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C5-morpholinomethylation of *N*1-sulfonylcytosines by one-pot microwave assisted Mannich reaction

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Josipa Matić,[a,b,*] Irena Nekola,[c] Aleksandar Višnjevac,[d] Renata Kobetić,[a] Irena Martin-Kleiner,[e] Marijeta Kralj,[e,b] and Biserka Žinić[a,b,*]

A fast and efficient route for introduction of methylene bridged-amine (morpholinomethyl) functionality in the C5 position of the sulfonylated cytosine nucleobase has been developed. First, novel *N*1-sulfonylcytosine derivatives **3**-**6** were prepared by the condensation of silylated cytosine with selected sulfonyl chlorides. They were subsequently transformed to 5-morpholinomethyl-*N*1-sulfonylcytosine derivatives (**8**, **12**-**15**) using microwave irradiation. As a result of the cytosine ring opening in *N*1-tosylcytosine, depending on the reaction conditions, peculiar tosyl-urea derivative **9** has been isolated, which provided additional insight into reaction pathway. The influence of the C5-substituent on the antiproliferative activity has been evaluated by MTT test on U251, MCF-7 and MOLT-4 tumor cell-lines.

Introduction

Over the last few decades, in an extensive pursuit for the new chemotherapeutics, modified pyrimidine nucleobases and nucleosides have emerged as promising antitumor agents.[1,2,3] Numerous patent applications in the recent years prove that the interest in these compounds has not decreased.[4] On the other hand, sulfonyl moiety has been recognized as a potent pharmacophore in carbonic anhydrase and metalloprotease inhibitors, antifungal, antibacterial, antitumor and other biologically active compounds.[5,6] Since the sulfonyl-pyrimidines present an interesting combination of described pharmacophoric components, they have accordingly drawn quite attention of our research group.

Sulfonylated derivatives of cytosine, that we have described earlier, have shown strong antiproliferative activity against human tumor cell lines *in vitro*[7-12] and *in vivo*[13]. These findings encouraged us to further investigate modified pyrimidines. It has been known that different C5-substituted pyrimidine nucleosides often display antitumor activity.[14-17] One of the best known examples is 5-fluorouracil which is widely used in the treatment of cancer.[18] Moreover, it has been found that C5-carbonitrile pyrimidine derivatives modified with heterocyclic amines, like piperazine, piperidine and morpholine, could act as promoters of apoptosis in cancer cells through cyclin-dependent kinase 9 inhibition.[19] In addition, morpholine moiety is present in many drugs, like analgesic dextromoramide, antibiotic linezolid or antitumor drug gefitinib.[20]

We have decided to prepare a series of *N*1-sulfonylcytosines and an analogous series of *N*1-sulfonylcytosines with an amine (morpholine) substituent in the C5-position. Investigation of synthetic efforts for introduction of methylene-bridged morpholine moiety in the C5 position of *N*1-sulfonylcytosine is described herein. To the best of our knowledge, C5-morpholinomethylated derivatives of *N*1-sulfonylpyrimidine have not been reported so far. A secondary goal of the study was to evaluate the influence of C5-morpholinomethyl moiety on the biological activity of *N*1-sulfonylcytosines. Antiproliferative activity on U251, MCF-7 and MOLT-4 tumor cell-lines was examined by MTT test.

Results and discussion

Chemistry

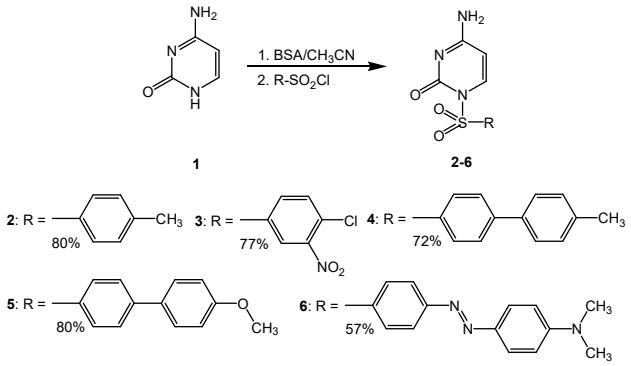
As a part of previous sulfonylcyclourea research, N1-sulfonylation of cytosine nucleobase was investigated and reaction conditions optimized throughout the preparation of *N*1-tosylcytosine (**2**; TsC).[21] The reaction was elegantly achieved by silylation with *N*,*O*-bis(trimethylsilyl)acetamide (BSA) in a dry acetonitrile and subsequent addition of appropriate sulfonyl chloride (Scheme 1). Novel *N*1-sulfonyl derivatives **3**-**6** were prepared in very good yield (57–80%). On the other hand, synthesis of 5-morpholinomethylcytosine **7** was described earlier by Prukała, and it was prepared by acid catalyzed Mannich reaction of cytosine with paraformaldehyde and morpholine in ethanol.[22]

In a quest for new pyrimidine analogs with potential medical application, we wanted to prepare cytosine derivatives which contain both N1-sulfonyl and C5-morpholinomethyl substituents.

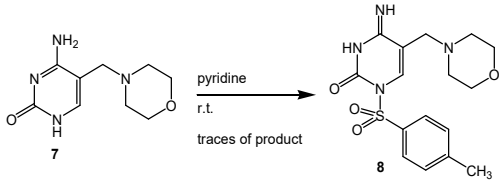
[a.] *Laboratory for Biomolecular Interactions and Spectroscopy, Division of Organic Chemistry and Biochemistry, Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia.*
[b.] *BioZyne Ltd., Bijenička cesta 54, 10000 Zagreb, Croatia.*
[c.] *Pliva Croatia TAPI R&D, Prilaz baruna Filipovića 25, 10000 Zagreb, Croatia.*
[d.] *Laboratory for chemical and biological crystallography, Division of Physical Chemistry, Bijenička cesta 54, Ruđer Bošković Institute, 10000 Zagreb, Croatia.*
[e.] *Laboratory of Experimental Therapy, Division of Molecular Medicine, Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia.*
Electronic Supplementary Information (ESI) available: ^{1}H and ^{13}C NMR spectra of all products, COSY NMR spectrum of **8**, additional reaction scheme, crystallographic data for the compound **9** (CCDC 1588667). See DOI: 10.1039/x0xx00000x

First we attempted the sulfonylation of **7** through several known methods: 1) activation/silylation of **7** with BSA in CH_3CN; 2) activation of **7** with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in *N*,*N*-dimethylformamide (DMF) and 3) activation of **7** with K_2CO_3 in DMF, 4) sulfonylation of **7** in pyridine.[21]

While method 4) provided traces of desired product **8** (Scheme 2), other methods resulted solely in isolation of starting compound **7** as a single product.



Scheme 1. Synthesis of *N*1-sulfonylcytosine derivatives.



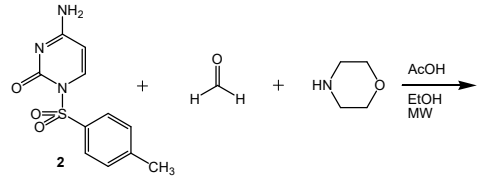
Scheme 2. N1-sulfonylation of 5-morpholinomethylcytosine **7** in pyridine.

