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Synthesis of novel derivatives of 5-carboxyuracil

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The synthesis and characterization of 13 new 5-carboxyuracil derivatives bearing ester and amide groups is described. Five previously synthesised 5-carboxyuracil esters were prepared by a new procedure using carbodiimide as a coupling reagent. The prepared compounds were tested as inhibitors of DNA polymerases, particularly M. MuLV and HIV-1 reverse transcriptases. In addition, it is shown that the methyl, ethyl, propyl, isopropyl, butyl, cyclohexyl, cyclooctyl, and prop-2-ynyl esters of 5-carboxyuracil are substrates of thymidine phosphorylase, thus allowing enzymatic synthesis of novel modified nucleosides.

Key words: 5-carboxyuracil, inhibition, reverse transcriptase, modified nucleosides

INTRODUCTION

Deoxyribonucleotides are the essential building blocks for the synthesis of DNA molecules and have important therapeutic and diagnostic applications such as PCR, real-time PCR, cDNA synthesis, primer extension, nick translation, DNA sequencing, and DNA labelling [1, 2]. Besides the common nucleic acid components, various modified nucleosides have been isolated from nature or resulted from synthetic approaches. Recently, new nucleobases – 5-hydroxymethylcytosine, 5-carboxycytosine and 5-formylcytosine, the oxidation products of 5-methylcytosine, have been discovered in DNA isolated from human and mouse cells, and the functional role of these nucleotides is being rigorously investigated [3, 4]. Modified nucleosides or nucleotides inhibit various enzymes, such as DNA and RNA polymerases, adenosine kinases, deaminases, and hydrolases and act both as anticancer or antiviral agents [5, 6]. In addition, novel nucleoside analogues such as the uracil derivatives are promising building blocks for aptamer synthesis. The purpose of this work is to synthesize various 5-carboxyuracil derivatives, which could be used in the biochemical research as the substrates or inhibitors of DNA/RNA enzymes.

In the present study we synthesized uracil analogues bearing ester or amide bounds at 5th position. The ability of modified 5-carboxyuracil derivatives to affect biosynthesis of DNA, carried on by several key reverse transcriptases, was investigated. In addition, an enzymatic activity of *Escherichia coli* thymidine phosphorylase (EC 2.4.2.4) towards the 5-carboxyuracil derivatives was analyzed.

EXPERIMENTAL

Chemicals and solvents purchased from Sigma-Aldrich and Fluka were of the highest purity available and used without further purification. Thin-layer chromatography (TLC) was carried out on 25 TLC aluminium sheets coated with silica gel

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60 F_{254} (Merck) and column chromatography on silica gel 60 (0.063–0.200 nm) (Merck). Reverse phase chromatography was carried out on Grace flash cartridges C-18. Purification of 5-carboxyuracil amides was carried out on diethylaminoethyl (DEAE) Sephadex A-25 columns with a linear (0.02–0.2 M) gradient of NaCl as a mobile phase. Melting points were determined with a MEL-TEMP (Electrothermal) melting apparatus in capillary tubes and are not corrected. ^{1}H NMR spectra were recorded in D_2O or DMSO-d_6 on Bruker Ascend 400, 400 MHz, and ^{13}C NMR were recorded on Bruker Ascend 400, 100 MHz. Chemical shifts are reported in parts per million relative to the solvent resonance signal as an internal standard. High-resolution mass spectra (HRMS) were recorded on an Agilent 6230 TOF mass spectrometer with electron spray ionization (JetStream ESI).

General procedure for the synthesis of 5-carboxyuracil esters 1–7

To a solution of uracil-5-carboxylic acid (109 mg, 0.7 mmol) in 3 ml DMF, 289 mg (1.4 mmol) *N,N′*-dicyclohexylcarbodiimide (DCC), 43 mg (0.35 mmol) 4-dimethylaminopyridine (DMAP), and 2.8 mmol of appropriate alcohol were added. The reaction mixture was stirred at room temperature overnight. The resulting precipitate was filtered and washed several times with chloroform. The solvent was removed under reduced pressure. The crude mixture was purified by column chromatography (25 ml silica gel, chloroform/methanol mixture, 10:0→10:1). The solvents were removed under reduced pressure to afford colourless solid reaction products **1**–**7**.

