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Synthesis, X-ray crystal structure study and antitumoral evaluations of 5,6-disubstituted pyrimidine derivatives

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ABSTRACT

5,6-Disubstituted pyrimidine derivatives (**3**–**20**) were prepared by intramolecular cyclization reaction of α-(1-carbamyliminomethylene)-γ-butyrolactone (**2**) with sodium ethoxide and subsequent chemical transformation of 2-hydroxy group in C-5 side chain as well as lithiation reaction for introduction of acyclic side chain at C-6. All compounds were characterized by ^{1}H NMR, ^{13}C NMR and mass spectra. Structures of compounds **4**, **7** and **14** were unambiguously confirmed by X-ray crystal structural analysis. Supramolecular structures of these three compounds differ significantly. Two N–H···O and one C–H···O hydrogen bonds in **4** form three-dimensional network. One O–H···N hydrogen bond and one π···π interaction self-assemble the molecules of **7** into sheets. In supramolecular aggregation of **14**, only π···π stacking interactions participate, so forming chains. The compounds were evaluated for their cytostatic activities against human malignant cell lines. Of all tested compounds, 2,4-dimethoxy-5-methoxytritylethylpyrimidine (**9**) and 2,4-dichloro-5-chloroethylpyrimidine (**14**) exhibited the most prominent inhibitory effects. Furthermore, compound **14** showed marked activity against human colon carcinoma (IC_{50} = 0.4 μM).

1. Introduction

Uracil derivatives substituted either at C-5 or C-6 positions, as well as their nucleosides, have significant status in the field of chemotherapy. 5-Substituted uracil analogs have been investigated extensively for use in cancer[1,2] and viral chemotherapy,[3,4] as enzyme inhibitors[5–8] and in the synthesis of modified nucleotides.[9,10] In addition, pyrimidines substituted at position C-6, as for example, 1-[(2-hydroxyethyl)-methyl]-6-(phenylthio)thymine (HEPT)[11,12] or 3,4-dihydro-2-alkoxy-6-benzyl-4-oxopyrimidines (DABOs)[13] and their derivatives[14–16] exhibited a potent and selective activity against human immunodeficiency virus type-1 (HIV-1). Although these compounds are relatively simple from a structural point of view, they have been the subject of great deal of chemical work.

'Suicide' gene therapy is one of the most promising approaches in cancer therapy.[17] The most intensively studied 'suicide' gene is herpes simplex virus type 1 thymidine kinase (HSV-1 TK),[18] which can activate prodrugs, such as nucleoside analogs, to form toxic drugs that may kill the cell.[19] For example, 5-(2-fluoroethyl)deoxyuridine, labeled with radioisotopes has an application in positron emission tomography (PET) to monitor the successful transfection of gene product in vivo during cancer-prodrug therapy.[20]

Recently, we have reported that some C-5 and/or C-6 substituted pyrimidine derivatives exhibited antiviral and cytostatic activities.[21–24] Syntheses and biological evaluations of C-6 alkylated pyrimidines as model compounds for development of tracer molecules in PET have been also reported.[25,26] Thus, we found that pyrimidine derivatives containing 2,3-dihydroxypropyl and 1,3-dihydroxyisobutyl side chain can be efficiently phosphorylated by HSV-1 TK and thus fulfill the necessary requirements for in vivo imaging of the HSV-1 TK gene expression.[25]

Therefore, biological activities by both 5-substituted and 6-substituted uracil derivatives encouraged us to explore chemistry and biological activities of novel 5,6-disubstituted uracil derivatives. In this paper we present syntheses, X-ray crystal structural study and cytostatic evaluations of pyrimidine derivatives (**3**–**20**) bearing substituents at positions C-5 and C-6 of pyrimidine ring (Figure 1).

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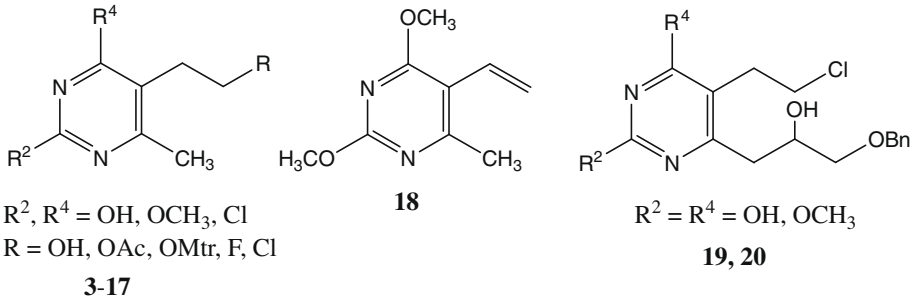
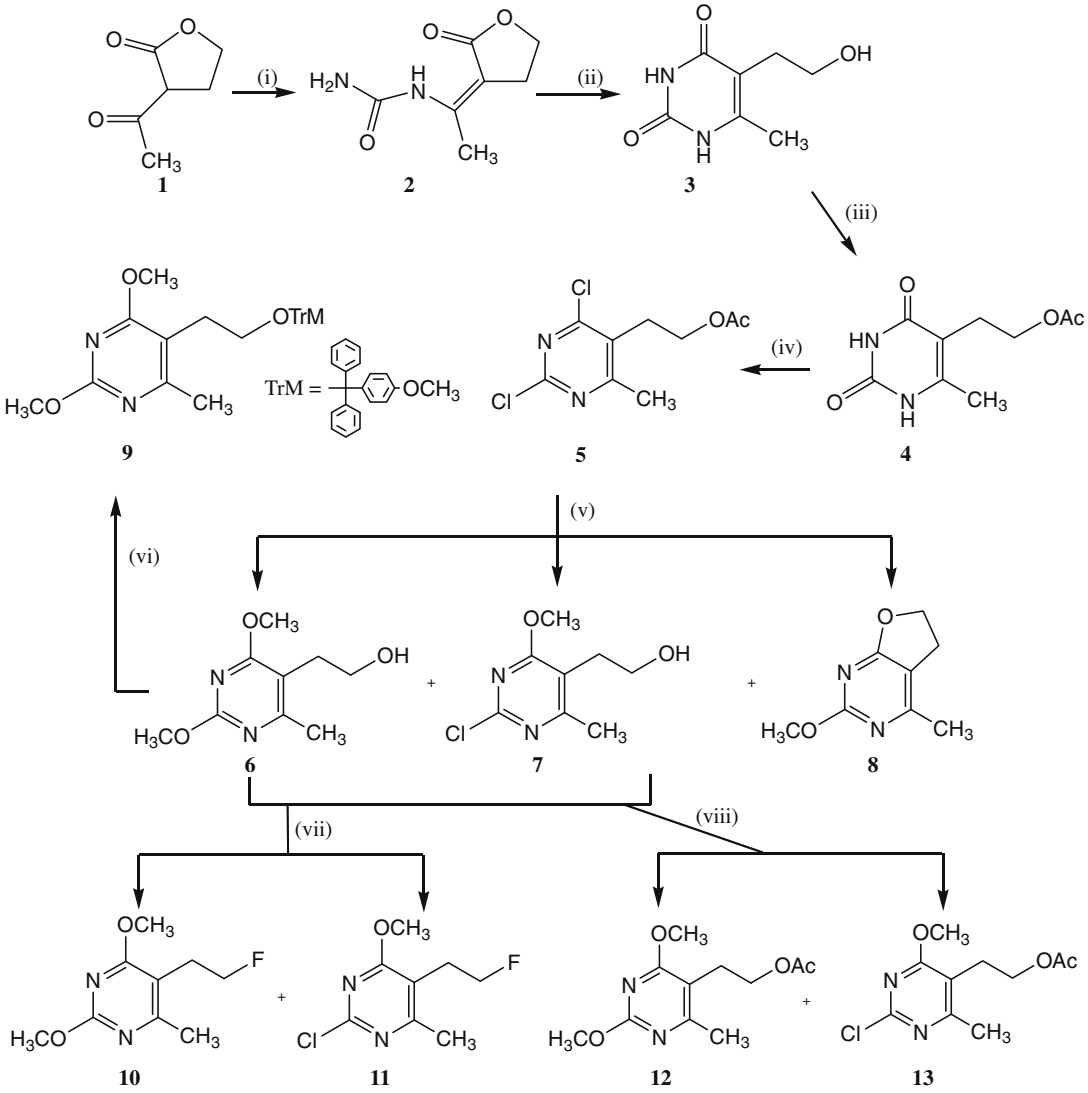


Figure 1. The new 5,6-disubstituted pyrimidine derivatives (**3**–**20**).

