C5-morpholinomethylation of N1-sulfonylcytosines by one-pot microwave assisted Mannich reaction

Josipa Matić, Irena Nekola, Aleksandar Višnjevac, Renata Kobetić, Irena Martin-Kleiner, Marijeta Kralj and Biserka Žinie

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 N1-sulfonylcytosine derivatives 3-6 were prepared by the condensation of silylated cytosine with selected sulfonyl chlorides. They were subsequently transformed to 5-morpholinomethyl-N1-sulfonylcytosine derivatives (8, 12-15) using microwave irradiation. As a result of the cytosine ring opening in N1-sulfonylcytosine, 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.

Results and discussion

Chemistry

As a part of previous sulfonylcyclourrea research, N1-sulfonylation of cytosine nucleobase was investigated and reaction conditions optimized throughout the preparation of N1-tosylcytosine (2, TsC). The reaction was elegantly achieved by silylation with \( \text{N} \text{O}-\text{bisp}(\text{trimethylsilyl})\text{acetamide} \) (BSA) in a dry acetonitrile and subsequent addition of appropriate sulfonyl chloride (Scheme 1). Inspired by interesting biological results with antibiotic linezolid or antitumor drug gefitinib, we have decided to prepare a series of N1-sulfonylcytosines and an analogous series of N1-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 N1-sulfonylcytosine is described herein. To the best of our knowledge, C5-morpholinomethylated derivatives of N1-sulfonylcytosine 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 N1-sulfonylcytosines. Antiproliferative activity on U251, MCF-7 and MOLT-4 tumor cell-lines was examined by MTT test.

Introduction

Over the last few decades, in an extensive pursuit for the new chemotherapeutics, modified pyrimidine nucleobases and nucleosides have emerged as promising anticancer agents. Numerous patent applications in the recent years prove that the interest in these compounds has not decreased. 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. Since the sulfonylpyrimidines 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 and in vivo. These findings encouraged us to further investigate modified pyrimidines. It has been known that different C5-substituted pyrimidine nucleosides often display antitumor activity. One of the best known examples is 5-fluorouracil which is widely used in the treatment of cancer. Moreover, it has been found that C5-carbonitrile pyrimidine derivatives modified with heterocyclic amines, like piperazine, piperdine and morpholine, could act as promoters of apoptosis in cancer cells through cyclin-dependent kinase 9 inhibition. In addition, morpholine moiety is present in many drugs, like analgesic dextromoramide, antibiotic linezolid or antitumor drug gefitinib.

We have decided to prepare a series of N1-sulfonylcytosines and an analogous series of N1-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 N1-sulfonylcytosine is described herein. To the best of our knowledge, C5-morpholinomethylated derivatives of N1-sulfonylcytosine 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 N1-sulfonylcytosines. Antiproliferative activity on U251, MCF-7 and MOLT-4 tumor cell-lines was examined by MTT test.
dabsyl chloride (4-(dimethylamino)azobenzene-4′-sulfonyl chloride). In addition, due to its specific features, azobenzene moiety plays a crucial role in a sophisticated drug-delivery systems, described in the latest studies.

Novel N1-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.

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. First we attempted the sulfonylation of 7 through several known methods: 1) activation/silylation of 7 with BSA in CH3CN; 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 K2CO3 in DMF, 4) sulfonylation of 7 in pyridine.

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 N1-sulfonylcytosine derivatives.](image1)

![Scheme 2. N1-sulfonylation of 5-morpholinomethylcytosine 7 in pyridine.](image2)

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 (4 h 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 2. 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-SOCl 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. In our case, corresponding N-ethoxymethyl derivatives of Tsc and cytosine were isolated from the mixture as well. In addition, as a possible source of byproducts, formation of 1,4 addsucts in the reaction of cytosine with morpholine and formaldehyde has also been described previously.

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. 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.

![Table 1. Optimization of reaction conditions for the Mannich aminomethylation of N1-sulfonylpyrimidines.](image3)
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.30

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 2 after one hour. It is reasonable to assume that higher reaction temperatures promote formation of 5-morpholinomethyl-N1-tosylcytosine (8), by cyclization of tosylurea-derivative 9 into starting TsC 2.