***Methyl 2,4-dioxo-1H,3H-pyrimidine-5-carboxylate* (1).** Yield 59 mg (50%), colourless powder; mp 241–245 °C. R_f = 0.42 (chloroform/methanol, 9:1). ^{1}H NMR (DMSO-d_6, ppm): δ = 3.69 (s, 3H, CH_3), 8.14 (s, 1H, C=CH), 11.31 (s, 1H, NH), 11.60 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, ppm): δ = 163.66, 160.48, 151.04, 150.06, 103.38, 51.85. UV λ_{max}: 270 nm. HRMS: calcd. for $C_6H_6N_2O_4$ 193.0220 $[M+Na]^+$, found 193.0225. The physicochemical and spectral characteristics of the obtained compound correspond to those given in [7].

***Propyl 2,4-dioxo-1H,3H-pyrimidine-5-carboxylate* (2).** Yield 59 mg (42%), colourless powder; mp 220–225 °C. R_f = 0.53 (chloroform/methanol, 9:1). ^{1}H NMR (DMSO-d_6, ppm): δ = 0.92 (t, 3H, CH_3, J = 7.4 Hz), 1.59–1.69 (m, 2H, CH_2), 4.07 (t, 2H, CH_2, J = 6.6 Hz), 8.11 (s, 1H, C=CH), 11.29 (s, 1H, NH), 11.57 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, ppm): δ = 163.10, 160.49, 151.06, 149.74, 103.06, 65.83, 22.03, 10.80. UV λ_{max}: 270 nm. HRMS: calcd. for $C_8H_{10}N_2O_4$ 221.0533 $[M+Na]^+$, found 221.0534.

***Isopropyl 2,4-dioxo-1H,3H-pyrimidine-5-carboxylate* (3).** Yield 35 mg (25%), colourless powder; mp 245–247 °C. R_f = 0.51 (chloroform/methanol, 9:1). ^{1}H NMR (DMSO-d_6, ppm): δ = 1.23 (d, 6H, CH_3, J = 6.3 Hz), 4.98 (hept, 1H, CH, J = 6.3 Hz), 8.07 (s, 1H, C=CH), 11.28 (s, 1H, NH), 11.57 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, ppm): δ = 162.35, 160.53, 151.06, 149.50, 103.87, 67.71, 22.10. UV λ_{max}: 269 nm. HRMS: calcd. for $C_8H_{10}N_2O_4$ 221.0533 $[M+Na]^+$, found 221.0539.

***Butyl 2,4-dioxo-1H,3H-pyrimidine-5-carboxylate* (4).** Yield 71 mg (48%), colourless powder; mp 217–222 °C. R_f = 0.53 (chloroform/methanol, 9:1). ^{1}H NMR (DMSO-d_6, ppm): δ = 0.91 (t, 3H, CH_3, J = 7.4 Hz), 1.38 (2H, CH_2), 1.52–1.69 (m, 2H, CH_2), 4.12 (t, 2H, CH_2, J = 6.5 Hz), 8.11 (s, 1H, C=CH), 11.28 (s, 1H, NH), 11.35 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, ppm): δ = 163.15, 160.33, 151.09, 149.75, 103.57, 64.04, 30.69, 19.12, 14.05. UV λ_{max}: 270 nm. HRMS: calcd. for $C_9H_{12}N_2O_4$ 235.0689 $[M+Na]^+$, found 235.0696. The spectral characteristics of the obtained compound correspond to those given in [8].

***Cyclohexyl 2,4-dioxo-1H,3H-pyrimidine-5-carboxylate* (5).** Yield 101 mg (42%), colourless powder; mp 259–261 °C. R_f = 0.56 (chloroform/methanol, 9:1). ^{1}H NMR (DMSO-d_6, ppm): δ = 1.19–1.54 (m, 5H, CH_2), 1.66–1.73 (m, 3H, CH_2), 1.73–1.83 (m, 2H, CH_2), 4.79 (m, 1H, CH), 8.09 (s, 1H, C=CH), 11.27 (s, 1H, NH), 11.55 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, ppm): δ = 162.25, 160.50, 151.07, 149.53, 103.88, 72.14, 31.46, 25.42, 23.45. UV λ_{max}: 269 nm. HRMS: calcd. for $C_{11}H_{14}N_2O_4$ 261.0846 $[M+Na]^+$, found 261.0854.

***Cyclooctyl 2,4-dioxo-1H,3H-pyrimidine-5-carboxylate* (6).** Yield 45 mg (24%), colourless solid; mp 263–268 °C. R_f = 0.51 (chloroform/methanol, 9:1). ^{1}H NMR (DMSO-d_6, ppm): δ = 1.64–1.81 (m, 10H, CH_2), 1.41–1.61 (m, 4H, CH_2), 4.90 (m, 1H, CH), 8.07 (s, 1H, C=CH), 11.26 (s, 1H, NH), 11.50 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 162.20, 160.52, 151.07, 149.42, 104.01, 74.67, 31.35, 27.10, 25.28, 22.78. UV λ_{max}: 269 nm. HRMS: calcd. for $C_{13}H_{18}N_2O_4$ 289.1159 $[M+Na]^+$, found 289.1170.

