Antiviral and Antischistosomal Evaluation of Newly Synthesized Thioglycosides and their Acyclic Analogues

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Z. Naturforsch. **64c**, 483–489 (2009); received January 30/March 18, 2009

The pyrimidine thione derivatives **2a**–**d** were prepared by the reaction of thiourea, ethyl cyanoacetate and several aromatic aldehydes. The acyclic thioglycosides **4a**–**7d** were prepared by the reaction of the synthesized pyrimidine thiones **2a**–**d** with different alkyl halides, whereas the reaction of **2a**–**d** with 2,3,4,6-tetra-*O*-acetyl-*α*-D-glucopyranosyl bromide afforded the cyclic thioglycosides **8a**–**d** whose deprotection afforded **9a**–**d**. The obtained compounds were tested for their antischistosomal and antiviral activity against hepatitis B virus (HBV). Compounds **5a**, **5d**, **7a** showed high activity against HBV using the MTT assay; moreover compounds **5c**, **6d**, **7a**, **9a**, **9c** exhibited high activity as antischistosomal agents.

Key words: Pyrimidine Thione, Thioglycosides, Antischistosomiasis

Introduction

Pyrimidine thiones and their nucleosides are of considerable biological importance (Cheng, 1969). Various analogues of pyrimidine thiones possess effective antibacterial, antifungal, antiviral, insecticidal, and miticidal activities (Akkurt and Hiller, 1993; Ajitkumar and Cherayil, 1988). They are components of the tRNA of various microorganisms (Lipsett, 1965; Baczynskyj *et al*., 1968), yeasts (Feldmann and Falter, 1971), and mammalian cells (John *et al*., 1967) as a result of post-transcriptional modifications, and play a significant role in translation and its control (Lipsett, 1965; Krzysztof *et al*., 1993). On the other hand, thionucleosides exhibit antineoplastic properties, *e.g*. 4-thiouridine selectively inhibits the growth of Ehrlich ascites and mouse L1210 leukemia cells, 5-fluoro-4-thio-2′,3′-deoxyuridine inhibits the growth of L1210 cells and various hematopoietic human leukemia cells (Matthes *et al*., 1987). It was shown that 2′,3′-dideoxy-3′-fluoro-4-thiopyrimidine (Vince, 1981) is a potent and selective inhibitor of the retrovirus HIV. A variety of pyrimidine thione nucleosides have shown interesting biological activities including antitumour (Herdewijn, 1992), antiviral (Shealy and Clayton, 1969; Khodair *et al*., 1997; Goodchild *et al*., 1983; Wigerinck *et al*., 1993; Zorbach and Tipson, 1968; Antonio *et al*., 1992) and antischistosomal activities (Mitterbauer *et al*., 2002; Parker *et al*., 2000), and among such compounds are 3′-azido-3′-deoxythymidine (AZT, **1**) (Clercq, 1987) 2′,3′-dideoxycytidine (ddc, **2**) (Clercq, 1987), 3′-deoxy-3′-fluorothymidine (Fddthd, **3**) (Clercq, 1987) and 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T, **4**) (Fig. 1). Our attention has been directed to the synthesis of new pyrimidine thione glycosides and their acyclic analogues expecting that the resulting compounds might possess an interesting pharmacological effect.

Experimental

General

All melting points were recorded on a Büchi melting point apparatus and are uncorrected. Solvents were purified according to standard procedures. The IR spectra were recorded on a Perkin-Elmer model 1720 FTIR spectrometer using KBr discs. ^{1}H NMR spectra were recorded at 300 MHz. ^{13}C NMR spectroscopy was performed in the Chemistry Department, University of Leipzig, Germany. The progress of the reactions was monitored by TLC using aluminum silica gel plates 60 F 254. The detection was achieved by treatment with a solution of 15% H_2SO_4 in methanol, and heating at 150 °C. Elemental

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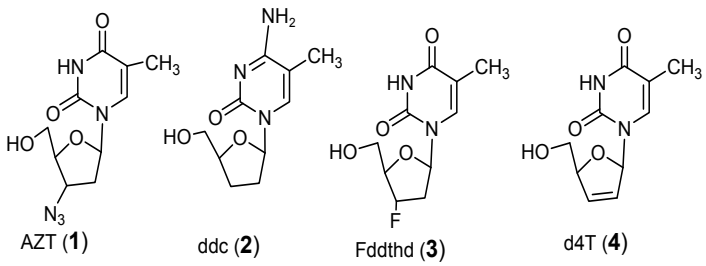

Fig. 1. Some pyrimidine thione nucleosides.

analyses were performed at the Cairo University, Egypt. Column chromatography was carried out using silica gel (0.040–0.063 mm) from Merck. Viral screening against HBV was conducted at the National Liver Institute, Menoufia University, Egypt. The antischistosomal activity study was carried out at Tudor Belharz Institute, Cairo, Egypt.