2. Results and discussion

2.1. Chemistry

α-(1-Carbamyliminomethylene)-γ-butyrolactone (**2**) was prepared by condensation of α-acetyl-γ-butyrolactone (**1**) with urea in ethanol. Intramolecular cyclization reaction occurred when compound **2** was treated with sodium ethoxyde to give pyrimidine derivative **3**[27,28] (Scheme 1). Primary hydroxyl group in **3** was protected to give 5-acetoxy-6-methyl substituted uracil derivative (**4**) which was subsequently chlorinated using phosphoryl chloride with *N*,*N*-diethylaniline yielding corresponding 2,4-dichloropyrimidine derivative **5**. Methoxylation of **5** gave a mixture of 2,4-dimethoxypyrimidine (**6**) and 2-chloro-4-methoxypyrimidine (**7**) containing 2-hydroxyethyl side chain, as well as bicyclic furo[2,3-*d*]pyrimidine derivative (**8**). Protection of hydroxyl group in



Scheme 1. Reagents and conditions: (i) urea, HCl, EtOH; (ii) NaOEt/EtOH; (iii) acetic anhydride, pyridine; (iv) $POCl_3$, *N*,*N*-diethylaniline, pyridine; (v) NaOMe/MeOH; (vi) 4-methoxytriphenylmethyl chloride, triethylamine, *N*,*N*-dimethylaminopyridine, DMF; (vii) DAST, CH_2Cl_2; (viii) acetic anhydride, pyridine.

compound **6** was performed with (*p*-methoxytriphenyl)methyl chloride in DMF in the presence of triethylamine and *N*,*N*-dimethylaminopyridine to give *p*-methoxytritylated derivative **9**. Transformation of hydroxyl group into fluorine was achieved with diethylaminosulfur trifluoride (DAST) as reagent. Thus, when a mixture of 5-(2-hydroxyethyl) substituted pyrimidine derivatives **6** and **7** reacted with DAST corresponding 5-(2-fluoroethyl) substituted pyrimidines (**10** and **11**) were obtained. Reaction mixture of **6** and **7** in acetic anhydride and dry pyridine gave acetylated **12** and **13**, respectively (Scheme 1).

The introduction of acyclic side chain at position C-6 in pyrimidine derivatives **9**, **10** and **12** by lithiation using lithium diisopropylamide (LDA) followed by addition of benzyloxyacetaldehyde to the lithiated intermediate failed. When compounds **10** and **12** were submitted to C-6 condensation defluorination and deacetylation occurred and 2,4-dimethoxy-5-(2-hydroxyethyl)-6-methylpyrimidine (**6**) was isolated as the product. On the other hand, in the same reaction of *p*-methoxytritylated derivative (**9**) starting compound **9** was isolated. This may be caused by spatial hindrance due to bulky trityl moiety in **9**.

Chlorination reaction of 5-(2-hydroxyethyl)-6-methylpyrimidine (**3**) proceeded smoothly and the desired trichlorinated derivative **14** was obtained (Scheme 2). In this reaction monochlorinated (**15**) and dichlorinated (**16**) pyrimidines were also isolated. Subsequent methoxylation of **14** using sodium methoxide yielded 2,4-dimethoxypyrimidine derivative **17** with chloroethyl substituent at C-5 of pyrimidine ring and its 5-vinyl analog **18** as a product of dehydrohalogenation. Lithiation of compound **17** with LDA afforded organometallic intermediate that reacted in situ with benzyloxyacetaldehyde to give **19** bearing the C-6 3-benzyloxy-2-hydroxypropyl side chain. Removal of 2,4-dimethoxy protecting groups with sodium iodide and trimethylchlorosilane (TMSCl) yielded corresponding pyrimidin-2,4-dione derivative **20**.

Newly prepared compounds **3–20** were characterized by ^{1}H NMR, ^{13}C NMR and mass spectra. The assignment of ^{1}H and ^{13}C NMR spectra was performed on the basis of the chemical shift, magnitude and multiplicity of H–H spin–spin coupling, as well as connectivities in two-dimensional homo and heteronuclear correlation spectra.

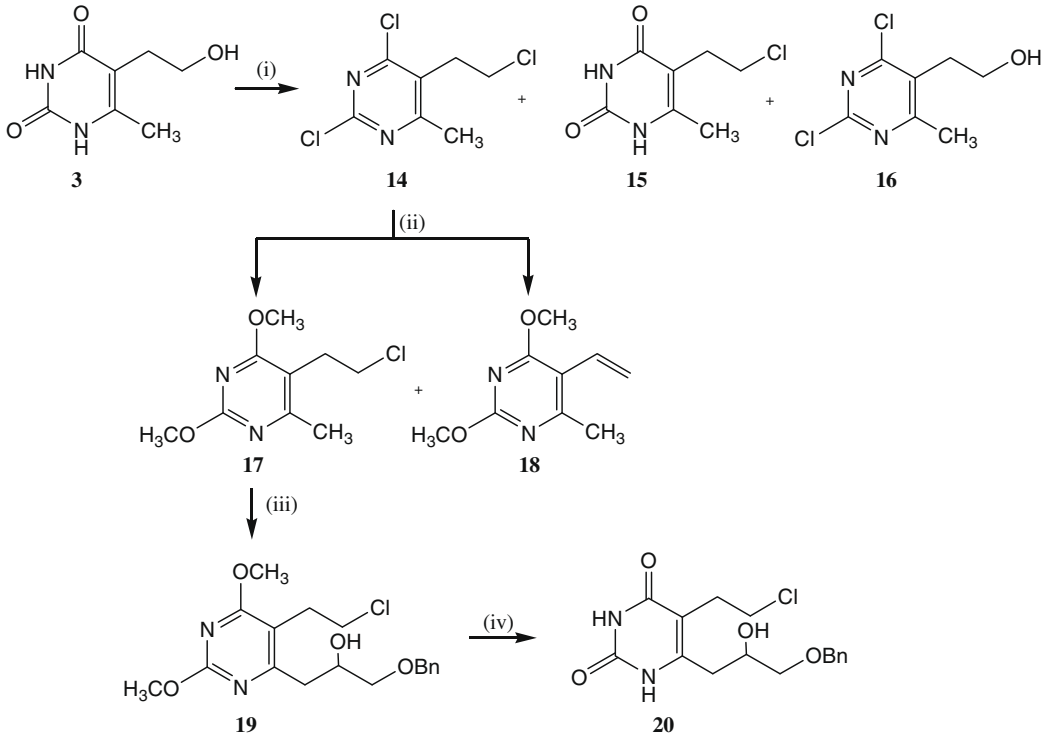
2.2. X-ray crystal structure analysis

The molecular structures of **7** and **14** are shown in Figs. 2 and 3, respectively. Two 2-chloro-6-methylpyrimidines differ in substituent at C-5 of pyrimidine ring, which is 2-hydroxyethyl group in **7** and 2-chloroethyl group in **14**. The geometrical parameters of pyrimidine ring in these two structures are very similar, and in accord with those in closely related 2-chloro-6-methylpyrimidines. The exception is N3–C4 bond in benzyl-(2-chloro-6-methylpyrimidin-4-yl)amine hemihydrate,[29] which is slightly longer (ca. 0.03 Å) due to the influence of the benzylamine group on the pyrimidine ring geometry.

The conformation of the alkyl chain in **7** and **14** is locked by intramolecular hydrogen bonds, C7···O1 in **7** and C7···Cl2 in **14** (Table 1; Figs. 2 and 3). Both intramolecular hydrogen bonds form five-membered rings which can be described by graph-set notation as $S(5)$.[30]

The hydroxyl hydrogen atom of 2-hydroxyethyl group in **7** participates in supramolecular aggregation via O2···N1 hydrogen bond. This intermolecular interaction forms C(7) chains[30] parallel to the *c* axis (Table 1; Fig. 4). Hydrogen-bonded chains are weakly linked by one π···π stacking interaction into (0 1 0) sheets.[31] The distance between the ring centroids of the coplanar pyrimidine rings [$\alpha = 0°$] is 3.5649(7) Å, the planes are separated by ca. 3.52 Å, and centroids offset amounts ca. 0.57 Å.