***Prop-2-ynyl 2,4-dioxo-1H,3H-pyrimidine-5-carboxylate* (7).** Yield 35 mg (13%), colourless solid; decomposes at ~330 °C. R_f = 0.43 (chloroform/methanol, 9:1). ^{1}H NMR (DMSO-d_6, ppm): δ = 3.56 (t, 1H, CH≡C, J = 2.45 Hz), 4.79 (d, 2H, CH_2, J = 2.45 Hz), 8.17 (s, 1H, C=CH), 11.34 (s, 1H, NH), 11.69 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, ppm): δ = 162.32, 160.36, 151.03, 150.73, 102.64, 79.12, 78.07, 52.02. UV λ_{max}: 270 nm. HRMS: calcd. for $C_8H_6N_2O_4$ 217.0220 $[M+Na]^+$, found 217.0227.

General procedure for the synthesis of 5-carboxyuracil amides 8-18

A mixture of uracil-5-carboxylic acid (218 mg, 1.4 mmol) and 1,1′-carbonyldiimidazole (454 mg, 2 mmol) was stirred in 5 ml of DMF 5 ml for 2 hours at room temperature. After carboxylic acid activation was completed (TLC), 2.8 mmol of appropriate amino acid was added and stirred overnight. The formed precipitate was filtered using a glass filter and washed with DMF. The solvent was removed under reduced pressure, and the residue was dissolved in 100 ml of H_2O. Purification was carried

out using ion exchange chromatography on DEAE-Sephadex A25 columns (20 ml) with a linear gradient (0.02–0.2 M) of NaCl as the mobile phase. The product was eluted with 0.15–0.2 M NaCl, the solvent was removed under reduced pressure and the residue was desalted using reverse phase chromatography (12 g C-18 cartridges). The solvent was removed under reduced pressure to afford white solid reaction products **9**–**18**. Oleyl 5-carboxyuracil amide **8** was purified by column chromatography (silica gel, chloroform/methanol, 10:0.5).

N-[(Z)-octadec-9-enyl]-2,4-dioxo-1H-pyrimidine-5-carboxamide (8). Yield 127 mg (31%), colourless solid; decomposes at ~320 °C. R_f = 0.5 (chloroform/methanol, 9:1). ^{1}H NMR (DMSO-d_6, ppm): δ = 0.85 (t, 3H, CH_3, J = 7.9 Hz), 1.06–1.37 (m, 22H, CH_2), 1.45 (p, 2H, CH_2, J = 6.6 Hz), 1.89–2.05 (m, 4H, CH_2), 3.20–3.25 (t, 2H, CH_2), 5.27–5.41 (m, 2H, CH=CH), 8.10 (s, 1H, C=CH), 8.71 (s, 1H, NH), 11.53 (bs, 1H, NH), 11.57 (bs, 1H, NH). ^{13}C NMR (DMSO-d_6, ppm): δ = 164.67, 162.14, 151.07, 148.10, 130.10, 130.08, 104.72, 38.72, 31.74, 29.62, 29.54, 29.53, 29.28, 29.25, 29.14, 29.13, 28.98, 29.04, 27.02, 26.86, 22.55, 14.40. UV λ_{max}: 270 nm. MS (ESI$^+$): *m/z* 406.25 $[M+H]^+$, 404.20 $[M-H]^-$.

***(R)-2-(2,4-dioxo-1H,3H-pyrimidine-5-carboxamido) propanoic acid* (9).** Yield 106 mg (33%), colourless solid; decomposes at 230 °C. R_f = 0.47 (1,4-dioxane/2-propanol/water/ammonia water, 4:2:2:1). ^{1}H NMR (D_2O, ppm): δ = 1.32 (d, 3H, CH_3, J = 7.2 Hz), 4.21 (q, 1H, CH, J = 7.2 Hz), 8.25 (s, 1H, C=CH). ^{13}C NMR (D_2O, ppm): δ = 180.44, 165.26, 164.58, 150.65, 146.53, 104.62, 36.75, 36.34. UV λ_{max}: 272 nm. HRMS: calcd. for $C_8H_9N_3O_5$ 228.0615 $[M+H]^+$, found 228.0614.

