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ScienceDirect
European Journal of Medicinal Chemistry 44 (2009) 1172–1179
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
http://www.elsevier.com/locate/ejmech

Original article

Synthesis of modified pyrimidine bases and positive impact of chemically reactive substituents on their in vitro antiproliferative activity

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Received 4 February 2008; received in revised form 26 May 2008; accepted 2 June 2008
Available online 11 June 2008

Abstract

The antiproliferative activity screening on human tumor cell lines of a series of modified uracil and cytosine bases as well as some corresponding acyclonucleosides, and comparison of structure–activity relationship revealed the importance of chemical reactivity of the substituent attached to the C5-position of uracil for the activity of studied compounds. Namely, the results obtained for the most active compounds, 5-(chloroacetylamino)uracil (**2**) and its acyclic sugar analogue **18**, suggest that formation of a covalent bond between reactive substituent and several possible targets within the thymidylate synthase mechanism (sulphur of the cysteine residue, basic part of the enzyme, *N*,*N*-methylene tetrahydrofolate or its reactive iminium forms) is the most probable mode of action. In addition, novel C5-substituted uracil derivative **6** (5-[bis-(2-*p*-methoxybenzylthioethyl)amine]acetylaminouracil) exhibited high antiproliferative activity against HeLa and MiaPaCa-2 cell lines, by an as yet unknown mechanism.

1. Introduction

In general, nucleoside analogues are structurally, metabolically, and pharmacodynamically related agents that nevertheless have diverse biological actions and therapeutic effects. This class of agents affects the structural integrity of DNA, usually after incorporation during replication or DNA excision repair synthesis, leading to stalled replication forks and chain termination. One of the remarkable features that is still unexplained about nucleic acid antagonists, is how drugs with such similar structural features, which share metabolic pathways and elements of their mechanisms of action, show such diversity in their clinical activities [1]. One of the most frequently used approaches to new antitumor drugs is a design of antimetabolites based on the similarity of structure to the naturally occurring pyrimidines and purines involved in the biosynthesis of DNA. Novel compounds should interfere with one or more biological applications of naturally occurring analogues.

The cytostatic agent 5-fluorouracil (**5-FU**), one of the most important agents for the treatment of colorectal, head and neck, pancreatic and breast carcinomas, is an antimetabolite exerting a cytotoxic effect primarily through inhibition of thymidylate synthase (TS) [2]. In the cascade of enzymatic conversions 5-fluorouracil related metabolites inhibit the action of thymidylate synthase, the only *de novo* source of thymidylate, one of the essential constituents of DNA and consequently lead to the thymineless death of cell [3]. Each of these conversion steps is a target of intensive research with the aim to improve bioactivity. Thus, a huge number of **5-FU** analogues were designed and studied with the aim of more efficient blocking of the (b) step (ternary complex of enzyme, substrate (ex-uracil) and coenzyme). Alternatively, antifolate

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doi:10.1016/j.ejmech.2008.06.002

inhibitors are achieving the same goal by interacting with the coenzyme [4].

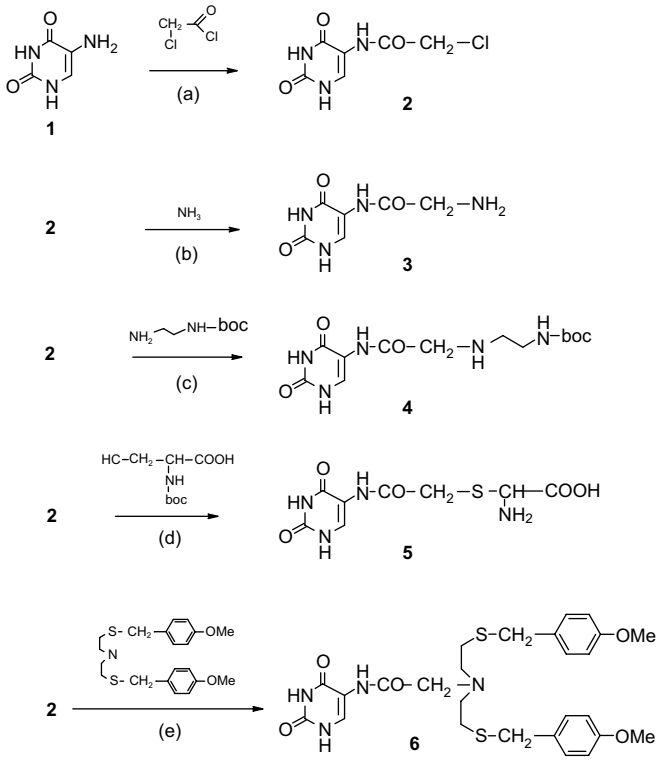
The higher activity of **5-FU** derivatives toward tumor cells in comparison to normal cells is based on higher division rates of tumors as well as on the fact that enzymes in normal cells degrade both uracil and **5-FU** but in tumor cells that degradation process does not work [5]. However, other mechanisms can also contribute to the cytostatic activity, as it is obvious from the metabolism complexity of **5-FU** and its prodrugs [6]. For example, **5-FU** is incorporated in DNA and RNA and the maturation process and metabolism of RNA is influenced [7].

In this work, we present the synthesis of a series of novel uracil derivatives, differing in properties of C5-positioned side chains, like chemical reactivity and bulkiness. Most of these compounds were also prepared in a form of N^1-substituted acyclic uridine analogues, to explore the impact of acyclic sugar analogues on biological activity. Furthermore, several cytosine analogues were prepared and studied to investigate if the uracil moiety is essential for the biological activity, and in line with recently observed differences in biological action between cytosine and uracil derivatives [7]. Bioactivity of all novel compounds was probed in vitro by screening cellular growth inhibition on several different human tumor cell lines. Most potent compounds were additionally studied by assessing their cell cycle perturbation impact.