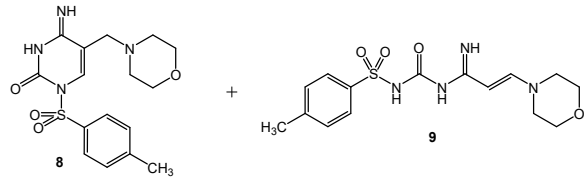
Since sulfonylation of **7** did not yield satisfactory results, we have decided to apply reversed synthetic approach. In this attempt, we planned to introduce a morpholinomethyl moiety to TsC (**2**). We applied the Prukała method (4h reflux), but a mixture of several byproducts was formed. Again, only traces of desired product **8** were detected, alongside with a large amount of starting TsC. Upon prolongation of the reaction time, complexity of the byproduct mixture increased (Supporting information, Figure S14). One of the reasons lies in the instability of N-SO_2 bond in the given conditions, namely several hours of heating in the acidic solution. Detection of cytosine (**1**) in the product mixture supports this assumption. Further on, it is well-known that cytidine reacts with formaldehyde in ethanol to provide the *N*-ethoxymethyl cytidine derivative.[23] In our case, corresponding *N*-ethoxymethly derivatives of TsC and cytosine were isolated from the mixture as well. In addition, as a possible source of byproducts, formation of 1,4 adducts in the reaction of cytosine with morpholine and formaldehyde has also been described previously.[24]

In an attempt to modify this method, we reasoned how to provide enough energy for a reaction to occur, but, at the same time, shorten the reaction time in order to prevent the formation of numerous byproducts. Following this logic, we have turned attention to microwave irradiation. Reaction conditions were explored with respect to temperature and time (Table 1). Aminomethylation of TsC (**2**) was studied as a model reaction. Two equivalents of morpholine and paraformaldehyde and four equivalents of acetic acid in respect to starting **2** were used in all entries, as described earlier.[22] Unlike previously described method, all reagents were added to reaction vial at once. The best yield on the compound **8** (65%), was obtained when the reaction mixture was heated 30 minutes at 100 °C. The product precipitated spontaneously from the reaction mixture upon cooling, which eliminated the need for a tedious isolation. Product was isolated simply by filtration, and purified by recrystallization from the methanol. Reasonably good results were obtained from reactions at higher temperature (120 °C). Although **8** was isolated in somewhat reduced yield (32-41%), the purity of the precipitated product was much higher and there was no need for recrystallization.

However, reactions at lower temperatures (80 °C) gave the unexpected results. As a result of cytosine ring opening, tosylurea derivative **9** was isolated in 45% yield, as a main product after 20 minutes. In addition, yield of **8** dropped significantly (Table 1). The amount of **9** decreased with prolongation of the reaction time, to disappear almost completely at higher temperatures. At 100 °C, **9** was isolated in only 2% yield after 20 minutes in a microwave reactor, while at longer reaction times merely traces were detected. Formation of similar acyclic analogues of uracil and cytosine was reported as a result of photoreactions in the presence of ethylamine.[25]

Table 1. Optimization of reaction conditions for the Mannich aminomethylation of *N*1-sulfonylpyrimidines.



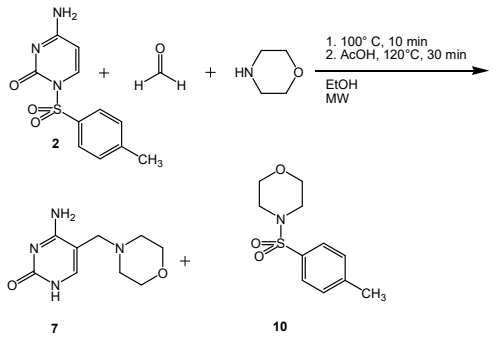


Entry	Temp.	Time	Yield of 8	Yield of 9
1	80 °C	20 min	23%	45%
2	80 °C	30 min	27%	30%
3	80 °C	40 min	48%	22%
4	80 °C	50 min	49%	17%
5	80 °C	60 min	49%	16%
6	100 °C	20 min	33%	2%
7	**100 °C**	**30 min**	**65%**	**traces**
8	100 °C	40 min	56%	traces
9	120 °C	20 min	41%	0%
10	120 °C	30 min	34%	0%
11	120 °C	40 min	32%	0%

In a separated experiment, we have proven that **9** undergoes ring closure upon heating. When **9** was heated for 1 hour in a microwave reactor at 100 °C, in pure ethanol, traces of TsC (**2**) were detected. Furthermore, heating of **9** in acidic ethanol solution resulted in almost complete conversion into TsC after one hour. It is reasonable to assume that higher reaction temperatures promote formation of

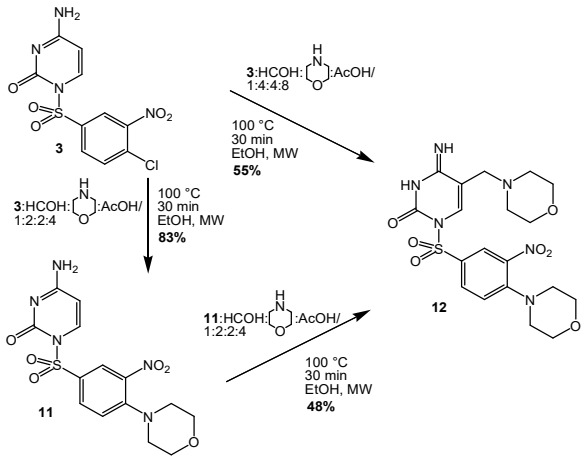
5-morpholinomethyl-*N*1-tosylcytosine (**8**), by cyclization of tosylurea-derivative **9** into starting TsC.

It is important to emphasize the significance of order in which the reagents were added. When we heated the reaction mixture prior to addition of acetic acid, as reported in described method, 5-morpholinomethylcytosine (**7**) was isolated as a main product.[22] This is due to the N-SO_2 bond breakage of TsC, which takes place in these conditions. In addition, as a result of the transamination reaction, an adduct **10** of morpholine and *p*-toluenesulfonic acid was isolated as well (Scheme 3).



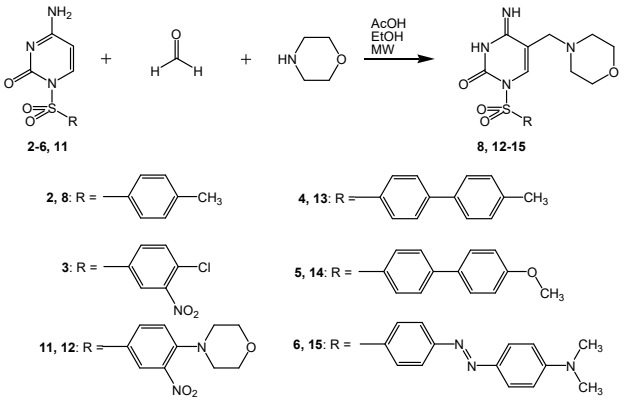
Scheme 3. Sequential addition of reagents leading to N-SO_2 bond breakage of TsC (**2**).

Once the optimal conditions for Mannich reaction were established, we have expanded the reaction scope on the series of *N*1-sulfonyl-aromatic and -polyaromatic cytosine derivatives **12**-**15** (Schemes 4 and 5, Table 2). Interestingly, the dominant reaction with the compound **3** was substitution of chlorine with morpholine (Scheme 4). Compound **11** was isolated in 83% yield. 5-morpholinomethyl-derivative **12** was isolated when the **11** was subsequently subjected to the established Mannich conditions, or alternatively, when the compound **3** was treated with double amount of reagents (four equivalents of morpholine and paraformaldehyde and eight equivalents of acetic acid).