***3-(2,4-dioxo-1H,3H-pyrimidine-5-carboxamido)propanoic acid* (10).** Yield 123 mg (39%), colourless solid; decomposes at ~320 °C. R_f = 0.44 (1,4-dioxane/2-propanol/water/ammonia water, 4:2:2:1). ^{1}H NMR (D_2O, ppm): δ = 2.37 (t, 2H, CH_2, J = 6.7 Hz), 3.46 (t, 2H, CH_2, J = 6.7 Hz), 8.27 (s, 1H, C=CH). ^{13}C NMR (D_2O, ppm): δ = 179.86, 164.75, 163.14, 152.05, 148.24, 105.05, 50.90, 17.98. UV λ_{max}: 272 nm. HRMS: calcd. for $C_8H_9N_3O_5$ 228.0615 $[M+H]^+$, found 228.0615.

***(S)-2-(2,4-dioxo-1H,3H-pyrimidine-5-carboxamido)-3-methylbutanoic acid* (11).** Yield 101 mg (29%), colourless solid; decomposes at ~310 °C. R_f = 0.56 (1,4-dioxane/2-propanol/water/ammonia water, 4:2:2:1). ^{1}H NMR (D_2O, ppm): δ = 0.84 (dd, 6H, CH_3, J = 11.2, 6.9 Hz), 2.10–2.19 (m, 1H, CH), 4.18 (d, 1H, CH, J = 4.6 Hz), 8.31 (s, 1H, C=CH). ^{13}C NMR (D_2O, ppm): δ = 178.76, 166.16, 164.76, 152.19, 107.36, 103.37, 60.27, 30.75, 19.11, 16.88. UV λ_{max}: 272 nm. HRMS: calcd. for $C_{10}H_{13}N_3O_5$ 256.0928 $[M+H]^+$, found 256.0926.

***(R)-2-(2,4-dioxo-1H,3H-pyrimidine-5-carboxamido)-3-methylbutanoic acid* (12).** Yield 100 mg, (28%), colourless solid; decomposes at ~310 °C. R_f = 0.56 (1,4-dioxane/2-propanol/water/ammonia water, 4:2:2:1). ^{1}H NMR (D_2O, ppm): δ = 0.83 (dd, 6H, CH_3, J = 11.2, 6.9 Hz), 2.08–2.17 (m, 1H, CH), 4.16 (d, 1H, CH, J = 4.6 Hz), 8.25 (s, 1H, C=CH); ^{13}C NMR (D_2O, ppm): δ = 178.09, 165.30, 163.30, 151.86, 149.19, 105.51, 60.38, 30.75, 19.10, 16.99. UV λ_{max}: 272 nm. HRMS: calcd. for $C_{10}H_{13}N_3O_5$ 256.0928 $[M+H]^+$, found 256.0929.

***(S)-2-(2,4-dioxo-1H,3H-pyrimidine-5-carboxamido)-4-methylpentanoic acid* (13).** Yield 100 mg (25%), colourless solid; decomposes at 275 °C. R_f = 0.60 (1,4-dioxane/2-propanol/water/ammonia water, 4:2:2:1). ^{1}H NMR (D_2O, ppm): δ = 0.84 (dd, 6H, CH_3, J = 10.1, 6.2 Hz), 1.53–1.64 (m, 3H, CH, CH_2), 4.26–4.30 (m, 1H, CH), 8.34 (s, 1H, C=CH). ^{13}C NMR (D_2O, ppm): δ = 180.21, 166.62, 165.16, 157.65, 155.83, 103.44, 53.79, 41.23, 24.81, 22.47, 21.07. UV λ_{max}: 272 nm. HRMS: calcd. for $C_{11}H_{15}N_3O_5$ 270.1084 $[M+H]^+$, found 270.1086.

***(R)-2-(2,4-dioxo-1H,3H-pyrimidine-5-carboxamido)-4-methylpentanoic* acid (14).** Yield 121 mg (30%), colourless solid; decomposes at 275 °C. R_f = 0.60 (1,4-dioxane/2-propanol/water/ammonia water, 4:2:2:1). ^{1}H NMR (D_2O, ppm): δ = 0.84 (dd, 6H, CH_3, J = 10.2, 6.2 Hz), 1.54–1.62 (m, 3H, CH, CH_2), 4.27–4.31 (m, 1H, CH), 8.36 (s, 1H, C=CH). ^{13}C NMR (D_2O, ppm): δ = 180.30, 167.02, 165.56, 158.90, 157.47, 103.13, 53.76, 41.27, 24.81, 22.48, 21.07. UV λ_{max}: 272 nm. HRMS: calcd. for $C_{11}H_{15}N_3O_5$ 270.1084 $[M+H]^+$, found 270.1083.