Preparation and culture of HepG2-2.2.15 cells

The required cell line was made by transfection of HepG2-2.2.15 cells with a plasmid containing multiple tandem copies of the HBV genome (subtypeayw) (Sells *et al.*, 1988). The HepG2-2.2.15 cell line was maintained in RPMI-1640 (Gltmax) (Gibco BRL Life Technologies, Paisly, Scotland) culture medium containing 100 IU/ml nystatin and 380 μg/ml G418 (genecin). The transferred HepG2-2.2.15 cell line was kept in a tissue culture flask at 37 °C and 5% CO_2. Subcultures were set up after one week by aspiration of the media from the culture flask. The cells were washed twice with PBS. 10% versene/trypsin was added and the cells were incubated for 1 min at 37 °C. The drug lamivudine which is a potent selective inhibitor of HBV replication (O'Banion *et al.*, 1992) has been used as a standard for the comparative studies.

DNA extraction

HBV-DNA extraction was done by mixing 10 μl of diluted supernatant (1:5 with PBS) in a reaction tube with 10 μl of 0.2 M NaOH and incubating at 37 °C for 1 h. Carefully, 9.6 μl of 0.2 M HCl were added followed by 90 μl of Tris-EDTA [2-amino-2-(hydroxymethyl)-1,3-propanediol-EDTA] (TE) buffer (Gibco BRL Life Technologies).

PCR-ELISA

The PCR reaction mixture contained 14 μl extracted supernatant, 4 mmol/l $MgCl_2$, 10 μmol/l DIG-11-dUTP, 190 μmol/l dTTP, 200 μmol/l dATP, dGTP, dCTP, 1.5 U Taq polymerase, 20 mmol/l HCl (pH 8.4), 50 mmol/l KCl, 1 μmol/l HCID-1 primer (5′CEGGA AAG AAG TCA GAA GGC A3′CE) and 1 μmol/l HCID-2 primer (5′CETTG GGG GAG GAG ATT AGG TT3′CE) (Roche, Munich, Germany) in a total volume of 50 μl. The PCR reaction conditions were: 32 cycles of 10 min at 94 °C, 30 s at 58 °C and 30 s at 72 °C with a 3 s increment for each cycle in a Perkin Elmer 480 thermal cycler (Perkin Elmer, Waltham, MA, USA).

Cytotoxicity assay

A colorimetric assay for living cells utilizes the colourless substrate 3-(3,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) that is modified to a coloured product by any living cell, but not by dead cells or tissue culture medium. The cytotoxic effect of the compounds was assessed by culturing the HepG2-2.2.15 cells in the presence of compounds using the MTT assay (Fouad *et al.*, 1998).

Calculation of IC50, CC50 and SI values

The 50% inhibitory concentration of antiviral drugs (IC50) was determined by interpolation from the plots of the amount of DNA copies versus antiviral drug concentration. The 50% cytotoxicity effect (CC50) was calculated from the average viability of the cells in proportion to the concentration of the drug. The selective index (SI) was calculated as CC50/IC50 (Fouad *et al.*, 1998).

In vitro test of the compounds for schistosomicidal activity

The method used for testing the compounds for their schistosomicidal effectiveness depends on the *in vitro* direct effect of these compounds on *Schistosoma mansoni* worms in culture. A stock solution of the compounds was first prepared by dissolving them in 100% DMSO, and successive dilutions were made utilizing both 10% DMSO and sterile demineralized water to get the required concentrations. In each well of a tissue culture plate, the tested solution was placed and completed to 2 ml with RPMI-1640 medium to reach the required concentration. Two wells were used for each concentration and three pairs of *Schistosoma* worms, males and females equally represented, were placed in each well using sterilized forceps. Positive (using the reference drug praziquantel) and negative controls were applied to the tested compounds to allow critical comparison of the effect. Test and control wells were incubated at (37 ± 0.5) ºC. Then, after the viability of the worms was determined daily for 2 d using a stereomicroscope, the rate of worm mortality was calculated for each compound and each case. Decreasing concentrations of the effective compounds were tested to determine the IC50 and IC90 values using statistical methods.