Scheme 4. Synthetic routes for the preparation of 5-morpholinomethyl derivative **12**.

However, 30 minute-reaction at 100 °C did not provide adequate results with compounds **4**-**6**, bearing sulfonylbiphenyl or azobenzene substituents at N1-position (Scheme 5). Major amount of starting compound (77-100%) was isolated after the reaction. Also, corresponding sulfonyl-urea derivative was isolated from reaction with methyl-biphenyl derivative **4**. Nevertheless, **13** and **14** were prepared in good yields (52 and 49%) when higher temperature (120 °C) was applied for 30 minutes (Table 2). Compound **15** was isolated in somewhat modest yield (30%) after 60 minutes at 120 °C.



Scheme 5. Synthesis of 5-morpholinomethyl derivatives **12**-**15**.

Table 2. Reaction conditions for the synthesis of 5-morpholinomethyl derivatives **8**, **12**-**15**.

*N*1-comp.	*N*1-C5-comp.	Temp.	Time	Yield
2	**8**	100 °C	30 min	65%
3	**12**	100 °C	30 min	55%
11	**12**	100 °C	30 min	48%
4	**13**	120 °C	30 min	52%
5	**14**	120 °C	30 min	49%
6	**15**	120 °C	60 min	30%

Characterization of the products

NMR spectra

Structure of 5-morpholinomethyl-*N*1-tosylcytosine (**8**) was confirmed by 2d NMR spectroscopy. Absence of characteristic C5-proton signal in ^{1}H NMR spectrum, as well as coupling interaction of C6-proton with methylene protons in COSY spectrum confirms that the aminomethylation occurred at the C5-position of cytosine base (Supporting information, Figure S13). According to the chemical shifts of NH-protons, aminomethylated cytosine base of the compound **8** occupies keto-imino tautomeric form.[26]

Protons of acyclic urea derivative **9** exhibit significant shifts in NMR spectra compared to TsC (**2**) and **8**, indicating dramatically different structure. Signals of tosyl-protons of **9** are shifted upfield for about 0.2 ppm, and morpholine N-CH_2 protons are 0.9 ppm shifted downfield, compared to **8**. The most pronounced shift of 1.2 ppm displays vinyl CH proton, compared to C5 proton of TsC.

The acyclic structure of **9** was not unambiguously determined by NMR in solution (DMSO-d6) and therefore, additional studies were performed in gas-phase (mass spectrometry) and solid phase (single crystal diffraction X-ray).

Mass Spectra

H/D exchange experiments were performed to prove open cytosine structure of **9**, with three fast exchangeable protons, in contrast to N1-substituted cytosine molecule, having only two exchangeable protons. The collision-induced fragmentation (CID) experiments of **9** showed completely different composition of the obtained fragments in regards to the open structure. N1-substituted cytosine molecular ion increases mass by 2 i.u while molecular ion of **9** increased by 3 i.u. Additionally, we observed that **9** has three not equivalent C-N bonds in the gas phase (Figure 1). Heterolytic bond cleavage of **9** molecular ion (*m/z* for $[M+H]^+$ 353 or $[M+D]^+$ 356) occurred at three positions. The variation of the collision energy (CE 5-20 eV) or the ionization mode (ES^+ or ES^-) yielded the major fragment formed upon the **a** bond cleavage. (e.g. the most abundant signal at *m/z* 182.1 in ES^+). Bond **a** was the most unstable C-N bond, followed by **c** and finally **b** (Figures 1 and 2).

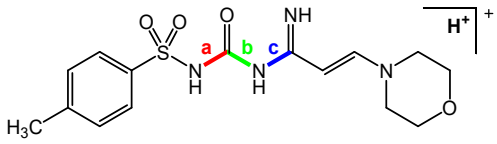


Figure 1. Marked up three C-N bonds that have similar but not the equivalent bonds in the gas phase. Bond **a** is the most unstable C-N bond, followed by **c** and finally **b**.

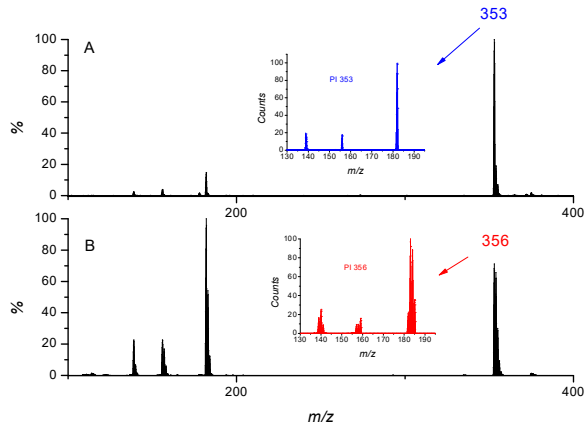


Figure 2. Full scan ES^+ spectrum for compound **9** dissolved in methanol (A) and deutero methanol (B) at about 10^{-6} mol dm^{-3}. Inserted spectrum is PI for molecular ion signals *m/z* for $[M+H]^+$ 353 (CH_3OH) and *m/z* 356 for $[M+D]^+$ (CD_3OD).

Molecular and crystal structure

Molecular structure of **9** reveals no peculiarities, with the exception of an unexpected proton migration from N8 to N12 and formation of a zwitterionic molecule that was discovered by the X-ray single crystal diffraction studies. Morpholine unity is in a usual chair conformation (Figure 3).

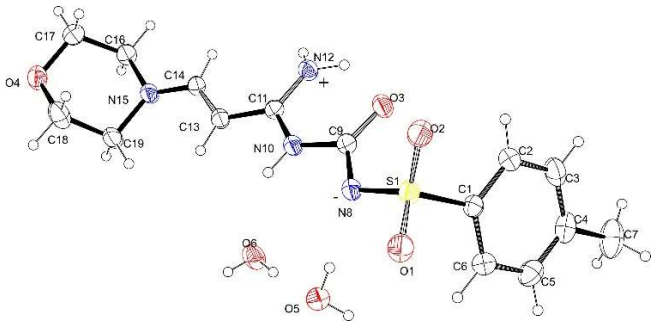


Figure 3. ORTEP drawing of compound **9** with the atom numbering. Displacement parameters are scaled to 50 % probability value.

Crystal packing of **9** is characterized by intensive networks of hydrogen bonds in which the co-crystallized water molecules of O5 and O6 are heavily involved (Table 3, Figures 4 and 5).

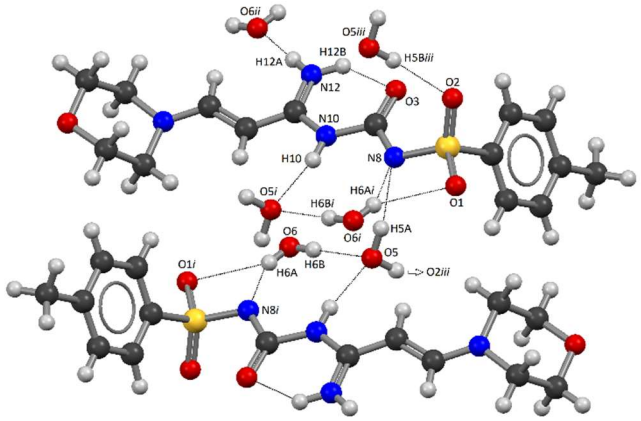


Figure 4. Hydrogen bonding pattern in the structure of **9**.