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.30 This is due to the N-SO2 bond breakage of TsC 2, 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).

Once the optimal conditions for Mannich reaction were established, we have expanded the reaction scope on the series of N1-sulfonyl-aromatic cytosine derivatives 12-15 (Schemes 4 and 5, Table 2). Beside biological activity, we wanted to assess the robustness of the method regarding diverse N1-sulfonylcytosine derivatives. 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).

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

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<td>2</td>
<td>8</td>
<td>100 °C</td>
<td>30 min</td>
<td>65%</td>
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<tr>
<td>3</td>
<td>12</td>
<td>100 °C</td>
<td>30 min</td>
<td>55%</td>
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<td>11</td>
<td>12</td>
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<tr>
<td>4</td>
<td>13</td>
<td>120 °C</td>
<td>30 min</td>
<td>52%</td>
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<tr>
<td>5</td>
<td>14</td>
<td>120 °C</td>
<td>30 min</td>
<td>49%</td>
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<tr>
<td>6</td>
<td>15</td>
<td>120 °C</td>
<td>60 min</td>
<td>30%</td>
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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 sulfonylurea derivative was isolated from reaction with methylbiphenyl 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.

Characterization of the products

NMR spectra
Structure of 5-morpholinomethyl-N1-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 aminemethylated cytosine base of the compound 8 occupies keto-imino tautomeric form.$^{35}$

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-C$_2$H$_4$ 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 2.

The acyclic structure of 9 was not unambiguously determined by NMR in solution (DMSO-$d_6$) 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 (i.e. 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.

<table>
<thead>
<tr>
<th>D-H $\cdots$ A</th>
<th>D-H (Å)</th>
<th>H $\cdots$ A (Å)</th>
<th>D $\cdots$ A (Å)</th>
<th>D-H $\cdots$ A (°)</th>
<th>symm op. on A</th>
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<tr>
<td>O5-H5A $\cdots$ N8</td>
<td>0.90 (4)</td>
<td>2.10 (4)</td>
<td>2.992 (2)</td>
<td>174 (3)</td>
<td>1</td>
</tr>
<tr>
<td>O5-H5B $\cdots$ O2ii</td>
<td>0.85 (3)</td>
<td>2.02 (3)</td>
<td>2.855 (2)</td>
<td>168 (3)</td>
<td>1+x, y, z</td>
</tr>
<tr>
<td>O6-H6A $\cdots$ O1i</td>
<td>0.75 (3)</td>
<td>2.35 (3)</td>
<td>3.047 (3)</td>
<td>155 (3)</td>
<td>1-x, 1-y, 1-z</td>
</tr>
<tr>
<td>O6-H6A $\cdots$ N8i</td>
<td>0.75 (3)</td>
<td>2.49 (4)</td>
<td>3.103 (2)</td>
<td>139 (3)</td>
<td>1-x, 1-y, 1-z</td>
</tr>
<tr>
<td>O6-H6B $\cdots$ O5</td>
<td>0.78 (3)</td>
<td>2.05 (3)</td>
<td>2.830 (2)</td>
<td>174 (4)</td>
<td>1</td>
</tr>
<tr>
<td>N10-H10 $\cdots$ OSi</td>
<td>0.89 (2)</td>
<td>2.19 (2)</td>
<td>3.075 (2)</td>
<td>171 (2)</td>
<td>1-x, 1-y, 1-z</td>
</tr>
<tr>
<td>N12-H12A $\cdots$ O6ii</td>
<td>0.87 (3)</td>
<td>2.01 (3)</td>
<td>2.875 (2)</td>
<td>172 (2)</td>
<td>1-x, 2-y, 1-z</td>
</tr>
<tr>
<td>N12-H12B $\cdots$ O3</td>
<td>0.89 (3)</td>
<td>2.02 (3)</td>
<td>2.657 (2)</td>
<td>128 (2)</td>
<td>1 (intra)</td>
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Crystal packing of 9 is characterized by intensive networks of hydrogen bonds in which the co-crystallized water molecules of OS and O6 are heavily involved (Table 3, Figures 4 and 5).