Results and Discussion

Chemistry

The reaction of ethyl cyanoacetate with the corresponding aldehydes in EtOH at reflux temperature afforded the inseparable arylidine derivatives **1a**–**d** (Abdou and Strekowski, 2000; Li *et al.*, 2005) which on treatment with thiourea in EtOH at room temperature (r.t.) gave the corresponding pyrimidine thiones **2a**–**d** (Fig. 2, Table I). The IR spectra showed absorption bands in the range 1670–1680 cm^{-1} characterizing the presence of a C=O group in addition to bands at 2206–2211 cm^{-1}, corresponding to C≡N groups, and bands at 3447–3470 cm^{-1} for NH groups. The ^{1}H NMR spectra showed signals of aromatic protons in the range δ 6.72–8.18 ppm in addition to signals in the range δ 10.30–11.88 ppm corresponding to NH groups. The structures of these pyrimidine thione derivatives were confirmed by

Table I. Aromatic substitutions in thioglycosides.

1a–**9d**	R
a	Thiophen-2-yl
b	Furan-2-yl
c	Cyclohexyl
d	Naphthalene-2-yl

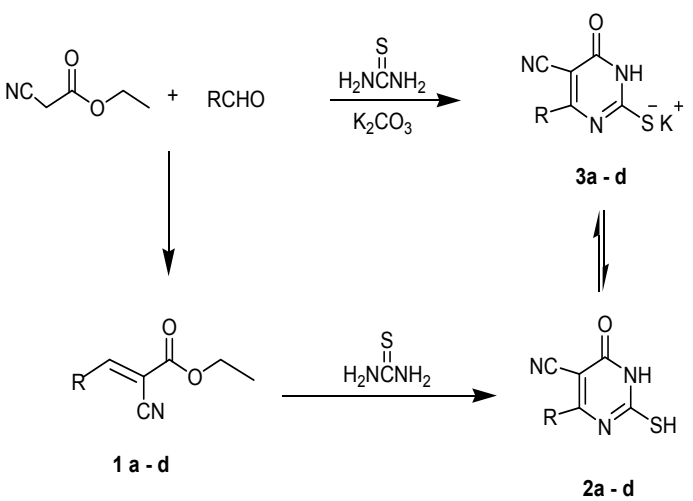

Fig. 2. Preparation of (*E*)-ethyl-2-cyano-3-(aryl)acrylates **1a**–**d**, 1,6-dihydro-2-mercapto-6-oxo-4-(aryl)pyrimidine-5-carbonitriles **2a**–**d**, potassium salts of 1,6-dihydro-2-mercapto-6-oxo-4-(aryl)pyrimidine-5-carbonitriles **3a**–**d**.

IR, ^{1}H NMR and mass spectra. The ^{1}H NMR spectrum of **2c**, as representative example, revealed signals at (δ in ppm): 1.98 (m, 2H, cyclohexyl-H1′), 2.03 (m, 4H, cyclohexyl-H2′,6′), 2.06 (m, 4H, cyclohexyl-H3′,5′), 2.20 (m, 1H, cyclohexyl-H4′), 10.50 (s, 1H, NH).