2. Synthesis

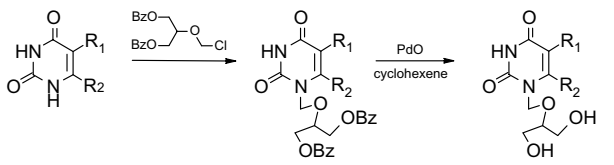
At first, uracil has been modified at the C5-position. Starting from 5-aminouracil **1**, the corresponding 5-(chloroacetyl) aminouracil **2** – already known to show antiviral activity [8] – was synthesized by treatment of chloroacetyl chloride in 1 M sodium hydroxide solution at 0 °C. After acidification with hydrochloric acid the product was obtained in high purity by repetitive crystallization from water. Compound **2** was converted into 5-(aminoacetyl)-aminouracil **3** with concentrated ammonia. The product was purified by column chromatography on Sephadex G10 giving **3** in 81% yield. As shown in Scheme 1, other uracil derivatives have been also accessible by conversion of **2**. Thus, heating **2** with *N*-boc-ethylenediamine in methanol under pressure at 90 °C led to 5-(*N*-boc-ethylenediaminoacetyl)aminouracil **4**. The reaction product was separated by column chromatography on Sephadex G10. Coupling of **2** with boc-protected cysteine was performed in ethanol and 2 M sodium hydroxide. The protection group was then removed by the treatment with 1 M hydrochloric acid. Purification was carried out by MPLC yielding *S*-{2-[(2,4-dioxo-1,2,3,4,-tetrahydropyrimidine-5-yl)amino]-2-oxoethyl}-D-cysteine **5**. The 5-[bis-(2-*p*-methoxybenzylthioethyl) amine]acetylaminouracil **6** was synthesized by direct condensation of the persilylated 5-(chloroacetyl)aminouracil **2** with bis-(2-*p*-methoxybenzylthioethyl)amine [9] in acetonitrile followed by column chromatography on silica gel.

Scheme 2 shows the structure of some uridine derivatives recently described as potential substrates of herpes simplex virus type-1 thymidine kinase [10]. These compounds were



Scheme 1. Synthesis of C^5-substituted uracil derivatives **2**–**6**. Reagents and conditions: (a) $ClCH_2COCl$, $NaOH/H_2O$, 30 min, 0 °C; 90 min, 22 °C; recrystallization from H_2O (**2**: 62%); (b) **2**, $NH_{3(aq)}$, 24 h, 22 °C; Sephadex G10 (**3**: 81%); (c) **2**, $H_2NCH_2CH_2NH$-boc, H_2O/MeOH, 1 h, 90 °C, pressure; Sephadex G10 (**4**: 76%); (d) **2**, boc-cysteine, NaOH/EtOH, 2 h, 22 °C; MPLC (RP18, $CH_3CN/H_2O = 1/9$) (**5**: 76%); (e) **2**, $NH_4(SO_4)_2$, hexamethyldisilazane, reflux, 3 h; bis(2-*p*-methoxybenzylthioethyl)-amine, $(Et)_3N$, CH_3CN, 90 °C, 4 h; column chromatography (SiO_2, $CH_2Cl_2/MeOH = 10/1$) (**6**: 77%).

also investigated in vitro by screening cellular growth inhibition on several different human tumor cell lines. Briefly, modification of the N^1-position of the uracil molecule was accomplished by direct condensation of the appropriate persilylated base with the chloromethyl ether 1,3-dibenzyloxy-2-chloro-methoxypropane and tetrabutylammonium iodide as a catalyst. Thus the nucleic bases uracil **7**, 5-hydroxyuracil **8**, 5-aminouracil **1**, 5-iodouracil **9**, 6-methyluracil **10** and 5-iodomethyluracil **11** were coupled to 1,3-dibenzyloxy-2-chloro-methoxypropane in dry dimethyl formamide with



7. R_1 = H, R_2 = H	R_1 = H, R_2 = H	12. R_1 = H, R_2 = H
8. R_1 = OH, R_2 = H	R_1 = OH, R_2 = H	13. R_1 = OH, R_2 = H
1. R_1 = NH_2, R_2 = H	R_1 = NH_2, R_2 = H	14. R_1 = NH_2, R_2 = H
9. R_1 = I, R_2 = H	R_1 = I, R_2 = H	15. R_1 = I, R_2 = H
10. R^1 = H, R_2 = CH_3	R^1 = H, R_2 = CH_3	16. R^1 = H, R_2 = CH_3
11. R_1 = I, R_2 = CH_3	R_1 = I, R_2 = CH_3	16. R_1 = I, R_2 = CH_3

Scheme 2. Constitutions of uridine derivatives **12**–**17** [10].

triethylamine yielding the *O*-benzylated precursors. Removal of the benzyl protection groups with palladium oxide in cyclohexene1-[(1,3-dihydroxy-2-propoxy)methyl]uracil (Acyclur) **12**, 5-hydroxy-1-[(1,3-dihydroxy-2-propoxy)methyl]-uracil (Hydracyclur) **13**, 5-amino-1-[(1,3-dihydroxy-2-propoxy)methyl]uracil (Amino-acyclur) **14**, 5-iodo-1-[(1,3-dihydroxy-2-propoxy)-methyl]-uracil (Iodacyclur) **15**, 6-methyl-1-[(1,3-dihydroxy-2-propoxy)methyl]uracil (Metacyclur) **16** and 5-iodo-6-methyl-1-[(1,3-dihydroxy-2-propoxy)methyl]uracil (Iodmetacyclur) **17**.

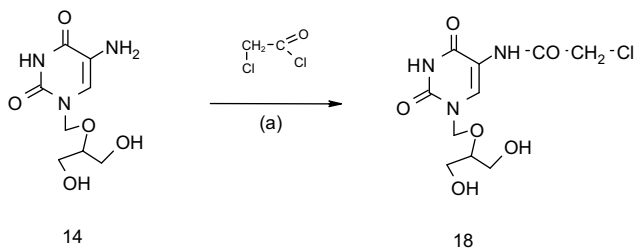
Of special interest is the chloroacetylated uridine derivative **18** (Scheme 3) which allows a direct comparison of biological activity with the corresponding uracil compound **2**. The preparation of **18** was similar to 5-(chloroacetyl)aminouracil **2**. Amino-acyclur **14** was treated with chloroacetyl chloride in aqueous sodium hydroxide at 0 °C for 90 min. The purification of the reaction product was carried out using reverse phase MPLC.