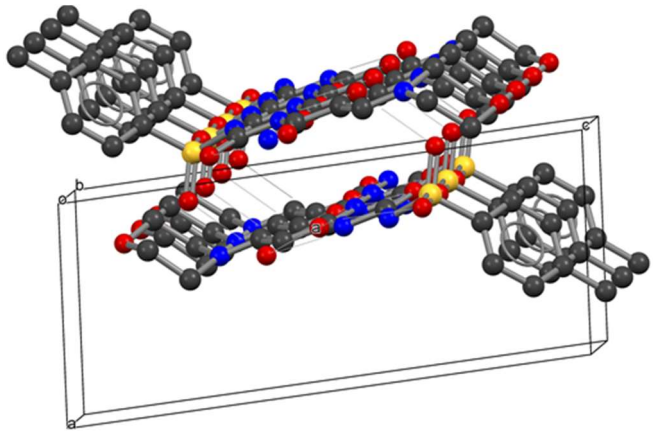


Figure 5. The H-bonded molecular tube a motif of the crystal packing in the structure of **9**.

The water molecule of O5 acts as a hydrogen bonding bridge between two main molecules (mutually related by the symmetry operation 1+x, y, z) being H-bonded to the deprotonated N8 of the first main molecule by its hydrogen H5A, and to the sulphonyl oxygen of the second one by its remaining hydrogen, H5B. The

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Table 3. Hydrogen bonds in the structure of **9**.

D-H ···*A*	D-H (Å)	H ···*A* (Å)	D···*A* (Å)	D-H ···*A* (°)	symm op. on *A*
O5-H5A···N8	0.90 (4)	2.10 (4)	2.992 (2)	174 (3)	1
O5-H5B···O2*iii*	0.85 (3)	2.02 (3)	2.855 (2)	168 (3)	1+x, y, z
O6-H6A···O1*i*	0.75 (3)	2.35 (3)	3.047 (3)	155 (3)	1-x, 1-y, 1-z
O6-H6A···N8*i*	0.75 (3)	2.49 (4)	3.103 (2)	139 (3)	1-x, 1-y, 1-z
O6-H6B···O5	0.78 (3)	2.05 (3)	2.830 (2)	174 (4)	1
N10-H10···O5*i*	0.89 (2)	2.19 (2)	3.075 (2)	171 (2)	1-x, 1-y, 1-z
N12-H12A···O6*ii*	0.87 (3)	2.01 (3)	2.875 (2)	172 (2)	1-x, 2-y, 1-z
N12-H12B···O3	0.89 (3)	2.02 (3)	2.657 (2)	128 (2)	1 (intra)

remaining water molecule (of O6) connects through its hydrogen atom H6B to the water molecule of O5, while its second hydrogen, H6A acts as a double donor forming two hydrogen bonds, towards the sulfonyl oxygen O1*i* and deprotonated nitrogen N8*i*, of the neighboring main molecule (related by the symmetry operation 1-x, 1-y, 1-z). Hence, the deprotonated nitrogen N8 acts as a double acceptor of the hydrogen bonds, connecting the main molecule to both of the co-crystallized water molecules. Finally, the protonated imino-nitrogen N12 effectuates a single intra-molecular H-bond in this structure, via its hydrogen H12B to the carbonyl oxygen O3. With its remaining hydrogen, H12A, N12 forms the hydrogen bond towards the co-crystallized water molecule of O6*ii*. All these interactions contribute to a construction of a two dimensional molecular tube of the (roughly measured) dimensions 4.58 Å x 6.24 Å, stretching parallel to the 100 crystallographic plane (Figure 5).

In vitro screening of antitumor activity

Antiproliferative activity of **2** (tosyl-derivative) on various tumor cell lines was extensively reviewed in previous studies, showing moderate activity on MCF-7 cells and no activity towards MOLT-4 cells.[7,11] The activity of new compounds was tested using MTT assay. Compounds showed diverse effect on proliferation of U251, MCF-7 and MOLT-4 tumor cell lines (Table 4). Among *N*1-sulfonylcytosine derivatives, compounds **3** (4-chloro-3-nitrophenyl-derivative), **4** (methylbiphenyl-derivative) and **5** (methoxybiphenyl-derivative) showed low micromolar IC_{50} activities, especially on MCF-7 and MOLT-4 cell lines.

Introduction of C5-morpholinomethyl group produced very heterogeneous effect on the antitumor activity, strongly depending on the N1-substituent. Tosyl-derivative **8** and azobenzene-derivative **15** showed very modest activity. Introduction of C5-morpholinomethyl moiety completely deteriorated antitumor activity in **15**, compared to its moderately active *N*1-sulfonyl analogue **6**. Introduction of two morpholine units also diminished activity in the compound **12**, which showed moderate activity, compared to very active *N*1-sulfonyl analogue **3**. However, insertion of C5-morpholinomethyl moiety had favorable influence on the antitumor activity of biphenyl-derivatives. Compounds **13** (methylbiphenyl-derivative) and **14** (methoxybiphenyl-derivative) exhibited even more pronounced antiproliferative activity toward U251 cell line than their *N*1-sulfonyl analogues **4** and **5**. At the same, **13** and **14** preserved remarkable potency toward MCF-7 and MOLT-4 cell lines.

Table 4. Antiproliferative activity of the new compounds.

Compound	IC_{50}[a]/μM		
	U251	**MCF-7**	**MOLT-4**
3	3 ± 2	4 ± 3	2 ± 1
4	22 ± 1	3 ± 0.2	1 ± 0.1
5	23 ± 2	3 ± 1	5 ± 2
6	8 ± 3	30 ± 11	>100
8	≥100	≥100	≥100
12	61±1	28±3	18±2
13	4±0.6	2±0.2	2±0.1
14	3±0.2	2±0.5	2±0.1
15	46±38	>100	>100

[a]IC_{50}; the concentration that causes 50% growth inhibition

Experimental

Materials and apparatus

Solvents were distilled from appropriate drying agents shortly before use. Microwave assisted reaction was conducted in a borosilicate glass vials sealed by reusable snap-cap with PTFE coated silicone septum. The microwave heating was performed in the Anton Paar microwave synthesis reactor Monowave 300. After completed irradiation, the reaction tube was cooled with high-pressure air until the temperature had fallen below 55 °C. TLC was carried out on DCplastikfolien Kieselgel 60 F254 and preparative thin layer (2 mm) chromatography was done on Merck 60 F254. NMR spectra were recorded on 600 and 300 MHz spectrometers. Mass spectrometry was performed on the Agilent 6410 Triple Quad mass spectrometer (Agilent Technologies). High resolution mass spectra (HRMS) were obtained using a MALDI-TOF/TOF mass spectrometer 4800 Plus MALDI TOF/TOF analyzer (Applied Biosystems Inc., Foster City, CA, USA). FT-IR spectra of the samples in KBr pellets were recorded at

resolution of 4 cm^{-1} on an ABB Bomem MB102 single-beam spectrometer. The electronic absorption spectra of newly prepared compounds were measured on a Varian Cary 100 Bio spectrometer.