When the 6-aryl-5-cyano-4-oxo-2-thioxo-3,4-dihydropyrimidine derivatives **2a**–**d** were stirred with anhydrous potassium carbonate in dry acetone for one hour, the corresponding potassium salts **3a**–**d** were obtained. It was interesting to note that **3a**–**d** were successfully prepared by one-pot reaction of thiourea with ethyl cyanoacetate and corresponding aldehydes in the presence of anhydrous potassium carbonate. The structures of compounds **3a**–**d** were confirmed by IR, ^{1}H NMR, ^{13}C NMR and mass spectra. The IR spectra showed absorption bands in the range 1668–1680 cm^{-1}, characterizing the presence of C=O groups, and absorption bands at 3448–3494 cm^{-1} for NH groups. The ^{1}H NMR spectra showed signals of aromatic protons in the range δ 6.66–8.68 ppm and signals in the range of δ 10.50–11.57 ppm, corresponding to NH groups. The ^{13}C NMR spectra showed characteristic peaks at δ 122.93–154.75 ppm for aromatic carbon atoms, in addition to peaks at δ 162.65–164.62 ppm for C≡N groups and signals at δ 182.25–182.68 ppm, corresponding to C=O groups. The ^{1}H NMR spectrum for compound **3a**, as an example, showed signals at (δ in ppm): 7.22 (d, 1H, thiophenyl-H1′), 7.84 (m, 1H, thiophenyl-H2′), 8.12 (d, 1H, thiophenyl-H3′), 11.57 (s, 1H, NH), whereas its ^{13}C NMR spectrum showed signals at (δ in ppm): 119.19 (C–N), 128.39 (thiophenyl-C2′), 129.32 (thiophenyl-C3′), 132.09 (thiophenyl-C4′), 141.97 (thiophenyl-C5′), 158.31 (C=N), 162.56 (C≡N), 182.25 (C=O).

The acyclic thioglycosides **4a**–**7d** were prepared by the reaction of the pyrimidine thiones **2a**–**d** and their potassium salts **3a**–**d** with different alkyl halides (Fig. 3). The structures of compounds **4a**–**7d** were proved by spectral data. The IR spectra showed absorption bands in the range 1668–1680 cm^{-1}, characterizing the presence of a C=O group, and absorption bands in the range 3356–3498 cm^{-1} for NH groups. The ^{1}H NMR spectra showed signals in the range δ 1.21–5.93 ppm for aliphatic side chain protons and signals at δ 9.04–13.50 ppm for NH groups. The ^{13}C NMR spectra showed characteristic peaks in the region 25.39–39.08 ppm, corresponding to aliphatic side chain carbon atoms, and peaks at δ 114.91–132.72 ppm for aromatic carbon atoms. The ^{1}H NMR spectrum of **4c**, as an example, showed signals at (δ in ppm): 1.21 (t, 3H, CH_2CH_3), 1.34 (m, 2H, cyclohexyl-H1′), 1.52 (m, 4H, cyclohexyl-H2′,6′), 1.69 (m, 4H, cyclohexyl-H3′,5′), 1.76 (m, 1H, cyclohexyl-H4′), 3.07 (q, 2H, CH_2CH_3), 12.98 (s, 1H, NH), whereas its ^{13}C NMR spectrum showed signals at (δ in ppm): 25.39 (CH_3), 30.29 (CH_2), 39.5 (cyclohexyl-C1′), 39.75 (cyclohexyl-C2′), 39.91 (cyclohexyl-C3′), 40.33 (cyclohexyl-C4′), 40.75 (cyclohexyl-C5′), 44.43 (cyclohexyl-C6′), 115.76 (C–N), 154.42 (C=N), 167.48 (C≡N), 177.28 (C=O).

The reaction of pyrimidine thiones **2a**–**d** with 2,3,4,6-tetra-*O*-acetyl-*α*-D-glucopyranosyl bromide in the presence of anhydrous potassium carbonate in dry acetone afforded the thioglycosides **8a**–**d** (Fig. 4), which were proven on the basis of their IR, ^{1}H NMR, ^{13}C NMR and mass spectra. The IR spectra showed absorption bands in the range 1744–1752 cm^{-1}, corresponding to the *O*-acetyl groups, and absorption bands at 3432–3490 cm^{-1}, characterizing the presence of NH groups. The ^{1}H NMR spectra showed signals in the range δ 5.31–5.59 ppm, characterizing the presence of anomeric protons of the sugar moiety. The ^{13}C NMR spectra showed characteristic peaks at 70.52–77.90 ppm, corresponding to the anomeric

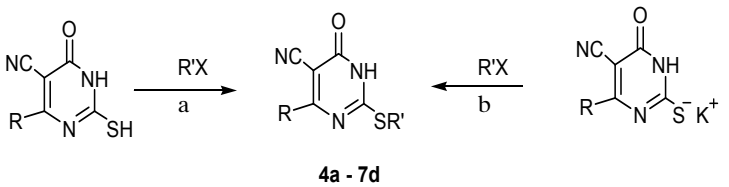

Fig. 3. Synthesis of the thioglycosides **4a**–**7d**. R = thiophen-2-yl, furan-2-yl, cyclohexyl, naphthalene-2-yl, R' = ethyl, allyl, methyloxirane and/or propane-1',2'-diole. Reagents and conditions: (a) potassium carbonate, dry acetone, r.t., 12 h; (b) EtOH, r.t., 24 h.