Furthermore, some cytosine derivatives modified at the N^1- and N^4-position have been investigated. Methylation of cytosine **19** and 5-hydroxymethylcytosine **20** [11] by the treatment with methyliodide and tetrabutylammonium hydroxide solution in DMF led smoothly to 1-methylcytosine **21** [12] and 1-methyl-5-hydroxymethylcytosine **22**. The methylation of 4-thiouracil **23** under the same reaction conditions yielded 4-methylthiouracil **24** [13]. By refluxing **24** with hydroxylamine hydrochloride in dry ethanol followed by treatment with concentrated ammonia, N^4-hydroxycytosine **25** [14] was obtained (Scheme 4).

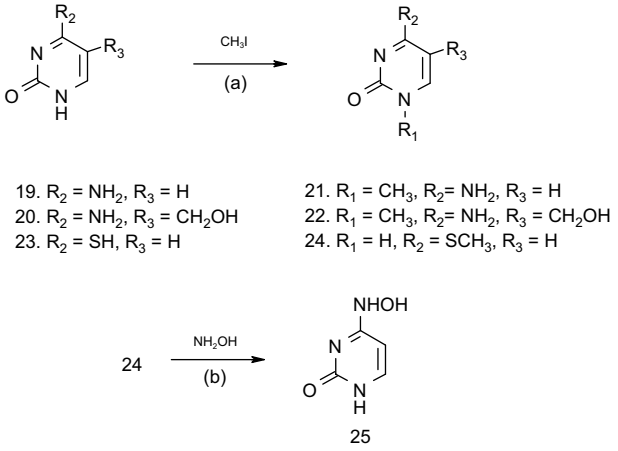
A tosyl group was introduced into both cytosine **19** and 1-methylcytosine **21**. Therefore, compounds **19** and **21** were treated with *p*-toluenesulfonyl chloride in dry pyridine yielding N^1-(*p*-toluenesulfonyl)cytosine **26** [15,16] and 1-methyl-N^4-(*p*-toluenesulfonyl)cytosine **27**. Pure products were obtained by column chromatography (Scheme 5).

3. Biological results and discussion

The tested compounds showed very different antiproliferative effects on the investigated panel of cell lines (Table 1 and Fig. 1). Uracil derivatives revealed a much broader range of cell growth inhibitory activity compared to cytosines. Compounds **3**, **12**–**17**, **24** did not show any or very weak antiproliferative effect. Compounds **4** and **5** showed moderate growth inhibitory activity mostly at the highest tested concentration



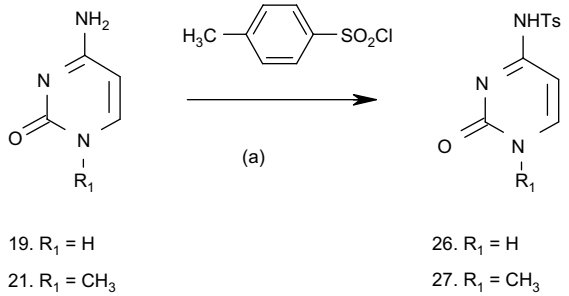
Scheme 3. Synthesis of C^5-chloroacetylated uridine derivative **18**. Reagents and conditions: (a) $ClCH_2COCl$, $NaOH/H_2O$, 30 min, 0 °C; 90 min, 22 °C; MPLC (RP18, $CH_3CN/H_2O = 1/4$) (**18**: 75%).



Scheme 4. Synthesis of cyctosine derivatives **21**, **22**, **25** and 4-methylthiouracil **24**. Reagents and conditions: (a) CH_3I, $[(C_4H_9)_4N]OH$, DMF, 2 h, 22 °C; recrystallization from EtOH (**21**: 86%); column chromatography (SiO_2, $CH_2Cl_2/MeOH = 1/1$), (**22**: 82%); MPLC (RP18, CH_3OH) (**24**: 84%); (b) NH_2OH, EtOH, 5 h, reflux; recrystallization from EtOH (**25**: 78%).

on HeLa, MiaPaCa-2 and SW620 cells but not on MCF-7 and H460 cells. On the contrary, compounds **2**, **6** and **18** showed rather strong antiproliferative and cytotoxic activity on the tested cell lines, comparable to 5-fluorouracil (**5-FU**). The most prominent activity was seen on HeLa and MiaPaCa-2 cells by **2** and **6** (Fig. 1), which prompted us to perform additional studies of the impact of **2** and **6** on the cell cycle of HeLa cells. Besides, we additionally tested **2**, **6** (Fig. 1), **18** and **5-FU** (data not shown) for their inhibitory activity on human skin keratinocytes HaCaT, as a model of nontumorigenic cell line. Interestingly, compound **6** strongly inhibited the growth ($IC_{50} = 9$ μM), while **2** and **18** did not (IC_{50} were >100 and ≥100, respectively). On the other hand **5-FU** markedly inhibited the growth of HaCaT cells ($IC_{50} = 0.4$ μM).

In contrast to uracil derivatives, in general cytosine derivatives did not show any growth inhibitory activity (**22**, **25**, and **27**). The only exceptions were 1-methylcytosine **21** causing a weak antiproliferative effect exclusively on the HeLa cell line and N^1-tosylated cytosine **26**, weakly inhibiting the growth of MCF-7 cell line. Further experiments are needed



Scheme 5. Synthesis of tosylated cyctosine derivatives **26** and **27**. Reagents and conditions: (a) TsCl, pyridine, 6 h, 22 °C; column chromatography (SiO_2, $CH_2Cl_2/MeOH = 50/1$) (**26**: 90%, **27**: 88%).