The molecules of **14**, arranged in herringbone fashion (Fig. 5), are self-assembled by two weak aromatic π···π stacking interactions between the pyrimidine rings of the neighboring molecules. An interplanar angle between the rings is 0.77°, interplanar spacing is ca. 3.54 Å, a centroid separations are 3.9066(16) and 3.9067(16) Å, and corresponding centroid-centroid offset ca. 1.66 Å. These π···π interactions link the molecules parallel to the *a* axis into chains.



Scheme 2. Reagents and conditions: (i) $POCl_3$, *N*,*N*-diethylaniline, pyridine; (ii) $NaOCH_3$/MeOH; (iii) LDA, benzyloxyacetaldehyde; (iv) trimethylchlorosilane, NaI, acetonitrile.

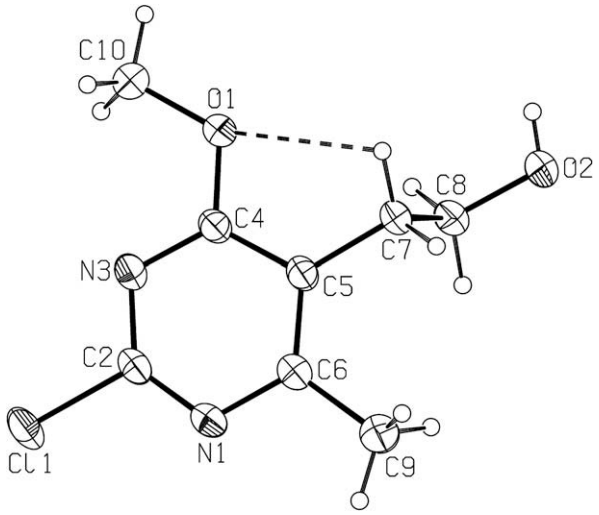


Figure 2. A molecular structure of **7**, with the atom-numbering scheme. Displacement ellipsoids for non-hydrogen atoms are drawn at the 30% probability level. Intramolecular hydrogen bond is shown dashed.

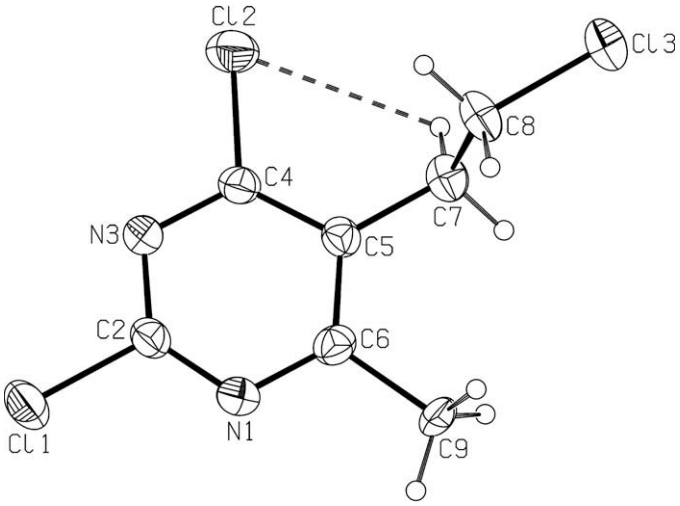


Figure 3. A molecular structure of **14**, with the atom-numbering scheme. Displacement ellipsoids for non-hydrogen atoms are drawn at the 30% probability level. Intramolecular hydrogen bond is shown dashed.

In compound **4** (Fig. 6), 2-acetoxyethyl chain is bonded to the pyrimidine ring C-5 atom. The geometry of pyrimidine-2,4-dione is in a good agreement with similar structures in which alkyl chain is bonded to C-5 atom of the pyrimidine ring.[32,33] The planes of the C8/O3/C10/O4/C11 atoms and pyrimidine rings' atoms are almost coplanar to each other. Their mean planes form an angle of 5.28(11)°.

The N1···O1 hydrogen bond in **4** generates $C(4)$ chains (Table 1; Fig. 7), while N3···O3 hydrogen bond forms $R_2^2(8)$ centrosymmetric dimers.[30] These two N–H···O hydrogen bonds are reinforced by

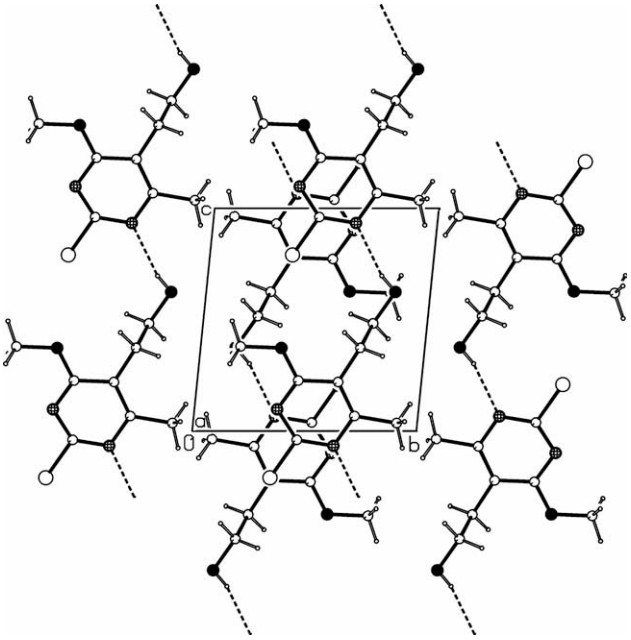


Figure 4. A crystal packing diagram of **7**, viewed along the *a* axis, showing O2···N1 hydrogen bond that link the molecules parallel to the *c* axis and stacking of the pyrimidine rings. Hydrogen bonds are indicated by dashed lines.

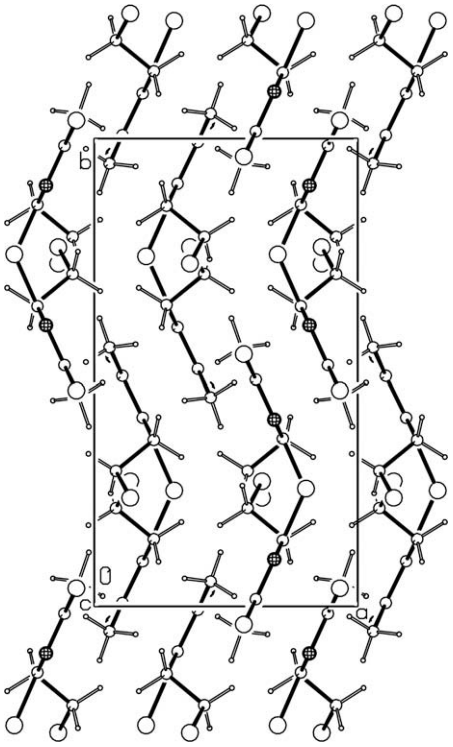


Figure 5. A crystal packing diagram of **14**, viewed along the *c* axis, showing the molecules arranged in herringbone fashion.