**Figure 2.** Full scan ES$^+$ spectrum for compound 9 dissolved in methanol (A) and deuterio methanol (B) at about 10$^{-6}$ mol dm$^{-3}$. Inserted spectrum is PI for molecular ion signals $m/z$ for [M+H]$^+$ 353 (CH$_3$OH) and $m/z$ 356 for [M+D]$^+$ (CD$_3$OD).

**Figure 3.** ORTEP drawing of compound 9 with the atom numbering. Displacement parameters are scaled to 50 % probability value.
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**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 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 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 O6ii. 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 In addition to *in vitro* studies, mechanism of action of 2 was analyzed in an extensive *in silico* study.11 The results revealed that 2 exhibits uncommon mechanism of cytostatic action, combining nucleic acid antimetabolite and a mechanism involving the activation of CYP1A1 and CYP1B1 genes by aryl hydrocarbon receptor, and a subsequent DNA damage response. Although a direct parallel cannot be drawn for all sulfonylcytosine derivatives, these assumptions present a valuable starting point for further analyses.

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 N1-sulfonylcytosine derivatives, compounds 3 (4-chloro-3-nitrophenyl-derivative), 4 (methylbiphenyl-derivative) and 5 (methoxybiphenyl-derivative) showed low micromolar IC50 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 N1-sulfonyl analogue 6. Introduction of two morpholine units also diminished activity in the compound 12, which showed moderate activity, compared to very active N1-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 N1-sulfonyl analogues 4 and 5. At the same time, 13 and 14 preserved remarkable potency toward MCF-7 and MOLT-4 cell lines.
As a well-known chemotherapeutic agent with pyrimidine structure, 5-fluourouracil (5-FU) was assayed for the comparison of the antitumor activity with the novel cytosine derivatives presented herein.\textsuperscript{18} Considering \textit{IC}_{50}, 5-FU showed superior activity than compound 8 (tosyl-derivative), 12 (4-morpholino-3-nitrophenyl-derivative) and compounds 6 and 15 (both azobenzene derivatives). However, compounds 3 (4-chloro-3-nitrophenyl-derivative), 13 (methylbiphenyl-derivative) and 14 (methoxybiphenyl-derivative) showed stronger activity on U251 cells, while they retained very similar, only slightly lower potency against MOLT-4 and MCF-7 cell-lines. Compounds 4 (methylbiphenyl-derivative) and 5 (methoxybiphenyl-derivative) also showed somewhat lower activity on all the tested cell lines, with the exception of 4 being equally active towards MOLT-4 cells, as the standard 5-FU.

### 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 30 °C. The microwave heating was performed at 15 min, with 30ºC/min heating rate. Then the vial was removed from the reactor and cooled to room temperature.

#### Synthesis

### General procedure for the preparation of 4'-sulfonylcytosine derivatives (3–6)

A mixture of cytosine (1 mmol) and BSA (3 mmol) was heated under reflux in dry acetonitrile (2 ml) for 30 min. The solution was then 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.

### N1-(4-chloro-3-nitrophenylsulfonyl)cytosine (3): Synthesis

N1-(4-chloro-3-nitrophenylsulfonyl)cytosine (3) was prepared according to the general procedure with 4'-chlorobenzensulfonyl chloride to give the product 3 (453 mg, 77%) as white crystals.