Table 1
Tumor cell growth inhibition presented as IC_{50} values (in μM)

IC_{50}[a] (μM)					
Compound	Cell lines				
	HeLa	MiaPaCa-2	SW620	MCF-7	H460
2	4 ± 2	17 ± 3	12 ± 9	15 ± 2	14 ± 1
3	>100	>100	>100	>100	>100
4	50 ± 46	50 ± 47	50 ± 44	>100	>100
5	86 ± 8	80 ± 18	70 ± 25	>100	>100
6	4 ± 3	3 ± 2	70 ± 25	≥100	76 ± 20
12	>100	>100	>100	>100	>100
13	>100	>100	>100	>100	>100
14	>100	>100	>100	>100	>100
15	30 ± 13	>100	>100	>100	>100
16	>100	>100	>100	>100	>100
17	73 ± 26	>100	>100	>100	>100
18	12 ± 1	18 ± 3	19 ± 3	32 ± 18	24 ± 3
21	12 ± 5	>100	>100	>100	>100
22	>100	>100	>100	>100	>100
24	>100	>100	>100	>100	>100
25	>100	>100	>100	>100	>100
26	>100	>100	>100	27 ± 5	>100
27	>100	>100	>100	>100	>100
5-FU[b]	4 ± 1	10 ± 3	9 ± 2	15 ± 2	2 ± 0.7

[a] IC_{50} – the concentration that causes 50% growth inhibition.
[b] **5-FU** – 5-fluorouracil.

to explain the unique selectivity of **21** and **26** in cell growth inhibition.

4. Cell cycle perturbations

Cell cycle and apoptosis are very important functional parameters to assess the cellular metabolism, physiology and pathology. The DNA content of the cell can provide a great deal of information about the cell cycle, and consequently the effect on the cell cycle of added stimuli, e.g., drug treatment. For example, the major mechanism of action of nucleoside analogues is through incorporation into DNA, and thus this class of agents shows specificity for cells in the S-phase. A very similar influence of the cell cycle was already described for **5-FU** [17]. **5-FU** is considered to be purely an S phase-active chemotherapeutic agent, with no activity when cells are in G0 or G1. Treatment of cells with **5-FU** induces G1/S phase arrest. Therefore, we assessed the influence of the most active compounds **2** and **6** and **5-FU** on the cell cycle, as well as the possible induction of apoptosis on the HeLa cell line, since the most prominent activity/selectivity was shown on this cell line. Interestingly, although the IC_{50} concentrations of these substances were quite similar, the cell cycle influences showed a different pattern. Compound **2** induced strong G1/S phase arrest, accompanied with a reduction of cells in G2/M, but with no obvious apoptosis activation (subG1 cells) (Fig. 2 and Table 2). Very similar, but somewhat more pronounced influence on the cell cycle can be seen after treatment with **5-FU**, without significant difference between 10 μM (Table 2) and 50 μM concentrations (data not shown). On the other hand, **6** induced a significant accumulation in the G1-phase of the cells at the expense of S phase population with an impressive increase in the number of subG1 cells (representing the apoptotic cells) after first 24 h, while during the next 48 h, about 60% of cells died by apoptosis.

5. Discussion of the results

The tested compounds are pyrimidine analogues substituted at different positions namely at C1, C4 and C5. Their antiproliferative activity depends considerably on the type of the substituents as well as the position on the pyrimidine derivatives. However, among uracil derivatives (**2**–**6**, **12**–**17**, and **24**), those which exhibit by far the highest activity are characterised by an amide linkage of the substituent attached to the C5-position of the uracil moiety (R_3, Scheme 4). It seems that the antiproliferative activity is controlled by the chemical reactivity of the substituent attached to the end of the amide spacer; e.g., good leaving groups as chloride (**2** and **18**) show high activity, while those which showed weak or no activity have low- or non-reactive groups at the end of the amide spacer (**3**, **4**, and **5**). The only exception is the high activity of **6** (Scheme 1), although the *p*-methoxybenzylthio-substituent on the end of the amide spacer is not expected to be a good leaving group.

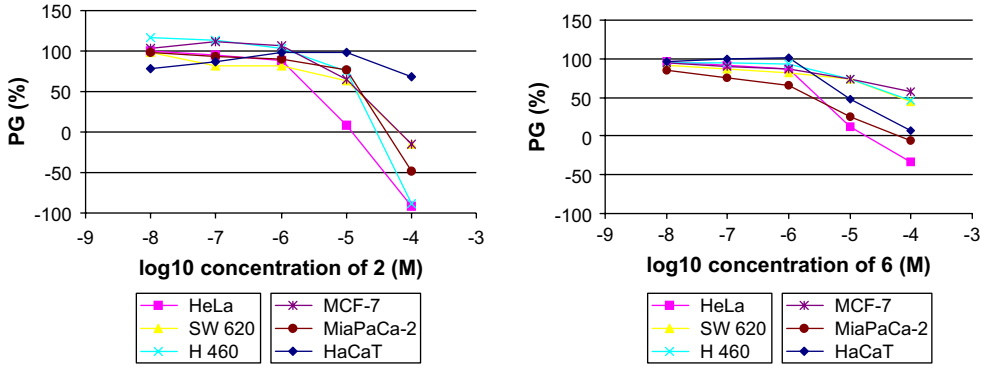


Fig. 1. Dose–response profiles for the most active compounds (**2** and **6**) tested on various human tumor cell lines in vitro. The cells were treated with the compounds at different concentrations, and percentage of growth (PG) was calculated. Each point represents a mean value of four parallel samples in three individual experiments.