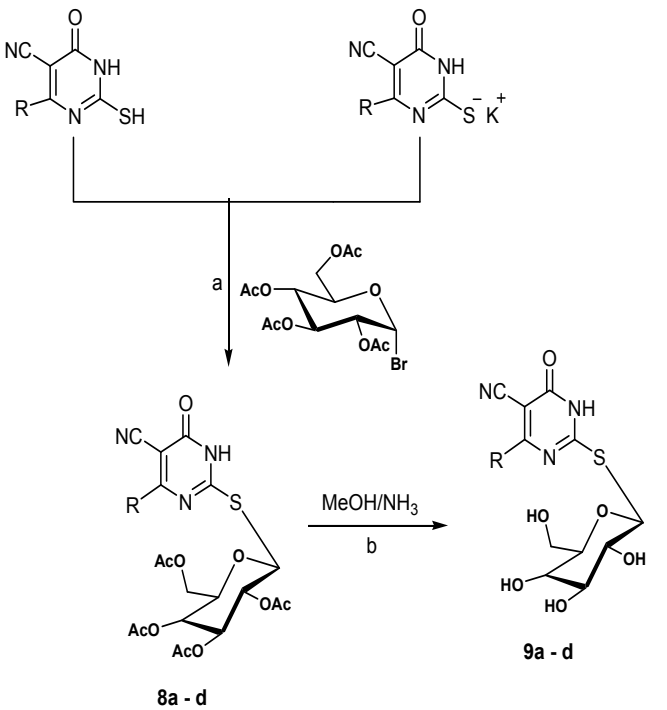

Fig. 4. Synthesis of the thioglycosides **8a**–**d**. Reagents and conditions: (a) 2,3,4,6-tetra-*O*-acetyl-*α*-D-glucopyranosyl bromide, anhydrous potassium carbonate, acetone, r.t., 24 h; (b) 1:1 MeOH/25% NH_3, r.t., 2 h.

C1′. It is obvious that the absence of peaks corresponding to a C=S group indicates that the attachment of a sugar unit has taken place at the sulfur atom and not at the nitrogen atom. This also agreed with the mode of their preparation. The ^{1}H NMR spectrum of **8d**, as representative example, showed signals at (δ in ppm): 1.80, 1.87, 1.91, 2.14 (4 s, 12H, 4 CH_3), 2.84 (dd, 1H, H-5′), 2.91 (d, 2H, CH_2), 3.81 (dd, 1H, J = 10.2 Hz, H-6″), 4.05 (dd, 1H, H-6′), 5.00 (t, 1H, H-4′), 5.20 (dd, 1H, H-3′), 5.25 (t, 1H, H-2′), 5.31 (d, 1H, H-1′), 5.73 (d, 1H, NH), 7.25 (m, 2H, naphthyl-H1′,3′), 7.44 (m, 1H, naphthyl-H4′), 7.84 (m, 2H, naphthyl-H7′,10′), 7.99 (m, 2H, naphthyl-H8′,9′), whereas its ^{13}C NMR spectrum showed signals at (δ in ppm): 15.38, 15.49, 20.76, 31.77, 36.80, (5 CH_3), 61.33 (CH_2), 65.47 (C-6′), 66.63 (C-5′), 74.48 (C-4′), 76.62 (C-3′), 77.26 (C-2′), 77.90 (C-1′), 125.21 (naphthyl-C-2″), 125.49 (naphthyl-C-1″), 126.49 (naphthyl-C-3″), 12.83 (naphthyl-C-4″), 130.69 (naphthyl-C-5″,6″), 134.04 (naphthyl-C-7″,8″), 134.29 (naphthyl-C-9″,10″), 164.10 (C=N), 170.06, 170.39, 172.10, 178.20 (4C=O).

