The 2-Thioanalogues of tRNA Components Derived from 5-Hydroxyuridine

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The synthesis of 5-cyanomethoxy-2-thiouracil (5b) is described. The acid catalysed ethanolysis of 5b leads to 5-ethoxycarbonylmethoxy-2-thiouracil (5a). Condensations of 5-methoxy-2-thiouracil (5) and 5a with the sugar component 7 using the "silyl method" yield peracylated 5-methoxy-2-thiouridine (8) and 5-ethoxycarbonylmethoxy-2-thiouridine (8a), which under treatment with sodium methanolate/methanol afford title 5-methoxy-2-thiouridine (1) (s'cmnnrU) and 5-methoxycarbonylmethoxy-2-thiouridine (2) (s'mcmnrU), respectively.

Introduction

5-Methoxyuridine (mo5U) (1a) and 5-methoxycarbonylmethoxyuridine (mcmnrU or mV) (2a) occupy the "first position" of several tRNA anticodons ("wobble position", U34) and recognize not only A and G but also U as "third letter" of cognate codons [1, 2]. Recently Yokoyama et al. [3, 4] have postulated post-transcriptional 2-thiation of U34 to be a main factor leading to the restriction in base pairing of such a modified unit with G and U.

Thus, considering the significance of the genetic code redundancy [5] one can expect 5-methoxy-2-thiouridine (1) and 5-methoxycarbonylmethoxy-2-thiouridine (2) to be unfunctional components in the formation of tRNA structures being capable of recognizing several synonymous codons. In fact, although the majority of uridines located at the "wobble position" have their own 2-thio-counterparts e.g.: mmn5U, s'mmn5U; mc5U, s'mcm5U;

Looking to allow reuse in the area of future scientific usage.

Results and Discussion

The use of the "silyl method" [21] for the synthesis of modified uridines depends on the preparation of suitable heterobases. A number of "wobble uridine" heterobases have been synthesized employing the general approach, which consists of the condensation of type 4 "active esters" with thiourea in the presence of an alkaline catalyst [13–18].

In this way 5-methoxy-2-thiouracil (5) has been obtained in good yield [18]. A direct adaptation of this method for the synthesis of (5a) failed, as the condensation of the intermediate 4a with thiourea under various experimental conditions did not provide 2-thiouracil derivatives not even in chromatographically detectable amounts.

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As an alternative it has been shown that, the analogous reaction of 3b leads to 5b, which by acid catalysed alcoholysis can be transformed into the desired 2-thiouracil derivative 5a. The compound 3b was prepared in high yield by alkylation of sodium ethyl glycolate with bromoacetonitrile [23] in dimethoxyethane. Next, 3b was condensed with ethyl formate in the presence of sodium hydride (5 °C, DME as solvent) to give 4b. The crude enol 4b was reacted with thiourea in refluxing ethanol. Unfortunately, after neutralization and evaporation of solvent 5-cyanomethoxy-2-thiouracil (5b) did not precipitate in cold water and the separation of the product mixture was accomplished using silica gel chromatography. The nitrile 5b and ester 5a (as the partial ethanolysis product of 5b) were eluated in 11% and 3% yield, respectively and their structures were confirmed by the spectral data. It is noteworthy that, the condensation of alternatively α-formylated ester 3b (at the nitrile group) with thiourea, leading to the respective derivative of cytosine, was not observed.

Ethanolysis of 5b was performed by refluxing the substrate with a mixture of hydrochloric acid and ethanol. The ester 5a was purified by crystallization and its spectral data and t.l.c. mobility were in agreement with those, recorded for the previously isolated sample.

The S,0-disilylated heterobases 6 and 6a were prepared by the reaction of 5 and 5a with trimethylsilyl chloride in the presence of triethylamine [19].

Although several more effective silylating agents are in common use, this method has proved to be of value for the synthesis of persilylated uracils, which are sensitive to ammonia or to heat [20].

The condensations of 6 and 6a with peracylated sugar 7 were performed using the described procedure [14, 15] and the reaction mixtures were worked up according to the usually practiced method [14, 15]. The N1-ribonucleosides 8 and 8a were isolated by means of silica gel chromatography in satisfactory yield as well as N1,N3-diribosides (ca. 5% yield) and N2-ribosides (ca. 10% yield), which were eluated as faster and slower moving zones in comparison with N1-regioisomers, respectively. The noticeable amount of N1,regioisomers and N1,N3-diribosides results from the formation of the strong σ complexes between the heterobases 6 and 6a having electron donating groups at their 5-positions and the catalyst employed [21].

The deprotection of the sugar moieties of 8 and 8a combined with the transestrification of 8a was carried out by treatment with 0.01 N sodium methanolate in methanol [13]. The crude products 1 and 2 were purified by crystallization in high yield and their structures confirmed by NMR and MS spectra. These data and the chromatographic mobilities of 2 in different solvents were identical to those obtained from authentic specimens prepared by the alternative method [22].