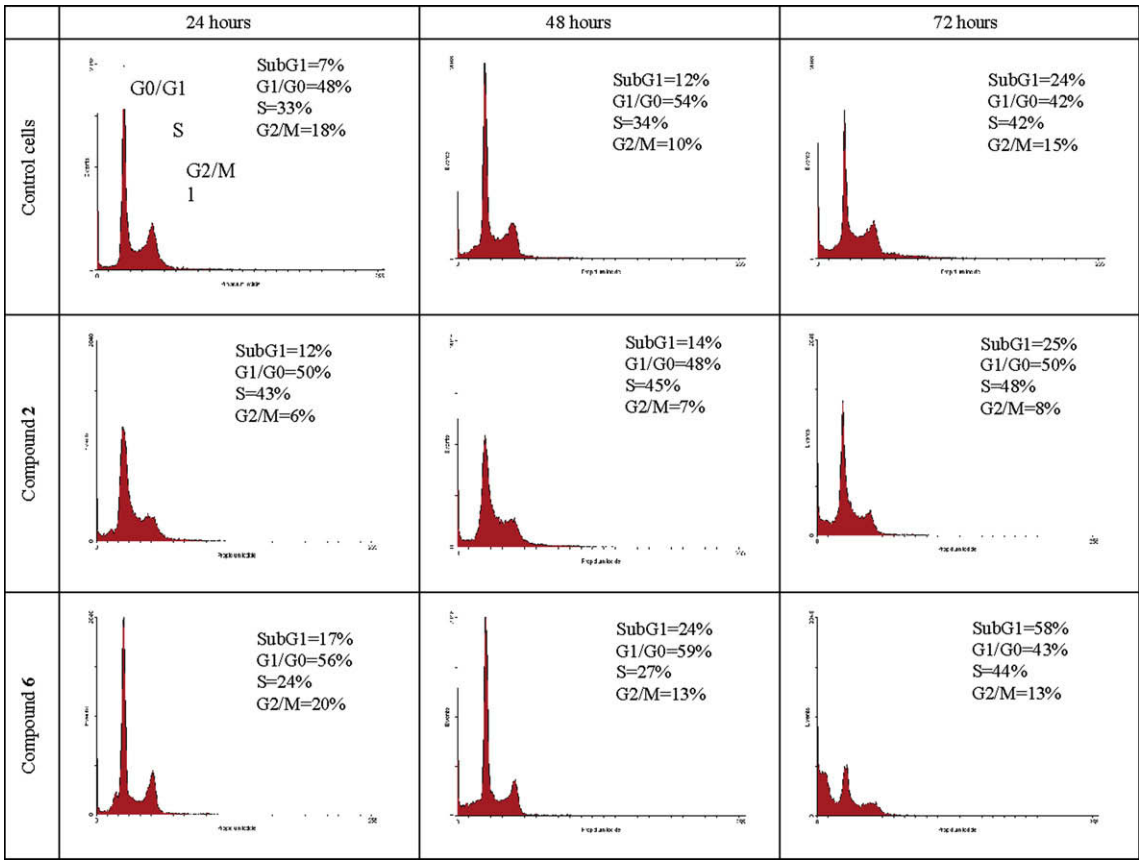


Fig. 2. DNA histograms obtained by flow cytometry (see Section 7), and the percentages of cells in subG1 (apoptotic cells), G0/G1, S and G2/M cell cycle phase, after the treatment of HeLa cells with **2** and **6** (at 2×10^{-5} M for 24 h, 48 h and 72 h).

Antiproliferative activity of **2** and its acyclic uridine analogue **18** is comparable, which is in line with comparable activity of **5-FU** and its 2′-deoxyribo derivatives [18]. Although some studies have shown that changes in the sugar part of uridine-like molecules can have strong biological impact, this is not noticeable for **2** and **18**, suggesting that reactivity of the substituent at C5-position of uracil moiety is mainly responsible for the observed activity.

If we consider thymidylate synthase (TS) as a possible target for uracil derivatives, there are several possibilities by which **2** and **18** can inhibit the enzyme. Upon positioning of the uracil moiety into the binding site of TS, usually sulphur of the cysteine residue undergoes Michael type addition to the C6-position of uracil [18]. However, competitive binding of the chemically reactive substituent attached to the C5-position of uracil (**2** and **18**) to the same sulphur of the cysteine residue is also likely to occur. To check this possibility, we have studied this reaction by mixing one of the most active compounds (**2**) with cysteine and have obtained in short reaction time and under comparatively mild conditions the product (**5**) in a quite high yield (Scheme 1). This experiment pointed out that reaction of **2** with cysteine residue of TS is likely to occur. Even more important, very low bioactivity of **5** (Table 1) suggested a high stability of this particular carbon–sulphur bond under biologically relevant experimental conditions,

Table 2
Flow cytometric analysis of HeLa cells treated with **2** and **6** ($c = 20$ μM) and 5-fluorouracil (5-FU, $c = 10$ μM)

Treatment	Cell cycle phase[a]	HeLa		
		24 h	48 h	72 h
Control	SubG1	7 ± 0.6	12 ± 1	24 ± 4
	G0/G1	48 ± 0.9	54 ± 2	42 ± 0.2
	S	33 ± 0.3	34 ± 1	42 ± 2
	G2/M	18 ± 1	10 ± 0.5	15 ± 1.8
2	SubG1	12 ± 5	14 ± 0.4	25 ± 0.5
	G0/G1	50 ± 2	48 ± 0.2[b]	50 ± 0.1[b]
	S	43 ± 0.3[b]	45 ± 1[b]	48 ± 1
	G2/M	6 ± 1[b]	7 ± 1[b]	8 ± 0.8[b]
6	SubG1	17 ± 3.5[b]	24 ± 0.4[b]	58 ± 2[b]
	G0/G1	56 ± 2.4[b]	59 ± 1[b]	43 ± 5
	S	24 ± 1.5[b]	27 ± 0.2[b]	44 ± 3
	G2/M	20 ± 1	13 ± 1	13 ± 1
5-FU	SubG1	10 ± 3.5	12 ± 2	26 ± 2
	G0/G1	60 ± 2.4[b]	59 ± 1	57 ± 3[b]
	S	39 ± 1[b]	41 ± 1[b]	43 ± 3
	G2/M	1 ± 1[b]	0 ± 0[b]	0 ± 0[b]

[a] The results are shown as percentages of cell population in each cell cycle phase. The experiment was repeated three times.
[b] Statistically significant at $p < 0.05$.

otherwise if cysteine of **5** would be a good leaving group, compound **5** should be as active as **2** and **18**. Therefore, it is possible that the sulphur of the cysteine residue of TS reacts with **2** and **18** in two different ways: either by forming a covalent bond at C6-position of uracil (common for enzymatic TS–uracil interaction), or by reacting with the substituent provided with a good leaving group. The latter reaction product would immediately block the TS activity.

Another possibility (which does not exclude the abovementioned) is that upon binding of a cysteine residue of TS at C6-position of uracil (common TS process) an enolate is formed, which then attacks the coenzyme (*N*,*N*-methylene tetrahydrofolate or some of its iminium forms), giving a ternary complex of enzyme–substrate–coenzyme [18]. In this case, a reactive side chain of **2** and **18** would be very close to the basic part on the enzyme which usually abstracts a proton from C5-position of the uracil moiety [18]. That should lead to the covalent bond formation between the **2** (or **18**) reactive side chain and the basic part on the enzyme. Such a covalently blocked enzyme active site would result in a permanently stabilized ternary complex of the enzyme, coenzyme and compound. Thus, the coenzyme will be blocked in a similar manner as in the case of **5-FU**.