Table 1
Hydrogen-bonding geometry for compounds **4**, **7** and **14**

	D···A	D–H (Å)	H···A (Å)	D···A (Å)	D–H···A (°)	Symmetry codes
4	N1···O1	0.87(2)	1.99(2)	2.846(2)	170(2)	$-x, -1/2+y, 1/2-z$
	N3···O2	0.89(2)	1.97(2)	2.858(2)	175(2)	$-x, 1-y, -z$
	C9···O4	0.96	2.41	3.252(3)	147	$1-x, ½+y, 1/2-z$
7	C7···O1	0.97	2.43	2.7832(15)	101	
	O2···N1	0.80(2)	2.15(2)	2.9314(15)	166(2)	$x, y, 1+z$
14	C7···Cl2	0.97	2.72	3.076(3)	103	

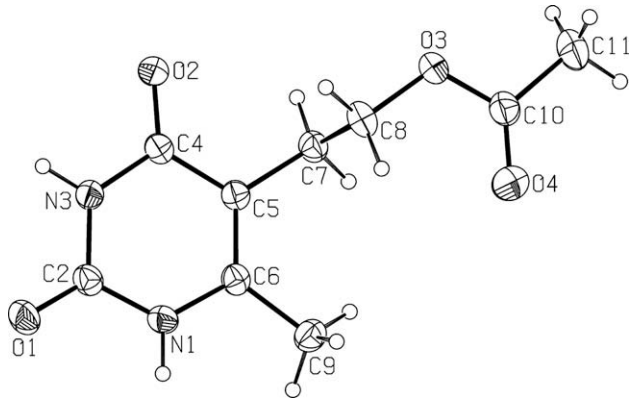


Figure 6. A molecular structure of **4**, with the atom-numbering scheme. Displacement ellipsoids for non-hydrogen atoms are drawn at the 30% probability level.

one C–H···O hydrogen bond, C9···O4, which link the molecules into *C*(9) chains.

2.3. Antitumoral activities

The compounds **4–15**, **17**, **19** and **20** were evaluated for their cytostatic activities against human malignant tumor cell lines: acute lymphoblastic leukemia (Molt-4), colon carcinoma (HCT 116 and SW 620), breast carcinoma (MCF-7) and lung carcinoma (H 460). Their activities were compared with those of 5-fluorouracil (5-FU) and cytosine arabinoside (Ara-C) (Table 2).

Of all compounds evaluated, 2,4-dimethoxy-5-methoxytritylethylpyrimidine (**9**) and 2,4-dichloro-5-chloroethylpyrimidine (**14**) derivatives exhibited the most pronounced inhibitory effect (IC_{50} in the range 0.4–50 μM). 5-Acetoxy-2,4-dimethoxypyrimidine (**12**) and 5-chloroethyl-2,4-dimethoxypyrimidine with 6-(3-benzyloxy-2-hydroxypropyl) side chain (**19**) showed moderate cytostatic activities (**12**: IC_{50} ~49 μM, **19**: IC_{50} ~31 μM). It is interesting to note that methoxytritylation and acetylation of hydroxyl group in **6** caused a significant increase of antitumor activity of **9** and **12**. On the contrary to this, their structural congener **6** with unprotected 5-hydroxyethyl substituent showed no inhibitory effect. This might be due to the greater lipophilicity of the methoxytritylated and acetylated derivatives. Thus, 2,4-dimethoxy-5-methoxytritylethylpyrimidine (**9**) showed the strongest inhibitory effect against human colon carcinoma (HCT 116: IC_{50} = 4 μM) and acute lymphoblastic leukemia (Molt-4: IC_{50} = 5 μM), while 5-acetoxy-2,4-dimethoxypyrimidine (**12**) displayed moderate cytostatic activity against all tested cell lines. However, pyrimidin-2,4-dione derivatives (**3**, **4**, **15** and **20**) did not exhibit any inhibitory effects (IC_{50} >100 μM). The presence and identity of halogen substituents have significant impact on biological activity. Thus, the formation of halogen bonds in molecules is recognized as a kind of intermolecular interaction that favorably contributes to the stability of ligand-target complexes.[34] Interestingly, increase in the chlorine content enhanced the antiproliferative effect. Therefore, compound **14** containing both aliphatic and aromatic chlorine atoms possessed the most significant inhibitory effect. This compound inhibited colon carcinoma SW 620 in submicromolar range (IC_{50} = 0.4 μM). Influence of bromine in 2,4-dimethoxypyrimidine derivatives on

Table 2
Inhibitory effects of compounds **3–15**, **17**, **19** and **20** on the growth of human malignant tumor cell lines

Compd	IC_{50}[a] (μM)				
	Molt-4	HCT 116	SW 620	MCF-7	H 460
3	>100	>100	>100	>100	>100
4	>100	>100	>100	>100	>100
5	>100	>100	>100	>100	>100
6	>100	>100	>100	>100	>100
7	N.T.	>100	>100	>100	>100
8	N.T.	>100	>100	>100	>100
9	5 ± 4	4 ± 0.03	20 ± 3	6 ± 3	50 ± 34
10	N.T.	>100	>100	>100	>100
11	N.T.	>100	>100	>100	>100
12	N.T.	40 ± 10	44 ± 2	65 ± 19	47 ± 15
13	>100	>100	>100	>100	>100
14	5 ± 3	2 ± 0.2	0.4 ± 0.2	8 ± 7	3 ± 0.1
15	>100	>100	>100	>100	>100
17	>100	>100	>100	>100	>100
19	19 ± 11	16 ± 1	20 ± 4	26 ± 12	76 ± 26
20	>100	>100	>100	>100	>100
5-FU[b]	0.03 ± 0.01	4 ± 0.9	9 ± 2	15 ± 2	2 ± 0.6
Ara-C[b]	1 ± 0.2	0.1 ± 0.1	30 ± 23	⩾100	0.1 ± 0.02

[a] IC_{50}—the concentration that causes 50% growth inhibition.
[b] 5-FU—5-fluorouracil, Ara-C—cytosine arabinoside.

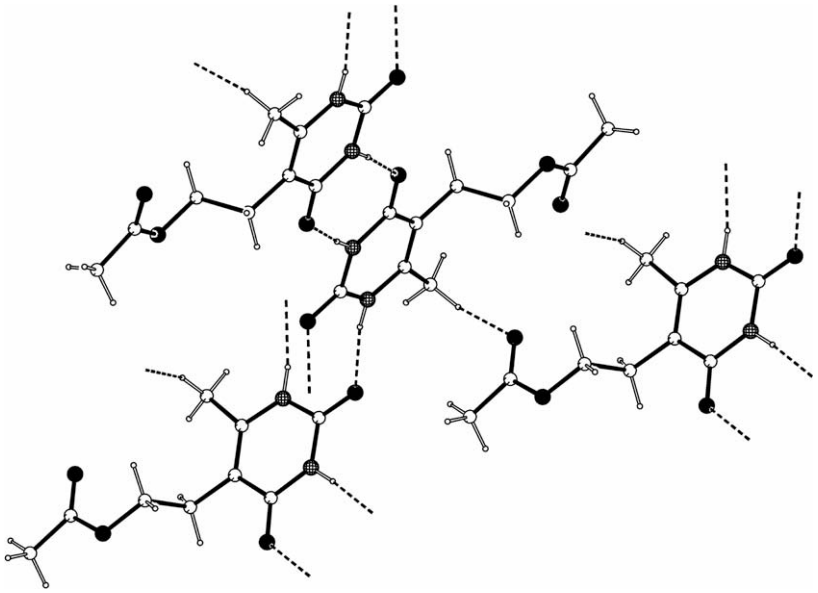


Figure 7. Part of the crystal structure of **4**, showing the N–H···O and C–H···O hydrogen bonds. Hydrogen bonds are indicated by dashed lines.

antitumoral activity was determined in our previous paper.[22] Thus, pyrimidine derivative that bears one aromatic and two aliphatic bromine atoms showed the highest inhibitory activity against all tested malignant tumor cell lines.

Comparison of cytostatic activities of novel compounds with those of 5-FU and Ara-C revealed that **14** inhibited more pronounced the growth of HCT 116 and MCF-7 cell lines than 5-FU. Furthermore, inhibitory effect of this compound was approximately 20-fold higher against SW 620 cell line than that of 5-FU. Besides, compounds **9**, **14** and **19** expressed better antiproliferative effects on SW 620 and MCF-7 cells than Ara-C.

3. Conclusions

Novel 5,6-disubstituted pyrimidine derivatives (**3**–**20**) were synthesized and characterized. The structures of compounds **4**, **7** and **14** were determined by X-ray crystal structural analysis. These three compounds built completely different supramolecular architectures. $\pi\cdots\pi$ stacking interactions participate in supramolecular aggregation of **14**, so forming chains. One O–H···N hydrogen bond and one $\pi\cdots\pi$ interaction self-assemble the molecules of **7** into sheets, while two N–H···O and one C–H···O hydrogen bonds in **4** form three-dimensional network.