**Experimental**

The melting points are uncorrected. UV spectra: Specord UV-VIS spectrometer. 1H NMR spectra: Bruker HX-72 instrument, tetramethylsilane was used as the internal standard. Electron impact mass
spectra (MS) at 15 eV: LKB 2091 spectrometer, abbrev. B-heterobase; s-sugar. The thin-layer chromatography (t.l.c.) was performed on Merck silica gel plates 60 F254 using the following systems (v/v): chloroform—methanol 9:1 (A); chloroform—methanol 85:15 (B); benzene—ethyl acetate 5:3 (C). The silica gel column chromatography was carried out on silica gel Merck 60 F (230–400 mesh). All evaporations were made under reduced pressure and bath temperature below 40 °C.

1. Ethyl cyanomethoxyacetate (3b)
To the stirred suspension of sodium hydride (3.95 g, 0.16 mol) in anhydrous dimethoxyethane (DME, 80 ml) ethyl hydroxyacetate (17.1 g, 0.16 mol) was added dropwise. The stirring was continued for 30 min, bromoacetonitrile (19.74 g, 0.16 mol) [24] was added, the reaction mixture refluxed for 6 h and left overnight at room temperature. The precipitate was filtered off, the filtrate evaporated and the residue distilled to give 3b (21.6 g, 90%) b.p. 98/15 mm.

NMR (CDCl3): 4.26 (q., J = 7 Hz, 2H, CH2); 4.21 (s., 2H, CH-CN); 1.25 (t., J = 7 Hz, 3H, CH3).
IR (film): 2250 (—CN), 1740 (—COOC2H5) cm⁻¹.

2. 5-Cyanomethoxy-2-thiouracil (5b)
To the stirred suspension of sodium hydride (3.95 g, 0.16 mol) in DME (60 ml) the mixture of ethyl cyanomethoxyacetate 3b (14.3 g, 0.1 mol) and ethyl formate (11.3 ml, 0.15 mol) was added dropwise at 0–5 °C. The stirring was continued for 2 h at room temperature and thiourea (5.23 g, 0.07 mol) in anhydrous ethanol (250 ml) was introduced in one portion. The reaction mixture was refluxed for 7 h, the solvent evaporated and the heavy oil was dissolved in water (70 ml). The solution was acidified with acetic acid to pH = 3.5 and evaporated to dryness. The residue was dissolved in ethanol (200 ml), impurities filtered off and to the filtrate silica gel (10 g) was added. Ethanol was evaporated, silica gel co-evaporated with toluene (3×50 ml), suspended in chloroform and applied on silica gel column (60 g).

The elution was performed with chloroform—methanol 97:3 (v/v) to give three fractions (a, b, c).

a: 5a (600 mg, 3%) Rf = 0.53 (A). This sample exhibits Rf value and the spectral data identical to that recorded for the authentic specimen prepared by ethanolysis of 5b.

b: 5b (2 g, 11%) Rf = 0.37 (A) m.p. 223–224 °C (from ethanol).
NMR (CD3SOCD3): 7.39 (s., H, H6), 4.92 (s., 2H, CH2—), 4.17 (s., 2H, CH-CN).
UV (H2O): pH = 2, λmax = 277; pH = 12, λmax = 229, 263.
IR (KBr): 2280 (—CN) cm⁻¹.
MS: m/e 183 (100%, M+), 156 (1.5%, M-CH3-CN), 143 (27%, M-CH3-CN).
c: Thiourea m.p. 182 °C, Rf = 0.11 (A).

3. 5-Ethoxycarbonylmethoxy-2-thiouracil (5a)
A solution the nitrile 5b (2.59 g, 0.014 mol) in the mixture of ethanol (50 ml) and concentrated hydro-
chloric acid (10 ml) was refluxed for 3 h (progress of the reaction was monitored by t.l.c.) and the solvent evaporated. The residue was co-evaporated with ethanol (3×40 ml) and applied on a silica gel column using the procedure described for the preparation of 3b. The heterobase 5a was eluted with chloroform–methanol 9:1 (v/v) in 60% yield (2.1 g) m.p. 169–170 °C (from ethanol).

NMR (CD$_3$SOCD$_3$): 7.50 (s., H, H$_3$), 4.95 (s., 2H, -O–CH$_2$–), 4.47 (q., $J = 7$ Hz, 2H, -CH$_2$CH$_3$), 1.50 (t., $J = 7$ Hz, 3H, -CH$_3$).

UV (H$_2$O): pH = 2, $\lambda$ max = 272; pH = 12, $\lambda$ max = 242, 268.

MS: m/e (230, 100%, M$^+$), 157 (85%, M–COOC$_2$H$_5$), 128 (5%, M–OCH$_2$COOC$_2$H$_5$).