As another possibility of a ternary complex blocking, the reactive side chain of **2** and **18** could also alkylate *N*,*N*-methylene tetrahydrofolate or more likely some of its open, chemically more reactive iminium forms. That is in line with our results, showing that the most active compound **2** is able to alkylate in a high yield primary aliphatic nitrogens (giving for example compound **4**), and even secondary aliphatic nitrogen atoms, giving compound **6** (Scheme 1), although in the latter case our reaction conditions are not biologically relevant.

Compound **6** also showed high antiproliferative activity toward some tumor cell lines (Table 1), comparable to the activity of compound **2**. Intriguingly, opposite to **2**, compound **6** is less effective toward MCF-7 and H 460 cell lines. That could be also correlated to the different results in cell cycle perturbation experiments obtained for **2** and **6**, respectively, whereby **6** induced an impressive increase in the number of apoptotic cells after first 24 h, while during the next 48 h, about 60% of cells died by apoptosis. These results point to a different mechanism of action of **6** compared to **2** and **18**. The thymidylate synthase (TS) is probably not the target of **6**, which is highly cytotoxic especially to HeLa and MiaPaCa-2, while other cell lines are rather resistant. However, to give any firm hypothesis about its mechanism of action, further research is needed.

6. Conclusions

Comparison of structure–activity relationship in a series of uracil derivatives revealed the importance of chemical activity of the substituent attached to the C5-position of uracil with regard to their antiproliferative activity on human tumor cell lines. The results obtained by compound **2** and its acyclic sugar analogue **18** pointed toward formation of a covalent bond between reactive substituent and several targets within the hypothetical mechanism of thymidylate synthase as the most probable mode of action. Compounds **2** and **18** are similarly active to 5-fluorouracil (**5-FU**), therefore, the same target (TS) along with several modes of action significantly differing from the mechanism of **5-FU** offer new and interesting research path for increasing the activity and also the selectivity of uracil derivatives aiming toward antitumor therapy.

Intriguingly, high antiproliferative activity of compound **6** toward HeLa and MiaPaCa-2 cell lines is comparable to the activity of 5-fluorouracil (**5-FU**) but the mechanism of action of **6**, as well as biological targets in the cells are not known. Because of that, compound **6** and its close analogues could be considered as a promising alternative to the numerous **5-FU** derivatives targeting TS pathway. Therefore, further, more detailed studies of **6** in order to determine the action pathways responsible for biological activity are of highest interest.

7. Experimental

7.1. General remarks

Proton ^{1}H NMR spectra were obtained on a Varian Inova-400 (400 MHz) instrument with DMSO-d_6 as an internal standard. The chemical shifts are given as δ in parts per million, the coupling constants as J in hertz. Elemental analyses were carried out with a LECO CHNS 932 elemental analyzer. Melting points were determined on a BOËTIUS melting point apparatus and are uncorrected. ESI mass spectra were recorded on a Quattro LC (Micromass Inc.). Chromatographic purifications were performed using either Silica gel 60 (Merck), RP-18 material (LiChroprep™), or Sephadex G10 (Pharmacia). A MPLC (middle-pressure-liquid-chromatography) system (Knauer) with two columns (silica gel: Eurosil Baselect, 25 × 200 mm, Merck; RP-18 material, 30 × 480, Kronlab) was used with pressures ranging from 2 to 5 bar.

7.1.1. Biological studies

7.1.1.1. Proliferation assay. The HeLa (cervical carcinoma), MiaPaCa-2 (pancreatic carcinoma), SW620 (colon carcinoma), MCF-7 (breast carcinoma), H460 (lung carcinoma) (all purchased from American Type Culture Collection – ATCC) and HaCaT (skin keratinocytes, kindly provided by Prof. Sonja Radaković, Department of Dermatology, Medical University of Vienna, General Hospital, Vienna, Austria) human cell lines were seeded into a series of standard 96-well microtiter plates on day 0, at 1×10^4–3×10^4 cells/mL, depending on the doubling times of the specific cell lines. Test compounds were then added in 5-, 10-fold dilutions (10^{-8}–10^{-4} M) and incubated for a further 72 h. Stock solutions were prepared in DMSO, ($c = 0.04$ M), while 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 (DMSO concentration never exceeded 0.25%). After 72 h of incubation the cell growth rate was evaluated by

performing the MTT assay, as described previously [19]. The results were expressed as IC_{50}, a concentration necessary for 50% of inhibition. The IC_{50} values for each compound were calculated from dose–response curves using linear regression analysis by fitting the test concentrations that give PG values above and below the respective reference value (e.g., 50 for IC_{50}). Therefore, a ''real'' value for any of the response parameters is obtained only if at least one of the tested drug concentrations falls above, and likewise at least one falls below the respective reference value. If however, for a given cell line all of the tested concentrations produce PGs exceeding the respective reference level of effect (e.g., PG value of 50), then the highest tested concentration is assigned as the default value, preceded by a ''>'' sign.

7.2. Cell cycle analysis

Cells were seeded (2×10^5 per well) in a 6-well plate. After 24 h the tested compounds were added at concentration of 50 μM. The attached cells were trypsinized, combined with floating cells, washed with phosphate buffer saline (PBS) and fixed in 70% ethanol 24, 48 and 72 h after the treatment with compounds. Immediately before the analysis, the cells were washed with PBS and stained with 2.5 μg/ml of propidium iodide (PI) with the addition of 0.2 μg/μl of RNase A. The stained cells were then analyzed with Becton Dickinson FACSCalibur flow cytometer (20 000 counts were measured). The percentage of the cells in each cell cycle phase was determined using ModFit LT™ software (Verity Software House) based on the DNA histograms. As a minimum two experiments were done in triplicates, and Student *T*-test ($p < 0.05$) was used to measure the statistical significance.