Synthesis

General procedure for the preparation of *N*1-sulfonylcytosine derivatives (3–6)

A mixture of cytosine **1** (1 mmol) and BSA (3 mmol) was heated under reflux in dry acetonitrile (2 mL) for 30 min. The solution was cooled to 0 °C and sulfonyl chloride (1 mmol) was added. After heating under reflux for 60 min a small amount of ammonia/methanol was added in the reaction mixture which immediately resulted in precipitation of crystals. Solid was filtered off and washed with cold methanol. Crude product was recrystallized from methanol to give analytically pure sample.

***N*1-(4-chloro-3-nitrophenylsulfonyl)cytosine** (**3**): Synthesis was performed according to the general procedure with 4-chloro-3-nitrobenzenesulfonyl chloride to give the product **3** (453 mg, 77%) as white crystals.

M.p. = 222–224 °C; R_f = 0.3 (CH_2Cl_2:CH_3OH/9:1); UV (MeOH): λ_{max}/nm: 225 and 247; log ε/ dm^3 mol^{-1} cm^{-1}: 4.21 and 4.21; IR (KBr) ν_{max}/cm^{-1}: 3381 (m), 3099 (m), 1672 (s), 1659 (s), 1593 (w), 1572 (w), 1535 (s), 1491 (m), 1387 (m), 1356 (s), 1283 (m), 1256 (w), 1234 (w), 1188 (s), 1111 (m), 1097 (m), 1058; (w), 1020 (w); ^{1}H NMR (DMSO-d6) δ/ppm: 8.66 (d, 1H, J = 2.2 Hz, Ar), 8.28 (dd, 1H, J_1 = 8.6 Hz, J_2 = 2.3 Hz, Ar), 8.15–7.95 (m, 4H, Ar, H-6, NH_2), 6.00 (d, 1H, $J_{5,6}$ = 7.9 Hz, H-5); ^{13}C NMR (DMSO-d6) δ/ppm: 166.1 (C_q), 150.8 (C_q), 147.2 (C_q), 139.2 (CH, C-6), 137.1 (C_q), 133.6 (CH, Ar), 132.7 (CH, Ar), 131.4 (C_q), 126.2 (CH, Ar), 98.0 (CH, C-5). (see Supporting information Fig. S1) ESI-MS: calcd. for $C_{10}H_7ClN_4O_5S$: 330.0; found $[M+H]^+$ at *m/z* 331.0.

***N*1-(4'-methylbiphenyl-4)sulfonylcytosine** (**4**): Synthesis was performed according to the general procedure with 4'-methylbiphenyl-4-sulfonyl chloride to give the product **4** (217 mg, 72%) as white crystals.

M.p. = 244–245 °C; R_f = 0.5; UV (MeOH): λ_{max}/nm: 284; log ε/ dm^3 mol^{-1} cm^{-1}: 2.58; IR (KBr) ν_{max}/cm^{-1}: 3377 (m), 3107 (m), 3069 (w), 3030 (w), 1676 (s), 1595 (w), 1524 (s), 1483 (s), 1373 (m), 1362 (m), 1283 (m), 1232 (w), 1171 (s), 1148 (w), 1115 (w), 1092 (m), 1024 (w), 1005 (w); ^{1}H NMR (DMSO-d6) δ/ppm: 8.15 (d, $J_{6,5}$ = 7.9 Hz, 1H, H-6), 8.04–7.99 (m, 2H, Ar), 7.93–7.87 (m, 4H, NH_2, Ar), 7.65 (d, J = 8.2 Hz, 2H, Ar), 7.33 (d, J = 8.0 Hz, 2H, Ar), 5.98 (d, $J_{5,6}$ = 7.9 Hz, 1H, H-5); ^{13}C NMR (DMSO-d6) δ/ppm: 165.9 (C_q), 150.9 (C_q), 145.8 (C_q), 139.3 (CH, C-6), 138.5 (C_q), 135.4 (C_q), 135.2 (C_q), 129.7 (CH, Ar), 129.3 (CH, Ar), 127.0 (CH, Ar), 126.8 (CH, Ar), 97.4 (CH, C-5), 20.7 (CH_3). (see Supporting information Fig. S2) ESI-MS: calcd. for $C_{17}H_{15}N_3O_3S$: 341.1; found $[M+H]^+$ at *m/z* 342.1.

***N*1-(4'-methoxybiphenyl-4)sulfonylcytosine** (**5**): Synthesis was performed according to the general procedure with 4'-methoxybiphenyl-4-sulfonyl chloride to give the product **5** (283 mg, 80%) as white crystals.

M.p. = 227–228 °C; R_f = 0.4; UV (MeOH): λ_{max}/nm: 246; log ε/ dm^3 mol^{-1} cm^{-1}: 4.00; IR (KBr) ν_{max}/cm^{-1}: 3366 (m), 3099 (m), 1670 (s), 16076 (m), 1522 (s), 1487 (s), 1356 (m), 1286 (m), 1250 (m), 1169 (s), 1140 (w), 1115 (m), 1092 (m), 1024 (w); ^{1}H NMR (DMSO-d6) δ/ppm: 8.14 (d, $J_{6,5}$ = 7.9 Hz, 1H, H-6), 8.02–7.96 (m, 2H, Ar), 7.93–7.85 (m, 4H, Ar, NH_2), 7.75–7.68 (m, 2H, Ar), 7.11–7.04 (m, 2H, Ar), 5.97 (d, $J_{5,6}$ = 7.9 Hz, 1H, H-5); ^{13}C NMR (DMSO-d6) δ/ppm: 165.9 (C_q), 160.0 (C_q), 150.9 (C_q), 145.5 (C_q), 139.4 (CH, C-6), 134.8 (C_q), 130.3 (C_q), 129.4 (CH, Ar), 128.5 (CH, Ar), 126.5 (CH, Ar), 114.6 (CH, Ar), 97.4 (CH, C-5), 55.3 (CH_3). (see Supporting information Fig. S3) ESI-MS: calcd. for $C_{17}H_{15}N_3O_4S$: 357.1; found $[M+H]^+$ at *m/z* 358.1.

***N*1-(4-(dimethylamino)azobenzene-4'-sulfonylcytosine** (**6**): Synthesis was performed according to the general procedure with 4-(dimethylamino)azobenzene-4'-sulfonyl chloride to give the product **6** (212 mg, 57%) as purple crystals.

M.p. = 263–265 °C; R_f = 0.5; UV (MeOH): λ_{max}/nm: 267 and 454; log ε/ dm^3 mol^{-1} cm^{-1}: 4.12 and 4.31; IR (KBr) ν_{max}/cm^{-1}: 3373 (m), 3115 (m), 2920 (w), 1655 (s), 1605 (s); 1585 (m), 1520 (m), 1483 (m), 1367 (s), 1358 (s), 1313 (w), 1279 (m), 1234 (w), 1182 (m), 1140 (s), 1121 (m), 1084 (m), 1020 (w); ^{1}H NMR (DMSO-d6) δ/ppm: 8.15 (d, 1H, $J_{6,5}$ = 7.9 Hz, H-6), 8.09 (d, 2H, J = 8.7 Hz, Ar), 7.99–7.88 (m, 4H, Ar, NH_2), 7.84 (d, 2H, J = 9.1 Hz, Ar), 6.85 (d, 2H, J = 9.2 Hz, Ar), 5.98 (d, 1H, $J_{5,6}$ = 7.9 Hz, H-5); ^{13}C NMR (DMSO-d6) δ/ppm: 165.9 (C_q), 155.8 (C_q), 153.4 (C_q), 150.9 (C_q), 142.7 (C_q), 139.3 (CH, C-6), 136.3 (C_q), 130.1 (CH, Ar), 125. 7 (CH, Ar), 121.8 (CH, Ar), 111.6 (CH, Ar), 97.5 (CH, C-5), 39.8 (CH_3). (see Supporting information Fig. S4) ESI-MS: calcd. for $C_{18}H_{18}N_6O_3S$: 398.1; found $[M+H]^+$ at *m/z* 399.1.