4. S,O-Di-(trimethylsilyl)-5-methoxy-2-thiouracil (6a) and S,O-di-(trimethylsilyl)-5-ethoxycarbonylmethoxy-2-thiouracil (6a)

**General procedure**

To the suspension of 5 [18] or 5a (10 mmol) in anhydrous benzene (150 ml) trimethylsilyl chloride (2.61 g or 3.34 ml, 24 mmol) in anhydrous benzene (50 ml) trimethylsilyl chloride, water and dried. Chloroform was evaporated. The residue was co-evaporated with ethanol (3x40 ml) and applied on a silica gel column using benzene–ethyl acetate 5:2 (v/v) to give 6 (2.7 g, 90%) MS: m/e 359 (5.4%, M–CH$_3$).

5. 2',3',5'-Tri-O-benzoyl-5-methoxy-2-thiouridine (8)

Stannous tetrachloride (3.3 ml, 28 mmol) in anhydrous acetonitrile (60 ml) was added dropwise to the stirred solution of 1-O-acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranose 7 (10.1 g, 20 mmol) and the silylated heterobase 6 (6.64 g, 22 mmol) in acetonitrile (250 ml). The reaction mixture was left for 16 h at room temperature and acetonitrile evaporated to one third of the starting volume. The residue was poured into ice-cold 5% aqueous solution of sodium bicarbonate (400 ml) and the mixture extracted with chloroform (4×100 ml). The extracts were combined, washed with 10% aqueous solution of sodium chloride, water and dried. Chloroform was evaporated and the residue chromatographed on silica gel column using chloroform–methanol 98:2 (v/v) to give 8 (7.5 g, 62%) $R_f = 0.59$ (C).


**Analysis for C$_{37}$H$_{36}$N$_2$O$_5$S (602.54)**

Calcd C 61.80 H 4.40 N 4.05 S 5.31.
Found C 61.37 H 4.12 N 3.95 S 5.43.

6. 2',3',5'-Tri-O-benzoyl-5-ethoxycarbonylmethoxy-2-thiouridine (8a)

To the solution of 6a (2.4 g, 6 mmol) and 7 (3.3 g, 6.3 mmol) in acetonitrile (150 ml) stannous chloride (0.85 ml, 7.3 mmol) was added. The reaction mixture was worked up according to the procedure described for 8. The nucleoside 8a was eluted from silica gel column using benzene–ethyl acetate 5:2 (v/v) in 67% yield (2.46 g) $R_f = 0.68$ (B).

NMR (CDCl$_3$): 8.19–7.18 (m., 15H, H-aromat.), 5.75 (d., $J = 3.5$ Hz, H, H$_3'$), 4.75 (s.,2H, -OCH$_2$–), 4.07 (q.,$J = 7$ Hz, 2H, -CH$_2$–CH$_3$), 1.12 (t.,$J = 7$ Hz, 3H, -CH$_3$).

**Analysis for C$_{38}$H$_{38}$N$_2$O$_5$S (674.6)**

Calcd C 60.53 H 4.48 N 4.15 S 4.74.
Found C 60.72 H 4.21 N 4.32 S 4.95.

7. 5-Methoxycarbonylmethoxy-2-thiouridine (2)

The nucleoside 8a (1.68 g, 2.5 mmol) was dissolved in 0.01 N solution of sodium methanolate in methanol (250 ml) and the reaction was allowed to proceed for 48 h. The solution was neutralized with Dowex 50 W-X-4 (H$^+$ form), the resin filtered off and washed with methanol (2×50 ml). The filtrates were combined and methanol evaporated. The residue was extracted with hot diethyl ether (3×50 ml) and crystallized from methanol to give 2 (690 mg, 80%) m.p. 166–167 °C; $R_f = 0.58$ (B).

UV (H$_2$O): pH = 2, $\lambda$ max ($\epsilon$) = 282 (8300); pH = 12, $\lambda$ max ($\epsilon$) = 249 (15,500), 273 (9800).

NMR (CD$_3$OD): 8.17 (s., H, H$_3$), 6.58 (d., $J = 1.5$ Hz, H, H$_3'$), 4.69 (s., 2H, -OCH$_2$–), 3.76 (s., 3H, -CH$_3$).

MS: m/e 348 (0.5%, M$^+$), 256 (0.7%, B=+1) (24), 216 (100%, B+H), 157 (87%, B–COOCH$_3$).

8. 5-Methoxy-2-thiouridine (1)

The nucleoside 8 (4.2 g, 7 mmol) was treated with 0.01 N solution of sodium methanolate in methanol (700 ml) according to procedure described for 8a to give 1 (1.7 g, 83%) $R_f = 0.31$ (B) m.p. 229–230 °C (from methanol).

UV (H$_2$O): pH = 2, $\lambda$ max ($\epsilon$) = 287 (16,500); pH = 12, $\lambda$ max ($\epsilon$) = 249 (21,800), 273 (13,000).

NMR (CD$_3$SOCD$_3$): 8.36 (s., H, H$_3$), 6.86 (d., $J = 2$ Hz, H, H$_3'$), 3.95 (s., 3H, -CH$_3$).

MS: m/e 290 (1.7%, M$^+$), 158 (100%, B+H).

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