7.2.1. Reagents

All solvents and reagents were purchased from commercial sources and used without further purification. 5-Aminouracil **1**, uracil **7**, 5-hydroxyuracil **8**, 5-iodouracil **9**, 6-methyluracil **10**, 5-iodomethyluracil **11**, cytosine **19** and 4-thiouracil **23** were supplied by Serva. Uridine derivatives **12**–**17** [10], 5-hydroxymethylcytosine **20** [11], 1-methylcytosine **21** [12], 4-methylthiouracil **24** [13], N^4-hydroxycytosine **25** [14] and N^1-(*p*-toluenesulfonyl)-cytosine **26** [15,16] were prepared according to the literature. Reactions were monitored by thin-layer chromatography using TLC plates pre-coated with silica gel $60F_{254}$ (Merck).

7.2.2. 5-(Chloroacetylamino)uracil (*2*) [8]

5-Aminouracil **1** (6.5 g, 0.05 mol) was dissolved in 0.2 M sodium hydroxide solution (50 mL). To this solution, chloroacetyl chloride (5 mL, 0.06 mol) and 1 M aqueous sodium hydroxide solution (70 mL) were added within 30 min while stirring in an ice bath. Stirring was continued at 22 °C for 90 min, and then the solution was acidified with 5 M hydrochloric acid. The yellow precipitated crystals were separated and recrystallized from water giving **2** (6.4 g, 62%) (Found: C, 35.57; H, 2.81; N, 20.76. $C_6H_6N_3O_3Cl$ requires C, 35.40; H, 2.97; N, 20.64%); ^{1}H (400 MHz; DMSO-d_6) 4.34 (2H, s, CH_2), 8.09 (1H, s, C(6)H), 9.55 (1H, s, NH–CO), 10.72 (1H, br s, N(1)H), 11.53 (1H, br s, N(3)H); ESI-MS: *m/z* calculated for $C_6H_6N_3O_3Cl$ ($[M + H]^+$): 204.58; found: 204.18.

7.2.3. 5-(Aminoacetyl)aminouracil (*3*)

A solution of 6-methyluracil **10** (520 mg, 2.55 mmol) in concentrated ammonia (50 mL) was stirred at 22 °C for 24 h. The ammonia was evaporated and the residue was dissolved in water. Purification on a sephadex G10 column (25 mm × 100 mm) with water as eluent yielded **12** (381 mg, 81%) as a white powder (Found: C, 39.27; H, 4.55; N, 30.13. $C_6H_8N_4O_3$ requires C, 39.13; H, 4.38; N, 30.42%); ^{1}H(400 MHz; DMSO-d_6) 3.75 (2H, s, CH_2), 7.48 (2H, br s, NH_2), 8.06 (1H, s, C(6)H), 9.79 (1H, s, NH–CO), 10.77 (1H, br s, N(1)H), 11.54 (1H, br s, N(3)H); ESI-MS: *m/z* calculated for $C_6H_8N_4O_3$ ($[M + H]^+$): 185.15; found: 185.12.

7.2.4. 5-(N-Boc-ethylenediaminoacetyl)aminouracil (*4*)

To a suspension of **2** (120 mg, 0.6 mmol) in water (10 mL) and methanol (3 mL), a solution of *N*-boc-ethylenediamine (1.33 g, 8.3 mmol) in methanol (3 mL) was added and the mixture was heated in a pressure cylinder under stirring at 90 °C for 1 h. The solution was cooled down and the solvents were evaporated. Then the residue was dissolved in water, and purification on a sephadex G10 column with water as eluent afforded product **4** (146.6 mg, 76%). (Found: C, 47.62; H, 6.57; N, 21.26. $C_{13}H_{21}N_5O_5$ requires C, 47.70; H, 6.47; N, 21.39%); ^{1}H (400 MHz; DMSO-d_6) 1.35 (9H, s, 3 × Me), 2.53 (2H, m, CH_2–CH_2), 2.97 (2H, m, CH_2–CH_2), 3.20 (2H, s, CO–CH_2), 6.40 (1H, m, NH–CO), 6.76 (1H, m, CH_2–N*H*), 8.11 (1H, s, C(6)H), 9.32 (1H, s, C(5)NH–CO), 10.32 (1H, br s, N(1)H), 11.10 (1H, br s, N(3)H); ESI-MS: *m/z* calculated for $C_{13}H_{21}N_5O_5$ ($[M + H]^+$): 328.34; found: 328.42.

7.2.5. S-{2-[(2,4-Dioxo-1,2,3,4,-tetrahydropyrimidine-5-yl)amino]-2-oxoethyl}-D-cysteine (*5*)

Boc-cysteine (221.27 mg, 1 mmol) was added to a solution of **2** (203.58 mg, 1 mmol) in ethanol (5 mL) and 2 M aqueous sodium hydroxide (3 mL). The solution was stirred at 22 °C for 2 h and then acidified with 5 M hydrochloric acid to remove the protection group. The reaction product was dried at the rotary evaporator and the residue was dissolved in acetonitrile/water 1:9. Purification using MPLC on an RP18 column with acetonitrile/water 1:9 as eluent yielded **5** (219.1 mg, 76%). (Found: C, 37.35; H, 4.11; N, 19.39; S, 11.03. $C_9H_{12}N_4O_5S$ requires C, 37.50; H, 4.20; N, 19.43; S, 11.12%); ^{1}H (400 MHz; DMSO-d_6) 2.94 (2H, m, S–CH_2), 3.39 (2H, s, CO–CH_2), 3.44 (1H, m, CH), 3.42 (2H, m, NH_2), 8.06 (1H, s, C(6)H), 9.33 (1H, s, NH–CO), 10.68 (1H, s, N(1)H), 11.47 (1H, s, N(3)H); ESI-MS: *m/z* calculated for $C_9H_{12}N_4O_5S$ ($[M + H]^+$): 289.28; found: 289.18.