5-Morpholinomethyl-*N*1-sulfonylcytosine derivatives:

5-Morpholinomethyl-*N*1-tosylcytosine (**8**): *N*1-tosylcytosine (**2**) (200 mg; 0.75 mmol), paraformaldehyde (46 mg, 1.50 mmol, 97%), morpholine (131 µL, 1.50 mmol, 99%) and acetic acid (172 µL, 3.00 mmol) were suspended in absolute ethanol (5 mL). Reaction mixture was heated 30 minutes at 100 °C in a microwave reactor. Product **8** precipitated upon cooling of the mixture and it was purified by recrystallization from hot methanol (white crystals, 178 mg, 58%).

M.p. = 245–246 °C; R_f = 0.6 (CH_2Cl_2:CH_3OH/9:1); UV (MeOH): λ_{max}/nm: 247; log ε/dm^3 mol^{-1} cm^{-1}: 3.84; IR (KBr) ν_{max}/cm^{-1}: 3290 (m), 3098 (m), 3067 (m), 2959 (m), 2922 (m), 2841 (m), 1684 (s), 1517 (s), 1476 (m), 1451 (m), 1369 (m), 1353 (s), 1340 (m), 1291 (s), 1268 (w), 1240 (w), 1205 (w), 1188 (w), 1171 (s), 1116 (s), 1088 (s), 1008 (m); ^{1}H NMR (DMSO-d6) δ/ppm: 8.17 (s, 1H, NH), 8.00 (s, 1H, H-6), 7.86 (d, J = 8.3 Hz, 2H, Ar), 7.65 (s, 1H, NH), 7.44 (d, J = 8.1 Hz, 2H, Ar), 3.63–3.53 (m, 4H, O-CH_2), 3.30 (s, 2H, CH_2), 2.41 (s, 7H, N-CH_2, CH_3); 13C NMR (DMSO-d6) δ/ppm: 166.2 (C_q), 151.1 (C_q), 145.8 (C_q), 138.0 (CH, C-6), 134.7 (C_q), 130.0 (CH, Ar), 129.2 (CH, Ar), 104.9 (C_q, C-5), 66.6 (O-CH_2), 56.3 (CH_2), 53.0 (N-CH_2), 21.6 (CH_3). (see Supporting information Fig. S5) HRMS: *m/z*: calcd for $C_{16}H_{21}N_4O_4S^+$: 365.1284; found 365.1271 $[M+H]^+$.

5-Morpholinomethyl-*N*1-(4-morpholino-3-nitrophenylsulfonyl)cytosine (**12**):

a) *N*1-(4-morpholino-3-nitrophenylsulfonyl)-cytosine (**11**) (37 mg; 0.10 mmol), paraformaldehyde (7 mg, 0.23 mmol), morpholine (18 µL; 0.20 mmol) and acetic acid (23 µL, 0.40 mmol) were

suspended in absolute ethanol (2 mL). Reaction mixture was heated 30 minutes at 100 °C in a microwave reactor. Product **12** was isolated by thin-layer chromatography (eluent: 10% MeOH in CH_2Cl_2, yellow crystals, 23 mg, 48%).

b) *N*1-(4-chloro-3-nitrophenylsulfonyl)-cytosine (**3**) (196 mg, 0.59 mmol), paraformaldehyde 71 mg, 2.36 mmol), morpholine (206 µL, 2.36 mmol) and acetic acid (270 µL, 4.72 mmol) were suspended in absolute ethanol (5 mL). Reaction mixture was heated 30 minutes at 100 °C in a microwave reactor. Product **12** was isolated by thin-layer chromatography (eluent: 10% MeOH in CH_2Cl_2, yellow crystals, 155 mg, 55%).

M.p. = 272–273 °C; R_f = 0.6 (CH_2Cl_2:CH_3OH/9:1); UV (MeOH): λ_{max}/nm: 258 and 404; log ε/dm^3 mol^{-1} cm^{-1}: 4.58 and 3.72; IR (KBr) ν_{max}/cm^{-1}: 3420 (s), 3105 (m), 2951 (m), 1692 (s), 1662 (s), 1601 (s), 1556 (s), 1526 (s), 1478 (m), 1449 (w), 1413 (m), 1379 (m), 1367 (m), 1352 (m), 1340 (m), 1331 (w), 1296 (m), 1267 (w), 1235 (m), 1178 (s), 1118 (s), 1007 (m); ^{1}H NMR (DMSO-d6) δ/ppm: 8.37 (d, 1H, J = 2.4 Hz, Ar), 8.20 (brs, 1H, NH), 8.02 (dd, 1H, J_1 = 9.1, J_2 = 2.4 Hz, Ar), 7.97 (s, 1H, H-6), 7.69 (brs, 1H, NH), 7.43 (d, 1H, J = 9.2 Hz, Ar), 3.74–3.50 (m, 4H, O-CH_2), 3.57 (d, 4H, J = 4.0 Hz, O-CH_2), 3.30 (s, 2H, CH_2), 3.26–3.19 (m, 4H, N-CH_2), 2.39 (m, 4H, N-CH_2); ^{13}C NMR (DMSO-d6) δ/ppm: 165.7 (C_q), 150.7 (C_q), 148.4 (C_q), 137.5 (CH, C-6), 137.5 (C_q), 133.6 (CH, Ar), 128.8 (CH, Ar), 125.0 (C_q), 120.0 (CH, Ar), 104.4 (C_q, C-5), 66.1 (O-CH_2), 65.6 (O-CH_2), 55.8 (CH_2), 52.5 (N-CH_2), 50.2 (N-CH_2). (see Supporting information Fig. S6) HRMS: *m/z*: calcd for $C_{19}H_{24}N_6O_7SNa^+$: 503.1325; found 503.1342 [*M*+Na]$^+$.

5-Morpholinomethyl-*N*1-(4'-methylbiphenyl-4)sulfonylcytosine (**13**): *N*1-(4'-methylbiphenyl-4)-sulfonylcytosine (**4**) (101 mg, 0.30 mmol), paraformaldehyde (19 mg, 0.61 mmol, 97%), morpholine (52 µL, 0.60 mmol) and acetic acid (68 µL, 1.18 mmol) were suspended in absolute ethanol (3 mL). Reaction mixture was heated 30 minutes at 120 °C in a microwave reactor (stirring speed: 600 rpm). Product **13** was isolated by thin-layer chromatography (eluent: 10% MeOH in CH_2Cl_2, white crystals, 69 mg, 52%).