M.p. = 222–224 °C; R$_f$ = 0.3 (CH$_2$Cl$_2$:CH$_3$OH/9:1); UV (MeOH): $\lambda_{max}$/nm: 225 and 247; log $\varepsilon$/dm$^3$mol$^{-1}$cm$^{-1}$: 4.21 and 4.21; IR (KBr) $\nu_{max}$/cm$^{-1}$: 3381 (m), 3099 (m), 1672 (s), 1659 (s), 1593 (s), 1572 (w), 1535 (s), 1491 (m), 1387 (m), 1356 (s), 1283 (m), 1256 (w), 1234 (w), 1188 (s), 1111 (m), 1097 (m), 1058 (w); 1H NMR (DMSO-$d_6$) $\delta$/ppm: 8.66 (d, 1H, J = 2.2 Hz, Ar), 8.28 (dd, 1H, $J_{5,6}$ = 8.6 Hz, J = 2.3 Hz, Ar), 8.13–7.97 (m, 4H, Ar, H-6, NH$_2$), 6.00 (d, 1H, $J_{5,6}$ = 7.9 Hz, H-5); 13C NMR (DMSO-$d_6$) $\delta$/ppm: 166.3 (C), 150.8 (C$_q$), 147.2 (C$_q$), 139.2 (C$_q$), 133.6 (CH, Ar), 132.7 (CH, Ar), 131.4 (C$_q$), 126.2 (CH, Ar), 98.0 (CH, C-$q$). (see Supporting information Fig. S1) ESI-MS: calcd. for Cl$_2$H$_2$CIN$_2$O$_4$: 330.0; found [M+H]$^+$ at m/z 331.0.

### N1-(4'-methylbiphenyl-4-sulfonyl)cytosine (4): Synthesis

N1-(4'-methylbiphenyl-4-sulfonyl)cytosine (4) was synthesized 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): $\lambda_{max}$/nm: 284; log $\varepsilon$/dm$^3$mol$^{-1}$cm$^{-1}$: 2.58; IR (KBr) $\nu_{max}$/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); 1H NMR (DMSO-$d_6$) $\delta$/ppm: 8.15 (d, $J_{5,6}$ = 7.9 Hz, 1H, H-$q$), 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-$p$), 2.36 (s, 3H, CH$_3$); 13C NMR (DMSO-$d_6$) $\delta$/ppm: 165.9 (C$_q$), 150.9 (C$_q$), 145.8 (C$_q$), 139.3 (CH, C-$q$), 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-$p$), 20.7 (CH$_3$). (see Supporting information Fig. S2) ESI-MS: calcd. for C$_{17}$H$_{15}$N$_2$O$_5$: 341.1; found [M+H]$^+$ at m/z 342.1.

### N1-(4'-methoxybiphenyl-4-sulfonyl)cytosine (5): Synthesis

N1-(4'-methoxybiphenyl-4-sulfonyl)cytosine (5) was synthesized 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): $\lambda_{max}$/nm: 246; log $\varepsilon$/dm$^3$mol$^{-1}$cm$^{-1}$: 4.00; IR (KBr) $\nu_{max}$/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); 1H NMR (DMSO-$d_6$) $\delta$/ppm: 8.14 (d, $J_{5,6}$ = 7.9 Hz, 1H, H-$q$), 8.02–7.96 (m, 2H, Ar), 7.93–7.86 (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-$p$), 3.82 (s, 3H, OCH$_3$); 13C NMR (DMSO-$d_6$) $\delta$/ppm: 165.9 (C$_q$), 160.0 (C$_q$), 150.9 (C$_q$), 145.5 (C$_q$), 139.4 (CH, C-$q$), 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-$p$), 55.3 (OCH$_3$). (see Supporting information Fig. S3) ESI-MS: calcd. for C$_{21}$H$_{17}$N$_2$O$_7$: 357.1; found [M+H]$^+$ at m/z 358.1.
5-Morpholinomethyl-N1-(4'-methyldibenzophenyl-4)-sulfonylcystine (13): N1-(4'-methyldibenzophenyl-4)-sulfonylcystine (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 (elucent: 10% MeOH in CH3CN, white crystals, 69 mg, 52%).