7.2.6. 5-[Bis-(2-p-methoxybenzylthioethyl)amine]acetylaminouracil (*6*)

Compound **2** (305.37 mg, 1.5 mmol) and ammonium sulphate (30 mg) were dissolved in hexamethyldisilazane (7 mL) and refluxed for 3 h. The clear reaction mixture was

dried at a rotary evaporator. The residue was dissolved in acetonitrile (1.5 mL), treated with triethylamine (110 μL) and bis(2-*p*-methoxybenzylthioethyl)amine [9] (270 mg, 0.7 mmol) dissolved in acetonitrile (1 mL). This solution was stirred at 90 °C for 4 h. After cooling, water (2 mL) was added and the solvent was evaporated. The residue was dissolved in dichloromethane/methanol 10:1 (5 mL) and purified on a silica gel column with dichloromethane/methanol 10:1 as eluent yielding **6** (629.1 mg, 77%) (Found: C, 57.18; H, 6.01; N, 10.11; S, 11.59. $C_{26}H_{32}N_4O_5S_2$ requires C, 57.33; H, 5.92; N, 10.29; S, 11.77%); ^{1}H (400 MHz; DMSO-d_6) 2.45 (4H, m, 2 × S−CH_2), 2.59 (4H, m, 2 × N−CH_2), 3.14 (2H, s, CO−CH_2), 3.63 (4H, s, 2 × CH_2), 3.69 (6H, 2 × O−Me), 6.81 (4H, d, *J* 8.0 Hz, 4 × Ph), 7.17 (4H, d, *J* 8.0 Hz, 4 × Ph), 8.09 (1H, s, C(6)H), 9.12 (1H, s, NH−CO), 10.66 (1H, br s, N(1)H), 11.52 (1H, br s, N(3)H); ESI-MS: *m*/*z* calculated for $C_{26}H_{32}N_4O_5S_2$ ($[M + H]^+$): 545.68; found: 545.39.

*7.2.7. 5-(Chloroacetyl)amino-1-[(1,3-dihydroxy-2-propoxy)-methyl]uracil (**18**)*

Compound **14** (462 mg, 2 mmol) was dissolved in 1 M aqueous sodium hydroxide (30 mL) and cooled down to 0 °C. To this solution, chloroacetyl chloride (2 mL) and 1 M aqueous sodium hydroxide (25 mL) were added under stirring within 30 min. Stirring was continued at 22 °C for 1 h. After acidification with 5 M hydrochloric acid, the solvent was removed by rotary evaporation and the residue was purified using MPLC on an RP18 column with acetonitrile/water 1:4 as eluent giving **18** (461.1 mg, 75%) (Found: C, 39.18; H, 4.72; N, 13.49. $C_{10}H_{14}N_3O_6Cl$ requires C, 39.04; H, 4.59; N, 13.66%); ^{1}H (400 MHz; DMSO-d_6) 3.41 (4H, m, 2 × CH_2−OH), 3.51 (1H, m, CH), 4.35 (2H, s, CO−CH_2), 4.61 (2H, t, *J* 5.2 Hz, 2 × OH), 5.20 (2H, s, N−CH_2), 8.42 (1H, s, C(6)H), 9.63 (1H, s, NH−CO), 11.78 (1H, s, N(3)H); ESI-MS: *m*/*z* calculated for $C_{10}H_{14}N_3O_6Cl$ ($[M + H]^+$): 308.69; found: 308.33.

*7.2.8. 1-Methyl-5-hydroxymethylcytosine (**22**)*

Compound **22** was prepared starting from 5-hydroxymethylcytosine **20** (1.41 g, 10 mmol) suspended in DMF (100 mL). To this suspension, 1 M methanolic tetrabutylammonium hydroxide (10 mL) was added dropwise. After achieving a clear solution, methyliodide (1.3 mL, 20 mmol) dissolved in DMF (5 mL) was added within 10 min, and then the mixture was stirred at 22 °C for 2 h. The reaction mixture was dried by rotary evaporation. Column chromatography on silica gel with dichloromethane/methanol 1:1 as eluent yielded **22** (1.27 g,82%) as a white powder (Found: C, 46.61; H, 5.93; N, 27.18. $C_6H_9N_3O_2$ requires C, 46.45; H, 5.85; N, 27.08%); ^{1}H (400 MHz; DMSO-d_6) 3.20 (3H, s, Me), 4.14 (2H, d, *J* 4.8 Hz, CH_2), 4.98 (1H, t, *J* 5.2 Hz, OH), 7.53 (1H, s, C(6)H); ESI-MS: *m*/*z* calculated for $C_6H_9N_3O_2$ ($[M + H]^+$): 156.16; found: 156.24.

*7.2.9. 1-Methyl-N^4-(p-toluenesulfonyl)cytosine (**27**)*

A solution of 1-methylcytosine **21** (166.68 mg, 1.5 mmol) and tosyl chloride (1.29 g, 6.75 mmol) in dry pyridine (22.5 mL) was stirred at 22 °C for 6 h. The pyridine was evaporated and the residue was dissolved in acetic acid ethyl ester and extracted twice with water. The organic layer was dried with sodium sulfate, filtered and the solvent was evaporated. The dark brown oil was dissolved in dichloromethane (5 mL) and separated on a silica gel column with dichloromethane/methanol 50:1 as eluent. Thus **27** (327.4 mg, 88%) of high purity was obtained (Found: C, 51.48; H, 4.50; N, 15.14; S, 11.32. $C_{12}H_{13}N_3O_3S$ requires C, 51.60; H, 4.69; N, 15.04; S, 11.48%); ^{1}H (400 MHz; DMSO-d_6) 2.35 (3H, s, Me), 3.26 (3H, s, N−Me), 6.50 (1H, d, *J* 7.6 Hz, C(5)H), 7.34 (2H, d, *J* 8.0 Hz, Ph), 7.73 (2H, d, *J* 8.3 Hz, Ph), 7.81 (1H, d, *J* 7.6 Hz, C(6)H); *m*/*z* (ESI) 279.31 (M^+ 280.26). ESI-MS: *m*/*z* calculated for $C_{12}H_{13}N_3O_3S$ ($[M + H]^+$): 280.31; found: 280.26.

Acknowledgements

The authors are indebted to Brigitte Große for her careful experimental assistance, and Madlen Matterna for ESI-MS measurements. Support for this study was provided by Ministry of Science, Education and Sports of the Republic of Croatia (Projects 098-0982914-2918 and 098-0982464-2514).

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