M.p. > 300 °C (dec.); R_f = 0.5 (CH_2Cl_2/CH_3OH 9:1); UV (MeOH): λ_{max}/nm: 285; log ε/dm^3 mol^{-1} cm^{-1}: 4.69; IR (KBr) ν/cm^{-1}: 3440(s), 3097 (m), 1691 (s), 1654 (m), 1590 (w), 1518 (w), 1474 (w), 1365 (w), 1353 (w), 1340 (w), 1298 (w), 1172 (m), 1121 (w), 1089 (w); ^{1}H NMR (DMSO-d6) δ/ppm: 8.19 (brs, 1H, NH), 8.02 (d, 3H, J = 8.3 Hz, H-6, Ar), 7.90 (d, 2H, J = 8.6 Hz, Ar), 7.66 (d, 2H, J = 8.1 Hz, Ar), 7.60 (m, 1H, NH), 7.33 (d, 2H, J = 8.0 Hz, Ar), 3.58 (s, 4H, O-CH_2), 3.17 (s, 2H, CH_2), 2.46–2.23 (m, 7H, N-CH_2, CH_3); ^{13}C NMR (DMSO-d6) δ/ppm: 165.7 (C_q), 150.7 (C_q), 145.8 (C_q), 138.5 (C_q), 137.5 (CH, C-6), 135.4 (C_q), 135.2 (C_q), 129.7 (CH, Ar), 129.4 (CH, Ar), 127.0 (CH, Ar), 126.8 (CH, Ar), 104.5 (C_q, C-5), 66.1 (O-CH_2), 55.8 (CH_2), 52.5 (N-CH_2), 20.7 (CH_3). (see Supporting information Fig. S7) HRMS: *m/z*: calcd for $C_{22}H_{25}N_4O_4S^+$: 441.1597; found 441.1595 [*M*+H]$^+$.

5-Morpholinomethyl-*N*1-(4'-methoxylbiphenyl-4)sulfonylcytosine (**14**): *N*1-(4'-methoxylbiphenyl-4)-sulfonylcytosine (**5**) (54 mg, 0.15 mmol), paraformaldehyde (10 mg, 0.32 mmol), morpholine (26 µL, 0.30 mmol) and acetic acid (35 µL, 0.59 mmol) were suspended in absolute ethanol (3 mL). Reaction mixture was heated 30 minutes at 120 °C in a microvawe reactor. Product **14** was isolated by thin-layer chromatography (eluent: 10% MeOH in CH_2Cl_2, white crystals, 33 mg, 49%).

M.p. = 211–212 °C; R_f = 0.6 (CH_2Cl_2/CH_3OH 9:1); UV (MeOH): λ_{max}/nm: 299; log ε/dm^3 mol^{-1} cm^{-1}: 4.17; IR (KBr) ν/cm^{-1}: 3430(m), 1688 (s), 1662 (s), 1609 (m), 1592 (m), 1520 (s), 1489 (s), 1397 (w), 1368 (m), 1354 (m), 1340 (m), 1297 (s), 1270 (m), 1252 (m), 1174 (s), 1116 (m), 1090 (m); ^{1}H NMR (DMSO-d6) δ/ppm: 8.20 (brs, 1H, NH), 8.07–7.97 (m, 3H, Ar, H-6), 7.88 (d, 2H, J = 8.7 Hz, Ar), 7.72 (d, 2H, J = 8.8 Hz, Ar), 7.67 (brs, 1H, NH), 7.08 (d, 2H, J = 8.8 Hz, Ar), 3.82 (s, 3H, O-CH_3), 3.58 (s, 4H, O-CH_2), 3.32 (s, 2H, CH_2), 2.41 (s, 4H, N-CH_2); ^{13}C NMR (DMSO-d6) δ/ppm: 165.7 (C_q), 160.0 (C_q), 150.7 (C_q), 145.5 (C_q), 137.5 (CH, C-6), 134.8 (C_q), 130.3 (C_q), 129.4 (CH, Ar), 128.5 (CH, Ar), 126.4 (CH-Ar), 114.6 (CH, Ar), 104.4 (C_q, C-5), 66.1 (O-CH_2), 55.8 (CH_2), 55.3 (OCH_3), 52.5 (N-CH_2). (see Supporting information Fig. S8) HRMS: *m/z*: calcd for $C_{22}H_{25}N_4O_5S^+$: 457.1546; found 457.1529 [*M*+H]$^+$.

5-Morpholinomethyl-*N*1-(4-(dimethylamino)azobenzene-4'-sulfonylcytosine (**15**): *N*1-(4-(dimethylamino)azobenzene-4'-sulfonylcytosine (**6**) (60 mg, 0.15 mmol), paraformaldehyde (11 mg, 0.35 mmol), morpholine (26 µL, 0.30 mmol) and acetic acid (34 µL, 0.59 mmol) were suspended in absolute ethanol (5 mL). Reaction mixture was heated 60 minutes at 120 °C in a microwave reactor (stirring speed: 600 rpm). Product 15 was isolated by thin-layer chromatography (eluent: 10% MeOH in CH_2Cl_2, red crystals, 23 mg, 30%).

M.p. > 300 °C (dec.); R_f = 0.6 (CH_2Cl_2/CH_3OH 9:1); UV (MeOH): λ_{max}/nm: 272 and 455; log ε/dm^3 mol^{-1} cm^{-1}: 4.03 and 4.26; IR (KBr) ν/cm^{-1}: 3447 (s), 1607 (m), 1520 (w), 1367 (m), 1178 (m), 1142 (m), 1117 (m), 1084 (m); ^{1}H NMR (DMSO-d6) δ/ppm: 8.22 (brs, 1H, NH), 8.09 (d, 2H, J = 8.7 Hz, Ar), 8.04 (s, 1H, H-6), 7.91 (d, 2H, J = 8.7 Hz, Ar), 7.84 (d, 2H, J = 9.2 Hz, Ar), 7.70 (brs, 1H, NH), 6.87 (d, 2H J = 9.2 Hz, Ar), 3.58 (s, 4H, O-CH_2), 3.32 (s, 2H, CH_2+H_2O), 3.10 (s, 6H, CH_3), 2.41 (s, 4H, N-CH_2); ^{13}C NMR (DMSO-d6) δ/ppm: 165.7 (C_q), 155.8 (C_q), 153.4 (C_q), 142.7 (C_q), 137.4 (CH, C-6), 136.3 (C_q), 130.2 (CH-Ar), 125.7 (CH-Ar), 121.8 (CH-Ar), 111.6 (CH-Ar), 104.6 (C_q, C-5), 66.1 (O-CH_2), 55.7 (CH_2), 52.5 (N-CH_2). (see Supporting information Fig. S9) HRMS: *m/z*: calcd for $C_{23}H_{28}N_7O_4S^+$: 498.1923; found 498.1908 [*M*+H]$^+$.