By treatments of thioglycosides **8a**–**d** with methanolic ammonia at room temperature, the deacetylated thioglycosides **9a**–**d** were obtained in moderate yields (Fig. 4). The structures of the deacetylated thioglycoside derivatives were proven on the basis of their IR, ^{1}H NMR and mass spectra. Their IR spectra showed the disappearance of carbonyl acetyl absorption bands, in addition to the appearance of broad absorption bands in the range 3330–3440 cm^{-1} for OH groups. The ^{1}H NMR spectra showed signals in the range of δ 3.56–5.52 ppm, corresponding to a sugar moiety, and signals in the range of δ 5.64–5.85 ppm for NH groups. The ^{1}H NMR spectrum of **9b**, as representative example, showed signals at (δ in ppm): 3.38 (m, 1H, H-6′), 3.80 (dd, 1H, H-6″), 3.98 (m, 1H, H-5′), 4.08 (m, 1H, H-4′), 4.12 (t, 1H, H-3′), 4.16 (dd, 1H, H-2′), 4.22 (m, 1H, OH), 4.34 (d, 2H, CH_2), 4.52 (d, 1H, OH), 4.80 (d, 1H, OH), 5.22 (t, 1H, OH), 5.46 (t, 1H, NH), 5.86 (d, 1H, H-1′), 7.06 (d, 1H, furyl-H1′), 7.44 (m, 1H, furyl-H2′), 7.82 (d, 1H, furyl-H3′).

Antiviral activity

The synthesized products were tested for HBV inhibition using the hepatoplasma cell line HepG2-2.2.15, maintained in RPMI-1640 (Gltmax), as the target cell. The virion DNA can be detected in the cell culture medium using a PCR-ELISA technique (Korba and Gerin, 1992). From the results of antiviral activity measurements, it is clear that **4d**, **6b**, **7b**, **7d**, **8a**, **8d**, and **9d** exhibited relatively low toxicity and high reactivity (Table II).

Antischistosomal activity

The synthesized compounds were tested, as schistosomicidal effectiveness depends on the *in vitro* direct effect of these compounds, on *Schistosoma mansoni* worms in culture. From the results we found that **5c**, **6d**, **7a**, **9a** and **9c** exhibited high schistosomicidal activity (Table III).

Table II. The 50% inhibitory concentration (IC50) and selective index (SI) of the newly synthesized compounds. The 50% cytotoxicity effect (CC50) of all tested compounds is >100 μM.

Compound	IC50 [μM]	SI
Lamivudine	0.1	1000
4b	2.4	41.6
4c	3.0	33.3
4d	0.9	111.1
5a	2.9	34.4
5b	2.6	38.5
5 d	2.8	35.7
6a	1.8	55.5
6b	0.4	250
6c	1.1	90.1
7a	3.0	33.3
7b	0.7	142.9
7c	1.3	76.9
7d	0.4	2 50
8a	0.4	250
8b	1.2	83.3
8d	0.3	333.3
9a	0.9	111.1
9c	1.2	8 3.3
9d	1.0	100

Table III. Screening for schistosomicidal activity of pyrimidine thione compounds *in vitro* on *Schistosoma* worms using 100 ppm for male worms and 110 ppm for female worms.

Compound	Results (dead/total)				Mortality (%)
	1st		2nd		
	1/1	2/1	1/1	2/1	
4a	0/6	0/6	0/6	0/6	0
4c	2/6	4/6	2/6	4/6	50
5a	0/6	0/6	0/6	0/6	0
5b	0/7	0/5	3/6	0/6	25
5d	6/6	6/6	6/6	6/6	100
6d	0/6	0/6	0/6	0/6	0
6e	0/6	0/6	5/6	3/6	66.6
7a	0/6	0/6	0/6	0/6	0
7b	6/6	6/6	6/6	6/6	100
7d	0/6	0/6	0/6	0/6	0
7e	0/6	0/6	0/6	0/6	0
8a	0/6	0/6	4/6	0/12	33.3
8c	0/6	0/6	0/6	0/6	0
8d	0/6	0/6	0/6	0/6	0
9a	3/6	0/6	5/6	0/6	41.7
9c	0/6	0/6	6/6	3/6	75
9d	2/6	4/6	2/6	4/6	50
10a	2/6	0/6	6/6	6/6	100
10c	0/6	0/6	6/6	5/6	92

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