***(S)-2-(2,4-dioxo-1H,3H-pyrimidine-5-carboxamido)-4-(methylthio)butanoic acid* (15).** Yield 250 mg (62%), colourless solid; decomposes at 250 °C. R_f = 0.57 (1,4-dioxane/2-propanol/water/ammonia water, 4:2:2:1). ^{1}H NMR (D_2O, ppm): δ = 1.98 (s, 3H, CH_3), 1.94–2.07 (m, 2H, CH_2), 2.42–2.47 (m, 2H, S-CH_2), 4.35 (dd, 1H, CH, J = 7.6, 4.7 Hz), 8.26 (s, 1H, C=CH). ^{13}C NMR (D_2O, ppm): δ = 178.24, 165.45, 163.64, 150.96, 149.7, 103.56, 54.20, 31.58, 29.48, 14.17. UV λ_{max}: 273 nm. HRMS: calcd. for $C_{10}H_{13}N_3O_5S$ 288.0649 $[M+H]^+$, found 288.0645.

***(R)-2-(2,4-dioxo-1H,3H-pyrimidine-5-carboxamido)-4-(methylthio)butanoic acid* (16).** Yield 322 mg (80%), colourless solid; decomposes at 250 °C. R_f = 0.57 (1,4-dioxane/2-propanol/water/ammonia water, 4:2:2:1). ^{1}H NMR (D_2O, ppm): δ = 1.99 (s, 3H, CH_3), 1.95–2.08 (m, 2H, CH_2), 2.44 (m, 2H, S-CH_2), 4.36 (dd, 1H, CH, J = 7.6, 4.7 Hz), 8.27 (s, 1H, C=CH). ^{13}C NMR (D_2O, ppm): δ = 178.58, 164.99, 163.34, 150.54, 132.58, 103.41, 54.21, 31.57, 29.47, 14.17. UV λ_{max}: 273 nm. HRMS: calcd. for $C_{10}H_{13}N_3O_5S$ 288.0649 $[M+H]^+$, found 288.0645.

***(S)-2-(2,4-dioxo-1H,3H-pyrimidine-5-carboxamido)-3-phenylpropanoic acid* (17).** Yield 190 mg (45%), colourless solid; decomposes at 280 °C. R_f = 0.46 (1,4-dioxane/2-propanol/water/ammonia water, 4:2:2:1). ^{1}H NMR (D_2O, ppm):

δ = 2.88–3.09 (m, 2H, CH_2), 4.46 (dd, 1H, CH, J = 7.6, 4.9 Hz,), 7.10–7.21 (m, 5H, CH), 8.19 (s, 1H, C=CH); ^{13}C NMR (D_2O, ppm): δ = 178.21, 166.24, 164.89, 157.41, 155.58, 137.38, 129.31, 128.51, 126.77, 103.30, 56.28, 37.87. UV λ_{max}: 272 nm. HRMS: calcd. for $C_{14}H_{13}N_3O_5$ 304.0928 $[M+H]^+$, found 304.0929.

***(R)-2-(2,4-dioxo-1H,3H-pyrimidine-5-carboxamido)-3-phenylpropanoic acid* (18).** Yield 199 mg (47%), colourless solid; decomposes at 280 °C. R_f = 0.46 (1,4-dioxane/2-propanol/water/ammonia water, 4:2:2:1). ^{1}H NMR (D_2O, ppm): δ = 2.93–3.14 (m, 2H, CH_2), 4.50 (dd, 1H, CH, J = 7.6, 4.9 Hz,), 7.17–7.26 (m, 5H, CH), 8.27 (s, 1H, C=CH); ^{13}C NMR (D_2O, ppm): δ = 178.32, 166.73, 165.40, 158.82, 157.57, 137.45, 129.34, 128.51, 126.77, 102.58, 56.27, 37.92. UV λ_{max}: 272 nm. HRMS: calcd. for $C_{14}H_{13}N_3O_5$ 304.0928 $[M+H]^+$, found 304.0922.

Enzymatic synthesis of 5-modified uridines 20–27

Enzymatic synthesis of modified uridines was carried out in 1.5 ml tubes by adding 1 U of thymidine phosphorylase from *E. coli* (Sigma-Aldrich, USA) into 200 µl of the reaction mixture, containing 20 mM phosphate buffer (pH 6.8), 20 mM thymidine, and 40 mM of modified nucleobase, similarly to how it was described previously [9, 10]. The reaction mixture was incubated at 37 °C for 24 h. After incubation, the samples were prepared for HPLC-MS analysis by adding 200 µl of acetonitrile and centrifugation for 5 min at 10 000 rpm. The supernatant was transferred into new tubes and the remaining particulates were filtered out using Whatman™ Mini-UniPrep G2 syringless filters (GE Healthcare Life Sciences, UK).