Byproducts:

1-Imino-3-morpholinoallyl-3-tosylurea (**9**)

R_f = 0.4 (CH_2Cl_2/CH_3OH 9:1); ^{1}H NMR (DMSO-d6) δ/ppm: 9.90 (bs, 1H, NH), 9.74 (bs, 1H, NH), 8.69 (s, 1H, NH), 7.88 (d, J = 13.4 Hz, 1H, CH), 7.65 (d, J = 8.1 Hz, 2H, Ar), 7.22 (d, J = 8.0 Hz, 2H, Ar), 4.75 (d, J = 13.4 Hz, 1H, CH), 3.75–3.54 (m, 4H, O-CH_2), 3.30–3.18 (m, 4H, N-CH_2), 2.33 (s, 3H, CH_3).; ^{13}C NMR (DMSO-d6) δ/ppm: 162.6 (C_q), 157.0 (C_q), 151.5 (CH), 142.3 (C_q), 139.8 (C_q), 128.1 (CH-Ar), 126.6 (CH-Ar), 80.6 (CH), 65.4 (CH_2), 20.6 (CH_3). (see Supporting information Fig. S10) ESI-MS: calcd. for $C_{15}H_{20}N_4O_4S$: 352.1; found [M+H]$^+$ at *m/z* 353.0 and 351.0 at *m/z* [M-H]$^-$.

4-Tosylmorpholine (**10**)

R_f = 0.9 (CH_2Cl_2/CH_3OH 9:1); ^{1}H NMR (DMSO-d6) δ/ppm: 7.68–7.58 (m, 2H, Ar), 7.53–7.43 (m, 2H, Ar), 3.72–3.54 (m, 4H, O-CH_2), 2.91–2.78 (m, 4H, N-CH_2), 2.42 (s, 3H, CH_3); ^{13}C NMR (DMSO-d6) δ/ppm: 143.8 (C_q), 131.5 (C_q), 129.8 (CH-Ar), 127.6 (CH-Ar), 65.2 (O-CH_2), 45.8 (N-CH_2), 21.0 (CH_3). (see Supporting information Fig. S11) ESI-MS: calcd. for $C_{11}H_{15}NO_3S$: 241.1; found [M+H]$^+$ at *m/z* 241.9.

***N*1-(4-Morpholino-3-nitrophenylsulfonyl)cytosine** (**11**):

R_f = 0.3 (CH_2Cl_2/CH_3OH 9:1); ^{1}H NMR (DMSO-d6) δ/ppm: 8.36 (d, J = 2.4 Hz, 1H, Ar), 8.07 (d, $J_{6,5}$ = 7.9, 1H, H6), 8.01 (dd, J_1 = 9.1, J_2 = 2.4 Hz, 1H, Ar), 7.93 (s, 2H, NH_2), 7.43 (d, J = 9.2 Hz, 1H, Ar), 5.94 (d, $J_{5,6}$ = 7.9 Hz, 1H, H5), 3.74–3.66 (m, 4H, O-CH_2), 3.27–3.19 (m, 4H, N-CH_2); ^{13}C NMR (DMSO-d6) δ/ppm: 166.0 (C_q), 151.0 (C_q), 148.4 (C_q), 139.4 (CH), 137.3 (C_q), 133.6 (CH-C6), 128.8 (CH), 125.0 (C_q), 120.0 (CH), 97.3 (CH-C5), 65.6 (O-CH_2), 50.2 (N-CH_2). (see Supporting information Fig. S12) ESI-MS: calcd. for $C_{14}H_{15}N_5O_6S$: 381.1; found $[M+H]^+$ at *m/z* 382.0.

Crystalographic data

Crystal data, data collection and refinement parameters are summarized in Table 5. Data collection was performed at room temperature on an Oxford Diffraction Xcalibur Nova R diffractometer with the microfocusing Cu tube (λ = 1.54179 Å). Data reduction and cell refinement were carried out using the CRYSALIS PRO software.[27] Structure was solved by direct methods with SIR2014[28] and refined by a full matrix least-squares refinement based on F2, with SHELXL[29]. Molecular illustrations were prepared with ORTEP-3[30] and MERCURY[31] included in the WinGX package[32]. Calculations of molecular geometries and crystal packing parameters were performed with PLATON[33]. Hydrogen atoms were located in the Fourier map and refined freely. CCDC 1588667 contains the supplementary crystallographic data. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223-336-033; or e-mail: deposit@ccdc.cam.ac.uk.

Table 5. Crystallographic data

Structure	**9**
Brutto formula	$C_{15}H_{24}N_4O_6S$
Formula weight (gmol^{-1})	388.44
Crystal color and habit	colourless plate
Crystal dimensions (mm)	0.04 x 0.06 x 0.2
Space group	*P-1*
a (Å)	6.5612 (3)
b (Å)	9.2289 (4)
c (Å)	15.5715 (7)
α (°)	95.807 (4)
β (°)	94.598 (4)
γ (°)	98.710 (4)
V (Å^3)	922.86 (7)
Z	2
μ (CuK_α) (mm^{-1})	1.918
Absorption correction	multi-scan
F(000)	414
θ max (°)	75.881
No. refl. measured	7824
No. refl. unique	3769
No. refl. observed [I>2σ(I)]	3369
R_{int}	0.0309
R_σ	0.0489
Parameters	280
R_1 [I>2σ(I)]	0.0537
wR_2, all	0.1531
S	1.001
ρ_{max}, ρ_{min} (eÅ^{-3})	0.65; -0.32

Cell culturing

Human tumor cell lines U251 (glioblastoma) and MCF-7 (breast carcinoma) cells were cultured as monolayers, while MOLT-4 (acute lymphoblastic leukemia) cell line was maintained in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37˚C.

Proliferation assays

The cells were inoculated onto a series of standard 96-well microtiter plates on day 0, at 1×10^4 to 3×10^4 cells/ml, depending on the doubling times of specific cell line. Test agents were then added in five 10-fold dilutions (10^{-8} to 10^{-4} M) and incubated for a further 72 hours. Working dilutions were freshly prepared on the day of testing. After 72 hours of incubation the cell growth rate was evaluated by performing the MTT assay, as described previously.[11,34] Each test point was performed in quadruplicate in at least two individual experiments. The results were expressed as IC_{50}, a concentration necessary for 50% of inhibition. Each result is a mean value from at least two separate experiments.

Conclusions

Fast and efficient synthetic method for the synthesis of 5-morpholinomethyl-*N*1-sulfonylcytosine derivatives has been established. *N*1-sulfonylcytosine derivatives **3**–**6** were prepared by the condensation of pyrimidine bases with different sulfonyl chlorides, and then transformed to C5-substituted *N*1-sulfonylcytosines (**8**, **12**-**15**) in moderate to very good yield (up to 65%). Microwave assisted reaction significantly shortened the reaction time and favored formation of Mannich product over numerous possible byproducts. Shortened reaction time adequately addressed the problem of N-SO_2 bond instability. Moreover, it has been found that temperature controls the reaction pathway. As a result of the cytosine ring opening, interesting tosylurea derivative **9** has been isolated from the reaction at lower temperature (80 °C). At higher temperatures (100 °C and 120 °C), **9** undergoes acid catalyzed cyclization to form starting TsC and participates in the Mannich reaction. In this sense, elevated temperatures promote formation of the Mannich product. Presented method constitutes a robust tool for introduction of new pharmacophores, such as cyclic amine morpholine, into biologically active pyrimidines. Two of the newly synthesized compounds, **13** and **14**, showed a very strong (micromolar) antiproliferative activity on U251, MCF7 and MOLT-4 tumor cell lines. In addition, scope of this method could be expanded to various amine, as well as pyrimidine derivatives, and this issue will be studied furtherly.

Conflicts of interest

The authors declare the following competing financial interest(s): Marijeta Kralj and Biserka Žinić are minority shareholders in BioZyne Ltd. The other authors declare no competing interests.

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