol. mp 248°. TLC R_F 0.84(A), 0.56(C), 0.36(D). UV λ_{max} (H_2O): 237 (ε 32,700), 298 (8,300) nm. MS m/z: 373 (M^+), 358 (M-15), 219 (B, $C_9H_9N_5O_2$), 218 (B-1). NMR ($CDCl_3$): 1.40, 1.59 (s each, 6, CMe_2), 2.31 (d,J=1.2 Hz, 3, 6-CH_3), 4.12 (s, 3, N-4-CH_3), 4.13 (d, 1, H-5'b), 4.47 (dd, 1, H-5'a), 4.77 (s, 1, H-4'), 5.09 (s, 2, H-2' and 3'), 6.30 (s, 1, H-1'), 7.38 (d, J=1.22 Hz, 1, H-7). Anal.Calcd.for $C_{17}H_{19}N_5O_5$ (373.37): C, 54.69; H, 5.13; N, 18.76. Found: C, 54.69; H, 5.07; N,18.68.

Fractions 52-68 contained the main product, non-fluorescent N-5-methyl isomer 8, as a crystallizing colourless oil after evaporations. Yield 113.2 mg (60.6%). An analytical sample was crystallized from isopropanol. mp >300°. TLC R_F 0.78(A), 0.51(C), 0.28(D). UV λ_{max} (H_2O): 232 (32,800), 289 (10.200) nm. MS m/z: 373 (M^+), 358 (M-15), 219 (B), 218(B-1). NMR ($CDCl_3$): 1.40, 1.60 (s each, 6, CMe_2), 2.33 (d, J=1.22 Hz 3, 6-CH_3), 3.62 (s, 3, N-5-CH_3), 4.15 (d, 1, H-5'b), 4.46 (dd, 1, H-5'a), 4.69 (bs, 1, H-4'), 4.83 (d, 1, H-5'a), 5.12 (d, 1, H-3'), 6.32 (s, 1, H-1'), 7.39 (d, J=1.22 Hz, 1, H-7). Anal.Calcd.for $C_{17}H_{19}N_5O_5$ (373.37): C, 54.69; H, 5.13; N, 18.76. Found: C, 54.88; H, 5.20; N, 18.54.

Method B. To a vigorusly stirred at 75° suspension of 6 (180 mg, 0.5 mmol) in benzene (2 mL) was added saturated solution of diazomethane in benzene (10 mL) in aliquots of ca 2 mL, during a period of 3 min. Boiling reaction mixture was maintained at this temperature for next 2 min, then cooled

and evaporated to dryness.Products were isolated and analyzed like in method A; amounts of material were as follows: 7, 24.1 mg (12.9%); 8, 103.7 mg (55.5%) and unreacted 6, 11.8 mg (6.6%).

Methylation of 2',3',5'-tri-O-acetyl-4-desmethylwyosine

Triacetate of 2[8] (223.7 mg, 0.5 mmol) was methylated with diazomethane in benzene like in the procedure of synthesis of 7 and 8 (Method B). Short-column chromatography (3.2 x 12 cm) in chloroform - methanol 98:2 allowed to obtain (in order of elution): fluorescent triacetyl derivative of wyosine, 9.6 mg (4.2%) of a solid foam (R_F 0.66 in solvent C; respective N-5-methyl isomer, 186.7 mg (80.9%) as a white solid foam (R_F 0.62); and unreacted starting material, 23.8 mg (10.3%, R_F 0.51). These products were identical in all respects with those described in Ref.8.

Acidic hydrolysis of 2,5'-cyclo-2-oxo-2',3'-O-isopropylidenewyosine (7)

A). A crystalline sample of compound 7 (0.4 mg, ca 1 μmol) was dissolved in 0.1 M citrate buffer, pH 2.9, (0.3 ml) and incubated at 37°. No hydrolysis products were detected even after 24 h, as shown by TLC in solvents B, C, D, and unchanged UV-spectrum after neutralization.

B). 7 (0.4 mg, ca 1 μmol) was incubated at 25° in 0.1 M citrate buffer, pH 2.1 (0.3 ml) for 24 h. The starting material remained unchanged according to TLC and UV-spectrum.

C). 7 (0.4 mg, ca 1 μmol) was incubated in 0.1 M HCl at 25°. After 4 h TLC in solvent E showed traces of a new fluorescent product, R_F 0.64.

D). To a stirred suspension of 7 (7.5 mg, 0.02 mmol) in water (0.5 mL) was added 0.2 M HCl (0.5 mL) and this was incubated at 50°. After 20 h TLC showed a complete hydrolysis. Resulting solution was neutralized with 0.1 M KOH and a half of the reaction mixture was evaporated to dryness. A white residue was suspended in solvent C and applied on a silica gel column (2 x 4 cm). Elution was with a chloroform - methanol gradient. Fractions containig chromatographically pure fluorescent product were evaporated to leave a white solid. TLC R_F 0.29(C), 0.64(E). UV λ_{max} (H_2O): 234, 279 and 298 nm.

MS m/z: 219 (M^+), 218 (M-1), 204 (M-15), 190 (M-CHO).
NMR (DMSO-d_6): 2.23 (d, 3, 6-CH_3), 3.75 (s, 3, N-4-CH_3), 5.00 (b, ~1, OH), 7.34 (d, 1, H-7), 12.27 (b, 1, NH).

E). Determination of the rate of glycosidic bond cleavage: To a solution of cyclonucleoside 7 in water (1.5 mL, ca 1 A_{237}/mL; 45 nmol) was added 1.5 mL of 2 M HCl. Ultraviolet spectra of the reaction mixture were recorded in 10-min time intervals at 30°. Increase of absorbance at 235, 250 and 284 nm corresponded to formation of the product. Half-time of glycosidic bond hydrolysis (found graphically) was $t_{1/2}$ 30 min; k_{obs} 2.31 x 10^{-2} min^{-1}.

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