HPLC-MS analysis

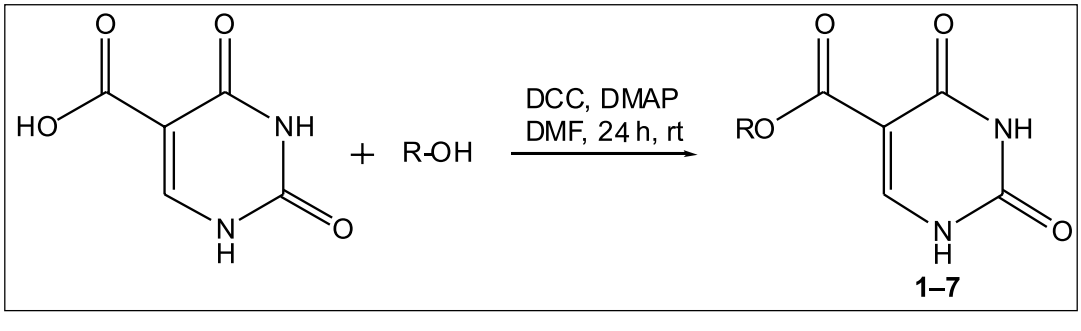
HPLC-MS analyses were performed using a high performance liquid chromatography system (CBM-20A controller, two LC-2020AD pumps, SIL-30AC auto sampler and CTO-20AC column oven; Shimadzu, Japan) equipped with a photo diode array (PDA) detector (SPD-M20A Prominence diode array detector; Shimadzu, Japan) and a mass spectrometer (LCMS-2020, Shimadzu, Japan) equipped with an ESI source. The chromatographic separation was conducted using a YMC Pack Pro column, 3 × 150 mm (YMC, Japan) at 40 °C and a mobile phase that consisted of 0.1% formic acid water solution (solvent A), and acetonitrile (solvent B) delivered in the gradient elution mode. Mass scans were measured from *m/z* 10 up to *m/z* 1 000, at 350 °C interface temperature, 250 °C DL temperature, ±4 500 V interface voltage, neutral DL/Qarray, using N_2 as nebulizing and drying gas. Mass spectrometry data was acquired in both the positive and negative ionization mode. The data was analyzed using the LabSolutions LCMS software.

RESULTS AND DISCUSSION

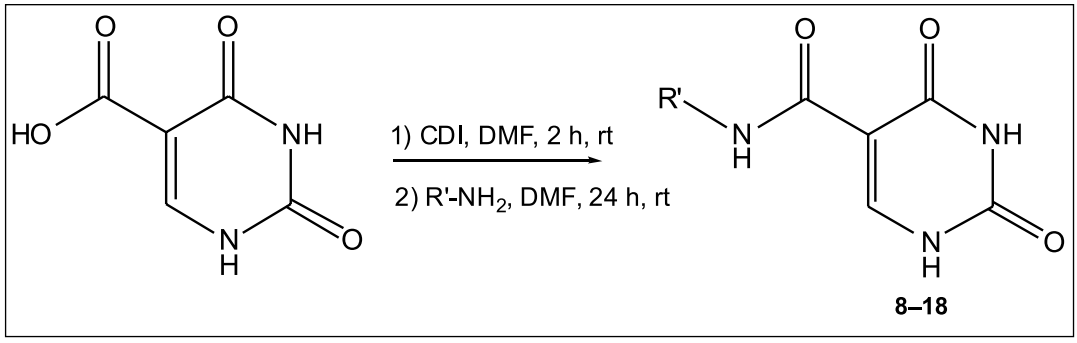
Chemical synthesis

Ester or amide bond formation between an acid and an alcohol or amine, respectively, is formally the condensation. The usual esterifications are an equilibrium reaction, and activation of carboxylic acid is necessary. The carboxyl group can be activated as acyl halides, acyl azides, acyl imidazoles, anhydrides, esters, etc. [11–15]. Methyl, propyl, isopropyl, butyl and cyclohexyl 5-carboxyuracil esters were synthesized before using $SOCl_2$ or concentrated sulfuric acid [7, 8, 16] for activation of uracil-5-carboxylic acid. Various carbodiimides are frequently used for ester bond formation [17, 18]. In this study, we employed dicyclohexyl carbodiimide (DCC) as a coupling agent for one-pot ester preparation under mild reaction conditions. The synthesis of the 5-carboxyuracylic esters (**1**–**7**) is outlined in Scheme 1. We used different primary (methyl, propyl, butyl, propargyl) or secondary (isopropyl, cyclohexyl, cyclooctyl) alcohols in the esterification reaction. The best yields were achieved with the primary alcohols. However, only 13% yield was obtained for ester **7**. It might be explained by the acidity of hydrogen atom to triple bonded carbon and the formation of the by-products during the esterification reaction.