Compounds were evaluated for their cytostatic activities against human malignant cell lines. By comparison of cytostatic activities of novel 5,6-disubstituted pyrimidine derivatives and 5-FU and Ara-C, we can infer that compound **14** exhibited higher inhibitory effects against SW 620, HCT 116 and MCF-7 cells than 5-FU, while compounds **9**, **14** and **19** revealed better antiproliferative effects against SW 620 and MCF-7 cells than Ara-C. Finally, compounds **9** and **14** emerged as the most interesting compounds with cytostatic activities that could be used for further structural optimization.

4. Experimental

4.1. General methods

Melting points (uncorrected) were determined with Kofler micro hot-stage (Reichert, Wien). Precoated Merck Silica Gel 60F-254 plates were used for thin layer chromatography (TLC) and the spots were detected under UV light (254 nm). Column chromatography (CLC) was performed using silica gel (0.063–0.2 mm) Fluka; glass column was slurry-packed under gravity. ^{1}H and ^{13}C NMR spectra were acquired on Bruker 300 MHz NMR spectrometer. All data were recorded in DMSO-d_6 at 298 K. Chemical shifts were referenced to the residual solvent signal of DMSO at δ 2.50 ppm for ^{1}H and δ 39.50 ppm for ^{13}C. Individual resonances were assigned on the basis of their chemical shifts, signal intensities, multiplicity of resonances and H–H coupling constants.

4.1.1. Procedures for the preparation of compounds

4.1.1.1. α-(1-Carbamyliminomethylmethylene)-γ-butyrolactone (2). To a solution of α-acetyl-γ-butyrolactone (**1**) (60 g, 0.46 mol) and urea (32 g, 0.54 mol) in ethanol (10 mL) 4–5 drops of 36% hydrochloric acid were added and the reaction mixture was heated at 70 °C for 20 h. The precipitate was collected by filtration to yield compound **2** as the white powder (51 g, 65%). Mp = 170–172 °C. ^{1}H NMR: δ 9.78 (1H, s, NH), 6.75 (2H, s, NH_2), 4.28 (2H, t, J = 7.72 Hz, OCH_2), 2.83 (2H, t, J = 7.67, CH_2), 2.27 (3H, s, CH_3) ppm. ^{13}C NMR: δ 172.63 (C=O lactone), 160.26 (C=O), 150.62 (C-2′), 97.09 (C-2), 65.66 (C-4), 25.72 (C-3), 19.08 (C-3′) ppm.

4.1.1.2. 5-(2-Hydroxyethyl)-6-methylpyrimidin-2,4-dione (3). To absolute ethanol (30 mL) sodium (0.87 g, 0.038 mol) was added in small portions followed by addition of compound **2** (5.1 g, 0.03 mol). The reaction mixture was refluxed for 18 h and water (15 mL) was added to the cooled reaction mixture. pH was adjusted to ~3 by adding few drops of 98% sulfuric acid. The solution was kept in refrigerator overnight, white crystals of compound **3** were filtered off and washed with anhydrous ether (5.1 g, 100%). Mp = 219–221 °C. ^{1}H NMR: δ 10.88 (1H, s, NH), 10.60 (1H, s, NH), 4.53 (1H, s, OH), 3.36 (2H, t, J = 6.73 Hz, H-2′), 2.37 (2H, t, J = 6.87 Hz, H-1′), 2.06 (3H, s, CH_3) ppm. ^{13}C NMR: δ 164.94 (C-4), 151.30 (C-2), 149.37 (C-6), 106.37 (C-5), 60.15 (C-2′), 28.47 (C-1′), 16.69 (CH_3) ppm.

MS m/z 171 $[MH]^+$. Anal. Calcd for $[C_7H_{10}N_2O_3]$: C, 49.41; H, 5.92; N, 16.46. Found: C, 49.50; H, 5.90; N, 16.43.

4.1.1.3. 5-(2-Acetoxyethyl)-6-methylpyrimidin-2,4-dione (4). Reaction mixture of compound **3** (200 mg, 1.2 mmol) and acetic anhydride (0.9 mL, 9.8 mmol) in anhydrous pyridine (5.5 mL) was stirred for 1 h under argon atmosphere. Water was then added and mixture was evaporated to dryness. Crude product was purified by column chromatography (CH_2Cl_2/CH_3OH = 20:1) to give colorless crystals of compound **4** (230 mg, 90%). Mp = 190–192 °C. ^{1}H NMR: δ 10.88 (1H, s, NH), 10.61 (1H, s, NH), 4.00 (2H, t, J = 6.83 Hz, H-2′), 2.53 (2H, t, J = 6.90 Hz, H-1′), 2.08 (3H, s, $COCH_3$), 1.98 (3H, s, CH_3) ppm. ^{13}C NMR: δ 170.72 (C=O), 164.72 (C-4), 151.17 (C-2), 149.85 (C-6), 105.17 (C-5), 62.75 (C-2′), 24.31 (C-1′), 21.13 ($COCH_3$), 16.45 (CH_3) ppm.

MS m/z 213 $[MH]^+$. Anal. Calcd for $[C_9H_{12}N_2O_4]$: C, 50.94; H, 5.70; N, 13.20. Found: C, 50.89; H, 5.71; N, 13.25.

4.1.1.4. 5-(2-Acetoxyethyl)-2,4-dichloro-6-methylpyrimidine (5). A mixture of compound **4** (1.3 g, 6.13 mmol), $POCl_3$ (12.1 mL), *N,N*-diethylaniline (1.2 mL) and pyridine was refluxed for 1 h. The solvent was evaporated to dryness, and the residue was diluted with iced water, washed with dichloromethane and dried over Na_2SO_4. The oily product was purified by column chromatography (CH_2Cl_2/CH_3OH = 50:1) to afford compound **5** as brown oil (1.16 g, 76%). ^{1}H NMR: δ 4.23 (2H, t, J = 6.66 Hz, H-2′), 2.53 (2H, t, J = 6.66 Hz, H-1′), 2.58 (3H, s, $COCH_3$), 1.98 (3H, s, CH_3) ppm. ^{13}C NMR: δ 172.56 (C=O), 170.70 (C-4), 161.95 (C-2), 156.58 (C-6), 128.06 (C-5), 61.47 (C-2′), 28.17 (C-1′), 22. 85 ($COCH_3$), 21.06 (CH_3) ppm.

MS m/z 248 250 252 $[M^+, M+2, M+4]^+$. Anal. Calcd for $[C_9H_{10}N_2O_2\ Cl_2]$: C, 43.40; H, 4.05; N, 11.25. Found: C, 43.36; H, 4.06; N, 11.22.

4.1.1.5. 5-(2-Hydroxyethyl)-2,4-dimethoxy-6-methylpyrimidine (6), 2-chloro-5-(2-hydroxyethyl)-4-methoxy-6-methylpyrimidine (7) and 2-methoxy-4-methyl-5,6-dihydrofuro[2,3-*d*]pyrimidine (8). To a solution of sodium (40 mg, 1.74 mmol) in absolute methanol (30 mL) compound **5** (200 mg, 0.8 mmol) was added and reaction mixture was refluxed for 3 h. The solvent was removed in vacuo and crude product was chromatographed on silica column (CH_2Cl_2/CH_3OH = 60:1) to afford oily compound **6** (53.8 mg, 34%), **7** (42.6 mg, 26%, mp = 101–103 °C) and bicyclic derivative **8** (28.3 mg, 21%, mp = 105–107 °C) as white solids.

Compound **6**: ^{1}H NMR: δ 4.64 (1H, t, J = 5.58 Hz, OH), 3.87 (3H, s, OCH_3), 3.83 (3H, s, OCH_3), 3.44 (2H, q, J = 6.60 Hz, H-2′), 2.63 (2H, t, J = 7.14 Hz, H-1′), 2.34 (3H, s, CH_3) ppm. ^{13}C NMR: δ 169.48 (C-4), 162.79 (C-6), 158.14 (C-2), 109.56 (C-5), 60.00 (C-2′), 55.20 (OCH_3), 55.07 (OCH_3), 28.64 (C-1′), 21.83 (CH_3) ppm.

MS m/z 199 $[MH]^+$. Anal. Calcd for $[C_9H_{14}N_2O_3]$: C, 54.53; H, 7.12; N, 14.13. Found: C, 54.42; H, 7.12; N, 14.14.