M.p. > 300 °C (dec.; Rf = 0.5 (CH3Cl2/CH3OH 9:1); UV (MeOH): λmax/nm: 285; log ε/dm3 mol−1 cm−1: 4.69; IR (KBr) ν/cm−1: 3440(s), 3097 (m), 1631 (s), 1518 (w), 1474 (w), 1365 (w), 1353 (w), 1298 (w), 1172 (m), 1121 (w), 1089 (w); 1H NMR (DMSO-d6) δ/ppm: 8.21 (s, 1H, NH), 8.07–7.99 (m, 4H, C-H, Ar), 7.90 (d, 2H, J = 8.6 Hz, Ar), 7.68 (bs, 1H, NH), 7.66 (d, 2H, J = 8.1 Hz, Ar), 7.33 (d, 2H, J = 8.0 Hz, Ar), 3.58 (s, 4H, O-CH2), 3.32 (s, 2H, CH2), 2.41 (bs, 4H, N-CH2), 2.37 (s, 3H, CH3); 13C NMR (DMSO-d6) δ/ppm: 165.7 (Cα), 150.7 (Cα), 145.8 (Cβ), 138.5 (Cγ), 137.5 (CH-C6), 135.4 (CH-C6), 135.2 (Cβ), 129.7 (C=), 129.4 (CH, Ar), 126.8 (CH, Ar), 104.5 (Cα-C5), 66.1 (O-CH2), 55.8 (CH2), 52.5 (N-CH2), 20.7 (CH3) (see Supporting information Fig. 57) HRMS: m/z: calcd for C23H19N2O7SNa+: 441.1597; found 441.1595 [M+H]+.

5-Morpholinomethyl-N1-(4'-methoxystyrylphenyl-4)-sulfonylcystine (14): N1-(4'-methoxystyrylphenyl-4)-sulfonylcystine (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 for 30 minutes at 120 °C in a microwave reactor. Product 14 was isolated by thin-layer chromatography (elucent: 10% MeOH in CH3CN, white crystals, 33 mg, 49%).

M.p. = 211–212 °C; Rf = 0.6 (CH3Cl2/CH3OH 9:1); UV (MeOH): λmax/nm: 299; log ε/dm3 mol−1 cm−1: 4.17; IR (KBr) ν/cm−1: 3430(s), 1688 (s), 1662 (s), 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); 1H NMR (DMSO-d6) δ/ppm: 8.21 (bs, 1H, NH), 8.07–7.97 (m, 3H, 3H, Ar), 7.68 (d, 2H, J = 8.7 Hz, Ar), 7.72 (d, 2H, J = 8.8 Hz, Ar), 7.67 (bs, 1H, NH), 7.08 (d, 2H, J = 8.8 Hz, Ar), 3.82 (s, 3H, O-CH3), 3.58 (s, 4H, O-CH2), 3.32 (s, 2H, CH2), 2.41 (s, 4H, N-CH2); 13C NMR (DMSO-d6) δ/ppm: 165.7 (Cα), 160.0 (Cα), 150.7 (Cγ), 145.5 (Cβ), 137.6 (CH-C6), 134.8 (Cβ), 130.3 (Cβ), 129.5 (CH, Ar), 128.5 (CH, Ar), 114.6 (CH, Ar), 104.5 (Cα-C5), 66.1 (O-CH2), 55.8 (CH2), 55.3 (OCH3), 52.5 (N-CH2). (see Supporting information Fig. 58) HRMS: m/z: calcd for C23H19N2O7SNa+: 457.1546; found 457.1529 [M+H]+.
SUMMARY OF THE MAIN RESULTS

- Table 5. Crystallographic data

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<th>Structure</th>
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<td>c (Å)</td>
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<tr>
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</table>

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% CO₂ at 37°C.

Proliferation assays

The cells were inoculated onto a series of standard 96-well microtiter plates on day 0, at 1×10⁴ to 3×10⁶ cells/ml, depending on the doubling times of specific cell line. Test agents were then added
in five 10-fold dilutions (10^4 to 10^8 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. 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-N1-sulfonylcytosine derivatives has been established. N1-sulfonylcytosine derivatives 3–6 were prepared by the condensation of pyrimidine bases with different sulfonyl chlorides, and then transformed to Cs-substituted N1-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-SO₂ 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 TsC₂ 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 further.

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.

Acknowledgements

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Notes and references

37 CrysAlis CCD, Version 1.171.32.29 (release 10-02008 CrysAlis171.NET), Oxford Diffraction Ltd.