In this work, we used carbonyldiimidazole (CDI) as a coupling reagent for one-pot amide formation. The synthesis route to the novel 5-carboxyuracil amides is shown in Scheme 2. The active acyl imidazole was formed in 2 hours. The completion of activation was monitored by TLC. Then, the amine R'-NH_2 was added, and the reaction mixture was stirred overnight. 5-Carboxyuracil amides **9**–**18** were purified by ion exchange chromatography on DEAE-Sephadex A25 columns with NaCl as a mobile phase. After the water was removed under reduced pressure, the residue was desalted and further purified using reverse phase chromatography to afford white solid reaction products **9**–**18** in



Scheme 1. Synthesis of 5-carboxyuracil esters. R = CH_3 (**1**); $(CH_2)_2CH_3$ (**2**), $CH(CH_3)_2$ (**3**), $(CH_2)_3CH_3$ (**4**), cyclohexyl (**5**), cyclooctyl (**6**), $CH_2C{\equiv}CH$ (**7**)



Scheme 2. Synthesis of 5-carboxyuracil amides. R′ = oleylamine (**8**), D-Ala (**9**), β-Ala (**10**), L-Val (**11**), D-Val (**12**), L-Leu (**13**), D-Leu (**14**), L-Met (**15**), D-Met (**16**), L-Phe (**17**), D-Phe (**18**)

a moderate yield. Oleyl 5-carboxyuracil amide **8** was purified by column chromatography on silica gel using chloroform/methanol mixtures as an eluent.

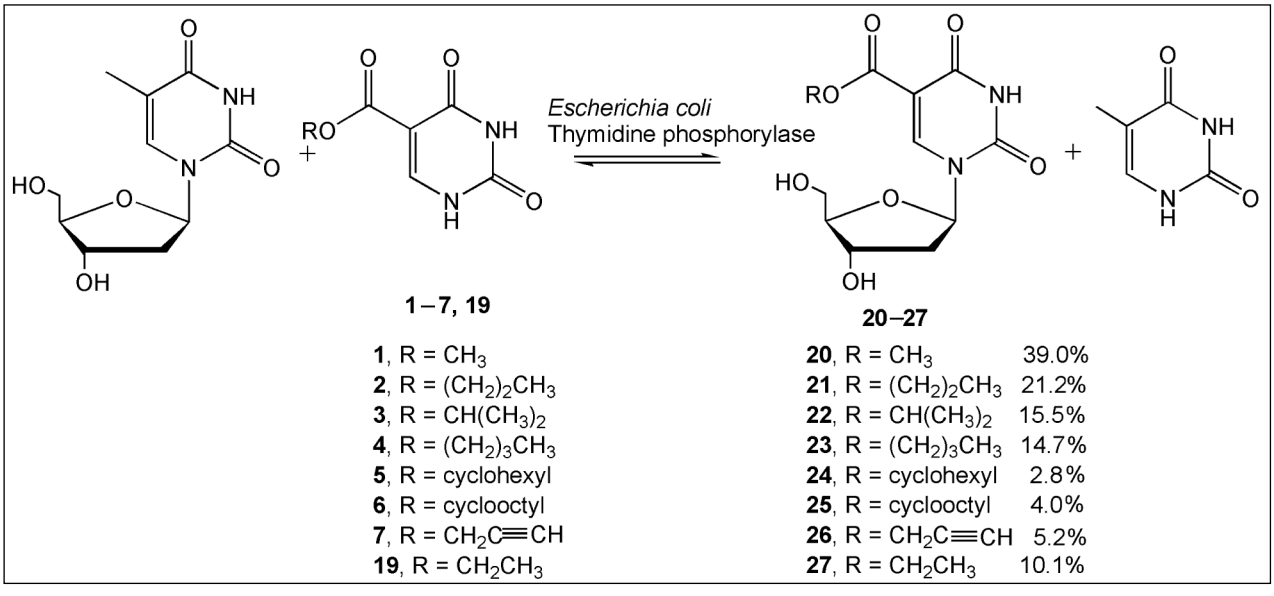
The identity and purity (>98%) of novel compounds were confirmed by NMR (^{1}H and ^{13}C) spectroscopy, high-resolution mass spectra (HRMS) and HPLC-MS analysis.