Compound **7**: ^{1}H NMR: δ 4.71 (1H, t, J = 5.61 Hz, OH), 3.92 (3H, s, OCH_3), 3.71 (2H, q, J = 6.64 Hz, H-2′), 2.69 (2H, t, J = 6.77 Hz, H-1′), 2.41 (3H, s, CH_3) ppm. ^{13}C NMR: δ 168.66 (C-4), 168.05 (C-6), 155.33 (C-2), 115.64 (C-5), 59.03 (C-2′), 54.74 (OCH_3), 28.26 (C-1′), 21.18 (CH_3) ppm.

MS *m/z* 202 204 [M^+, M+2]. Anal. Calcd for [$C_8H_{11}N_2O_2Cl$]: C, 47.42; H, 5.47; N, 13.82. Found: C, 47.37; H, 5.46; N, 13.83.

Compound **8**: ^{1}H NMR: δ 3.81 (3H, s, OCH_3), 4.65 (2H, t, J = 8.58 Hz, H-2′), 2.80 (2H, t, J = 6.87 Hz, H-1′), 2.35 (3H, s, CH_3) ppm. ^{13}C NMR: δ 166.44 (C-4), 168.34 (C-6), 159.38 (C-2), 108.26 (C-5), 66.20 (C-2′), 54.32 (OCH_3), 44.82 (C-1′), 21.97 (CH_3) ppm.

MS *m/z* 167 [MH]$^+$. Anal. Calcd for [$C_8H_{11}N_2O_2Cl$]: C, 57.82; H, 6.07; N, 16.86. Found: C, 57.94; H, 6.07; N, 16.88.

4.1.1.6. 2,4-Dimethoxy-5-[2-(4-methoxytriphenylmethoxy)ethyl]-6-methylpyrimidine (9). The reaction mixture of compound **6** (565 mg, 2.85 mmol), 4-methoxytriphenylmethyl chloride (2.19 g, 7.2 mmol), triethylamine (2.36 mL) and *N,N*-dimethylaminopyridine (40 mg, 0.33 mmol) in anhydrous DMF (5 mL) was stirred under argon atmosphere at 50 °C for 2 h. Solvents were evaporated and residual oil was purified by column chromatography (CH_2Cl_2/CH_3OH = 50:1) yielding oily compound **9** (515.4 mg, 39%). ^{1}H NMR: δ 7.26–7.27 (10H, m, Ph), 7.12 (2H, d, J = 8.82 Hz, Ph), 6.84 (2H, d, J = 8.85 Hz, Ph), 3.84 (3H, s, OCH_3), 3.80 (3H, s, OCH_3), 3.73 (3H, s, OCH_3), 3.12 (2H, t, J = 6.36 Hz, H-2′), 2.76 (2H, t, J = 6.24 Hz, H-1′), 2.30 (3H, s, CH_3) ppm. ^{13}C NMR: δ 169.50 (C-4), 166.82 (C-6), 162.93 (C-2), 158.56 (Cquat-OCH_3), 144.85 (Ph-quat), 135.60 (Cquat-Tr), 130.28 (CH-Ph), 128.22–128.26 (CH-Ph), 127. 25 (CH-Ph), 113.51 (CH-Ph-OCH_3 Tr), 109.44 (C-5), 86.30 (C-quat 3″), 61.99 (C-2′), 55.48 (OCH_3), 54.52 (OCH_3), 54.18 (OCH_3), 25.47 (C-1′), 21.80 (CH_3) ppm.

MS *m/z* 471 [MH]$^+$. Anal. Calcd for [$C_{29}H_{30}N_2O_4$]: C, 74.02; H, 6.43; N, 5.95. Found: C, 73.87; H, 6.45; N, 5.94.

4.1.1.7. 5-(2-Fluoroethyl)-2,4-dimethoxy-6-methylpyrimidine (10) and 2-chloro-5-(2-fluoroethyl)-4-methoxy-6-methylpyrimidine (11). The solution of compounds **6** and **7** (200 mg, 1.03 mmol) in dry CH_2Cl_2 (6 mL) was cooled to -78 °C and stirred for 15 min under argon atmosphere. Then, DAST (0.2 mL, 1.45 mmol) was added dropwise and reaction mixture was stirred at −78 °C for additional 15 min after which cooling bath was removed. After 3 h of stirring at room temperature, saturated aqueous solution of $NaHCO_3$ (5 mL) was added and reaction was partitioned. Organic layer was separated, dried over $MgSO_4$ and evaporated to dryness. Raw product was purified by column chromatography (CH_2Cl_2/CH_3OH = 30:1) to afford compounds **10** (108.3 mg, 59%) and **11** (9.3 mg, 41%) as pale yellow oils.

Compound **10**: ^{1}H NMR: δ 4.57 (1H, t, J_{H-F} = 6.44 Hz, H-2′), 4.41 (1H, t, J_{H-F} = 6.41 Hz, H-2′), 3.89 (3H, s, OCH_3), 3.84 (3H, s, OCH_3), 2.92 (1H, t, J_{H-F} = 6.45 Hz, H-1′), 2.85 (1H, t, J_{H-F} = 6.42 Hz, H-1′), 2.34 (3H, s, CH_3) ppm. ^{13}C NMR: δ 169.55 (C-4), 167.47 (C-6), 163.19 (C-2), 107.53 (d, J_{C-F} = 6.92 Hz, C-5), 82.46 (d, J_{C-F} = 165.86 Hz, C-2′), 54.55 (OCH_3), 54.37 (OCH_3), 26.08 (d, J_{C-F} = 21.16 Hz, C-1′), 21.75 (d, J_{C-F} = 1.32 Hz, CH_3) ppm.

MS *m/z* 201 [MH]$^+$. Anal. Calcd for [$C_9H_{13}N_2O_2F$]: C, 53.99; H, 6.54; N, 13.99. Found: C, 54.04; H, 6.55; N, 14.02.

Compound **11**: ^{1}H NMR: δ 4.64 (1H, t, J_{H-F} = 6.32 Hz, H-2′), 4.48 (1H, t, J_{H-F} = 6.33 Hz, H-2′), 3.94 (3H, s, OCH_3), 3.00 (1H, t, J_{H-F} = 6.32 Hz, H-1′), 2.92 (1H, t, J_{H-F} = 6.32 Hz, H-1′), 2.41 (3H, s, CH_3) ppm. ^{13}C NMR: δ 169.23 (C-4), 168.83 (C-6), 156.50 (C-2), 114.17 (d, J_{C-F} = 5.98 Hz, C-5), 82.08 (d, J_{C-F} = 165.75, C-2′), 55.43 (OCH_3), 26.26 (d, J_{C-F} = 21.22 Hz, C-1′), 21.56 (d, J_{C-F} = 0.91 Hz, CH_3) ppm.

MS *m/z* 204 206 [M^+, M+2]$^+$. Anal. Calcd for [$C_8H_{10}N_2OFCl$]: C, 46.96; H, 4.93; N, 13.69. Found: C, 46.91; H, 4.93; N, 13.72.

4.1.1.8. 5-(2-Acetoxyethyl)-2,4-dimethoxy-6-methylpyrimidine (12) and 5-(2-acetoxyethyl)-2-chloro-4-methoxy-6-methylpyrimidine (13). The compounds **6** and **7** (300 mg, 1.52 mmol) were dissolved in anhydrous pyridine (7 mL) and acetic anhydride (1.18 mL) was added. The reaction mixture was stirred at room temperature for 1 h. After addition of water solvents were removed under reduced pressure and crude product was purified by column chromatography (CH_2Cl_2/CH_3OH = 60:1) to afford oily products **12** (268 mg, 81%) and **13** (27 mg, 93%).

Compound **12**: ^{1}H NMR: δ 4.09 (2H, t, J = 6.84 Hz, H-2′), 3.89 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 2.80 (2H, t, J = 6.87 Hz, H-1′), 2.35 (3H, s, CH_3OAc), 1.97 (3H, s, CH_3), ppm. ^{13}C NMR: δ 171.12 (C═OAc), 160.63 (C-4), 165.23 (C-6), 164.91(C-2), 110.30 (C-5), 60.84 (C-2′), 56.16 (OCH_3), 55.84 (OCH_3), 28.22 (CH_3OAc), 27.73 (C-1′), 20.45 (CH_3) ppm.

MS *m/z* 241 [MH]$^+$. Anal. Calcd for [$C_{11}H_{16}N_2O_4$]: C, 54.99; H, 6.71; N, 11.66. Found: C, 55.15; H, 6.70; N, 11.68.

^{1}H NMR: δ 4.14 (2H, t, J = 6.63 Hz, H-2′), 3.94 (3H, s, OCH_3), 2.86 (2H, t, J = 6.66 Hz, H-1′), 2.42 (3H, s, CH_3), 1.97 (3H, s, CH_3OAc) ppm. ^{13}C NMR: δ 170.34 (C═OAc), 162.55 (C-4), 163.12 (C-6), 158.36 (C-2), 112.17 (C-5), 59.78 (C-2′), 58.33 (OCH_3), 30.49 (CH_3OAc), 26.44 (C-1′), 20.41 (CH_3) ppm

MS *m/z* 204 206 [M^+, M+2]. Anal. Calcd for [$C_{10}H_{13}N_2O_3Cl$]: C, 49.09; H, 5.36; N, 11.45. Found: C, 48.89; H, 5.34; N, 11.47.

4.1.1.9. 2,4-Dichloro-5-(2-chloroethyl)-6-methylpyrimidine (14), 5-(2-chloroethyl)-6-methylpyrimidin-2,4-dione (15) and 2,4-dichloro-5-(2-hydroxyethyl)-6-methylpyrimidine (16). The reaction mixture of compound **3** (1.155 g, 6.8 mmol), $POCl_3$ (13.45 mL) and *N,N*-diethylaniline (1.35 mL) was refluxed for 4 h. An excess of $POCl_3$ was removed under reduced pressure. The residue was poured onto ice, washed with dichloromethane and dried over Na_2SO_4. The crude product was purified by column chromatography to afford compound **14** (0.893 g, 59%, mp = 207–210 °C) as yellow solid, and oily compounds **15** (6 mg, 0.5%) and **16** (48 mg, 4%).

Compound **14**: ^{1}H NMR: δ 3.85 (2H, t, J = 7.21 Hz, H-2′), 3.23 (2H, t, J = 7.23 Hz, H-1′), 2.60 (3H, s, CH_3) ppm. ^{13}C NMR: δ 172.64 (C-4), 162.03 (C-2), 156.79 (C-6), 128.09 (C-5), 42.17 (C-2′), 31.46 (C-1′), 23.02 (CH_3) ppm.

MS *m/z* 224 226 228 230 [M^+, M+2, M+4, M+6]. Anal. Calcd for [$C_7H_7N_2Cl_3$]: C, 37.28; H, 3.13; N, 12.42. Found: C, 37.39; H, 3.13; N, 12.37.

Compound **15**: ^{1}H NMR: δ 11.03 (1H, s, NH), 10.75 (1H, s, NH), 3.65 (2H, m, H-2′), 2.67 (2H, t, J = 4.11 Hz, H-1′), 2.10 (3H, s, CH_3) ppm. ^{13}C NMR: δ 164.65 (C-4), 151.17 (C-2), 150.49 (C-6), 105.55 (C-5), 43.76 (C-2′), 28.28 (C-1′), 16.67 (CH_3) ppm.

MS *m/z* 188 190 [M^+, M+2]. Anal. Calcd for [$C_7H_9N_2O_2Cl$]: C, 44.58; H, 4.81; N, 14.85. Found: C, 44.71; H, 4.82; N, 14.84.

Compound **16**: ^{1}H NMR: δ 4.74 (1H, t, J = 8.61 Hz, OH), 3.61 (2H, t, J = 6.90 Hz, H-2′), 2.76 (2H, t, J = 7.29 Hz, H-1′), 2.34 (3H, s, CH_3) ppm. ^{13}C NMR: δ 170.58 (C-4), 161.84 (C-2), 155.47 (C-6), 116.23 (C-5), 54.20 (C-2′), 27.51 (C-1′), 22.99 (CH_3) ppm.

MS *m/z* 206 208 210 [M^+, M+2, M+4]. Anal. Calcd for [$C_7H_8N_2OCl_2$]: C, 40.60; H, 3.89; N, 13.53. Found: C, 40.48; H, 3.89; N, 13.56.

4.1.1.10. 5-(2-Chloroethyl)-2,4-dimethoxy-6-methylpyrimidine (17) and 2,4-dimethoxy-6-methyl-5-vinyl-pyrimidine (18). To a solution of sodium (45 mg, 1.95 mmol) in anhydrous MeOH (3 mL) compound **14** (190 mg, 0.84 mmol) was added and the mixture was heated at reflux for 3 h. After evaporation of solvents, water was added and the oily layer was extracted with CH_2Cl_2 and dried over Na_2SO_4. Filtered solution was concentrated, the residue was kept in refrigerator overnight and crystals of compound **17** were filtered off (81 mg, 45%, mp = 40–42 °C). Mother liquor was evaporated to dryness and the residue was purified on silica gel column (CH_2Cl_2/CH_3OH = 60:1) to afford compound **18** as colorless oil (56 mg, 37%).

Compound **17**: ^{1}H NMR: δ 3.93 (3H, s, OCH_3), 3.88 (3H, s, OCH_3), 3.70 (2H, t, J = 7.27 Hz, H-2′), 2.95 (2H, t, J = 7.27 Hz, H-1′), 2.41 (3H,

s, CH_3) ppm. ^{13}C NMR: δ 169.53 (C-4), 167.39 (C-6), 163.21 (C-2), 110.26 (C-5), 54.73 (OCH_3), 54.63 (OCH_3), 43.46 (C-2′), 28.26 (C-1′), 22.91 (CH_3) ppm.

MS *m/z* 216 218 [M^+, M+2]. Anal. Calcd for [$C_9H_{13}N_2O_2Cl$]: C, 49.89; H, 6.05; N, 12.93. Found: C, 50.04; H, 6.06; N, 12.92.

Compound **18**: ^1H NMR: δ 6.64 (1H, dd, J = 17.76, 11.85 Hz, CH-1′), 5.74 (1H, dd, J = 2.07, 17.76 Hz, CH-2′), 5.45 (1H, dd, J = 2.06, 11.84 Hz, CH-2′), 3.90 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 2.37 (3H, s, CH_3) ppm. ^{13}C NMR: δ 168.83 (C4), 166.53 (C-6), 162.61 (C-2), 127.95 (C1′), 119.71 (C2′), 108.74 (C-5), 54.69 (OCH_3), 54.44 (OCH_3), 21.88 (CH_3) ppm.

MS *m/z* 181 $[MH]^+$. Anal. Calcd for [$C_9H_{12}N_2O_2$]: C, 59.99; H, 6.71; N, 15.55. Found: C, 60.17; H, 6.70; N, 15.53.

4.1.1.11. 6-(3-Benzyloxy-2-hydroxypropyl)-5-(2-chloroethyl)-2,4-dimethoxypyrimidine (19).

The solution of compound **17** (100 mg, 0.46 mmol) in anhydrous THF (10 mL) was cooled at −70 °C and lithium diisopropylamide (LDA, 0.1 mL, 2 M in THF/heptane/ethylbenzene) was added dropwise to the reaction mixture. The temperature was then raised to -55 °C and the reaction mixture was stirred for 30 min. Benzyloxyacetaldehyde (0.1 g, 0.55 mmol) in THF (1 mL) was added and the mixture was additionally stirred for 3 h and then at room temperature for 2 h. The solution was neutralized with glacial acetic acid and stirred further for 15 min. The solvent was evaporated and the residual yellow oily product was extracted with CH_2Cl_2 and water. Organic layer was dried over $MgSO_4$ and purified by column chromatography (petroleum ether/ethyl acetate = 7:1). After column chromatography, compound **19** was isolated (132.3 mg, 78%) as pale yellow oil. ^1H NMR: δ 7.27–7.32 (5H, m, Ph), 5.58 (1H, dd, J = 2.28, 6.18 Hz, OH), 4.48 (4H, m, OCH_2), 3.90 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 3.82 (1H, m, CH), 3.77 (2H, m, CH_2-Pyr), 3.70 (2H, t, J = 7.29 Hz, H-2′), 2.95 (2H, t, J = 7.29 Hz, H-1′) ppm. ^{13}C NMR: δ 169.54 (C-4), 167.39 (C-2), 163.21 (C-6), 138.85 (Ph-quat), 127.78–128.88 (Ph), 107.42 (C-5), 97.63 (CH), 72.62 (OCH_2Ph), 66.16 (CH_2O), 54.63 (OCH_3), 54.44 (OCH_3), 43.47 (C-2′), 33.61 (Pyr-CH_2), 23.75 (C-1′) ppm.