Inhibition of DNA synthesis by the novel compounds

Synthesized 5-carboxyuracil derivatives **1**–**18** were evaluated as inhibitors in the assays of DNA biosynthesis, carried on two biologically relevant reverse transcriptases – M. MuLV and HIV-1. In short, the 23-nucleotide length primer was hybridized to the 35 nt DNA template strand to form a fully complementary duplex, facilitating primer extension using natural 2'-deoxynucleoside-5'-triphosphates. The presence of compounds **1**–**18** (up to 10 mM, up to 10 000 fold excess over dTTP) did not inhibit the incorporation of dTTP by both M. MuLV and HIV-1 reverse transcriptase (data not shown). Notably, the assay was extremely sensitive and allowed the detection of several consecutive nucleotide incorporation events (up to four in total) individually. Based on the obtained data, incompatibility of compounds **1**–**18** with the active centre of the tested reverse transcriptases could be concluded. However, this does not exclude affinity of the tested compounds to other DNA and/or RNA polymerases, not examined in the present work.

Enzymatic synthesis of C5 modified uridine nucleosides

Even though newly synthesized 5-carboxyuracil esters and amides have not inhibited DNA synthesis catalysed by reverse transcriptases, uridine compounds functionalized at position C5 are of great interest for the preparation of bioactive nucleoside derivatives as DNA synthesis inhibitors or non-natural nucleoside analogues for nucleic acid synthesis [19–22]. Moreover, it is expected that **1**–**18** based nucleosides might be more potent inhibitors of various polymerases. Unfortunately, the chemical synthesis of 5-carboxyuridine esters and amides is extremely complicated or even impossible. To overcome this bottleneck, one-pot



Scheme 3. Synthesis of modified nucleosides **20**–**27** via transglycosylation catalyzed by thymidine phosphorylase from *Escherichia coli*

reaction using thymidine phosphorylase and synthesized compounds **1**–**7, 9, 10, 13, 14** as well as commercially available 5-carbethoxyuracil **19** was carried out for the production of functionalized non-natural uridine nucleosides. This enzyme mediates the nucleobase-exchange reaction to convert thymidine to non-natural nucleosides possessing a functional group at position C5 [9, 10].

The HPLC-MS analysis of the reaction components showed that thymidine phosphorylase was active towards all tested 5-carboxyuridine esters (**1**–**7, 19)** forming the corresponding nucleosides **20**–**27** (Scheme 3). The highest yield of thymidine conversion to the corresponding uridine nucleoside was 39% using 5-carboxyuracil ester (methyl 2,4-dioxo-1*H*,3*H*-pyrimidine-5-carboxylate, **1**) as the second substrate. 5-Carboxyuracil esters possessing a group larger than a methyl group were less efficient substrates of the thymidine phosphorylase. The yield of thymidine conversion varied from 21.2% to 2.8% in the case of 5-carboxyuracil ester **2** and 5-carboxyuracil ester **7**, respectively. Meanwhile, no conversion was observed using the 5-carboxyuridine amides.

Further development and optimisation of this enzymatic method will provide new synthetic approaches to generate novel functionalized non-natural uridines, currently unavailable by known chemical procedures.

CONCLUSIONS

The present work describes the synthesis and characterization of 13 novel uracil compounds. In addition, five previously known 5-carboxyuracil esters were prepared by the new procedure using dicyclohexylcarbodiimide as a coupling reagent. For the first time, the enzymatic synthesis of modified nucleosides bearing the esters of 5-carboxyuracil as bases is presented.

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NAUJŲ 5-KARBOKSIURACILO DARINIŲ SINTEZĖ

S a n t r a u k a

Susintetinta ir apibūdinta 13 naujų 5-karboksiuracilo darinių, turinčių esterinę ir amidinę grupes. Ištirtas susintetintų junginių inhibitorinis poveikis DNR polimerazėms – M. MuLV ir HIV-1 atvirkštinėms transkriptazėms. Įrodyta, kad metilo, etilo, propilo, izopropilo butilo, cikloheksilo, ciklooktilo ir propin-2-ilo 5-karboksiuracilo esteriai yra timidino fosforilazės substratai naujų modifikuotų nukleozidų sintezėje.