MS *m/z* 366 368 [M^+, M+2]. Anal. Calcd for [$C_{18}H_{23}N_2O_4Cl$]: C, 58.93; H, 6.32; N, 7.64. Found: C, 59.05; H, 6.32; N, 7.65.

4.1.1.12. 6-(3-Benzyloxy-2-hydroxypropyl)-5-(2-chloroethyl)pyrimidin-2,4-dione (20).

A mixture of compound **19** (120 mg, 0.33 mmol), chlorotrimethylsilane (0.16 mL, 1.2 mmol) and NaI (180 mg, 1.2 mmol) in dry acetonitrile (6 mL) was stirred at room temperature under argon atmosphere for 18 h and then at 60 °C for 3 h. The solvent was evaporated under reduced pressure, and the residue was purified by column chromatography (CH_2Cl_2/CH_3OH = 15:1) to give compound **20** (40.5 mg, 36%) as white solid. Mp = 101–103 °C. ^1H NMR: δ 11.03 (1H, s, NH), 10.76 (1H, s, NH), 7.28–7.37 (5H, m, Ph), 5.76 (1H, s, OH), 4.48 (2H, m, CH_2Ph), 3.61 (2H, t, J = 7.25 Hz, CH_2Cl), 3.42–3.47 (2H, m, CH_2), 2.64 (2H, t, J = 7.15 Hz, CH_2), 2.10 (2H, s, pyr CH_2), 3.80 (1H, m, CH) ppm. ^{13}C NMR: δ 164.57 (C-4), 156.22 (C-6), 151.20 (C-2), 135.31 (Ph-quat), 127.81–128.56 (Ph), 109.12 (C-5), 98.44 (CH), 70.60 (CH_2Ph), 64.37 (CH_2O), 44.28 (C-2′), 32.89 (Pyr-CH_2), 22.80 (C-1′) ppm.

MS *m/z* 338 340 [M^+, M+2]. Anal. Calcd for [$C_{16}H_{19}N_2O_4Cl$]: C, 56.72; H, 5.65; N, 8.27. Found: C, 56.68; H, 5.65; N, 8.29.

4.2. X-Ray Determination of compounds 4, 7 and 14

Single crystals of **4** and **7** suitable for X-ray single crystal analysis were obtained at room temperature by partial evaporation from methanol solution. Single crystals of **14** were obtained from dichloromethane solution applying the same recrystallization method. The intensities were collected at 295 K on a Oxford Diffraction Xcalibur2 diffractometer using graphite-monochromated Mo$K\alpha$ radiation (λ = 0.71073 Å). CRYSALIS[35] programs were used for data collection and reduction. The intensities for **4** and **14** were corrected for absorption using the multi-scan absorption

Table 3
X-ray crystallographic data for compounds **4**, **7** and **14**

Compound	**4**	**7**	**14**
Formula	$C_9H_{12}N_2O_4$	$C_8H_{11}ClN_2O_2$	$C_7H_7Cl_3N_2$
Formula weight	212.21	202.64	225.50
Crystal size/mm	0.13 × 0.16 × 0.68	0.25 × 0.29 × 0.54	0.10 × 0.31 × 0.53
Crystal color, shape	Colorless, prism		
Crystal system	Monoclinic	Triclinic	Orthorhombic
Space group	$P\,2_1/c$	$P\bar{1}$	$P\,b\,c\,a$
Unit cell dimensions			
a/	11.7336(6)	7.5752(2)	7.8128(4)
b/	5.0612(3)	7.9618(2)	13.9748(5)
c/	18.2071(10)	7.9984(2)	17.1690(6)
α/°	90	82.878(2)	90
β/°	114.113(4)	79.812(2)	90
γ/°	90	81.739(2)	90
V/^{3}	986.90(9)	467.46(2)	1874.55(13)
Z	4	2	8
D_{calcd}/g cm^{-3}	1.428	1.440	1.598
Absorption coefficient μ/mm^{-1}	0.114	0.377	0.921
Scan mode	ω and ϕ	ω	ω
θ range/°	4.21–27.99	3.86–28.00	3.82–28.00
Index ranges	$-15 \leqslant h \leqslant 15$	$-9 \leqslant h \leqslant 9$	$-10 \leqslant h \leqslant 9$
	$-6 \leqslant k \leqslant 6$	$-10 \leqslant k \leqslant 10$	$-18 \leqslant k \leqslant 18$
	$-24 \leqslant l \leqslant 22$	$-10 \leqslant l \leqslant 10$	$-22 \leqslant l \leqslant 22$
Collected reflections No.	13,349	12,894	12,818
Independent reflections No./$R_{int.}$	2379/0.0471	2248/0.0198	2238/0.0384
Reflections No. $I \geqslant 2\sigma(I)$	1462	1785	1506
Data/restraints/parameters	2379/0/146	2248/0/124	2238/0/110
Goodness-of-fit on F^2, S	0.962	1.020	1.026
R [$I \geqslant 2\sigma(I)$]/R [all data]	0.0535/0.0899	0.0334/0.0425	0.0572/0.0793
wR [$I \geqslant 2\sigma(I)$]/wR [all data]	0.1408/0.1677	0.1130/0.1165	0.1764/0.1945
Max. and min. el. density/e^{-3}	0.344/−0.236	0.273/−0.155	0.794/−0.372

correction method (*CrysAlis Red*[35]). The crystal structures were solved by direct methods.[36] All non-hydrogen atoms were refined anisotropically by full-matrix least-squares calculations based on F^2.[36] The N1 and N3 hydrogen atoms in **4** and hydroxyl hydrogen atom in **7** were found in a difference Fourier map and their coordinates and isotropic thermal parameters have been refined freely. All other hydrogen atoms in **4** and **7**, as well as all hydrogen atoms in **14** were included in calculated positions as riding atoms with SHELXL97[36] defaults. PLATON[37] program was used for structure analysis and drawings preparation. Details of crystal data, data collection and refinement parameters are given in Table 3. CCDC 749761 (**4**), 749762 (**7**) and 749763 (**14**) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

4.3. Antitumor activity assays

The colon carcinoma (HCT 116 and SW 620), breast carcinoma (MCF-7) and lung carcinoma (H 460) cells were cultured as monolayers and maintained in Dulbecco's modified Eagle's medium (DMEM), while Molt-4 cells (acute lymphoblastic leukemia) were cultured in suspension in RPMI medium, both supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C.

The growth inhibition activity was assessed as described previously, according to the slightly modified procedure of the National Cancer Institute, Developmental Therapeutics Program.[21,22] Briefly, the cells were inoculated onto standard 96-well microtiter plates on day 0. Test agents were then added in five consecutive 10-fold dilutions (10^{-8} to 10^{-4} mol/L) and incubated for further 72 h. Working dilutions were freshly prepared on the day of testing. The solvent (DMSO) was also tested for eventual inhibitory activity by adjusting its concentration to be the same as in working concentrations (maximal concentration of DMSO was 0.25%). After 72 h of incubation, the cell growth rate was evaluated by performing the MTT assay[22] which detects dehydrogenase activity in viable cells. The absorbency (OD, optical density) was measured on a microplate reader at 570 nm.

Each test point was performed in quadruplicate in three individual experiments. The results are expressed as IC_{50}, which is the concentration necessary for 50% of inhibition. The IC_{50} values for each compound are calculated from dose–response curves using linear regression analysis by fitting the test concentrations that give PG (percentage of growth) values above and below the reference value (i.e., 50%). Each result is a mean value from three separate experiments.

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