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Compound instability in dimethyl sulphoxide, case studies with 5-aminopyrimidines and the implications for compound storage and screening

Eliška Procházková, Petr Jansa, Anna Březinová, Lucie Čechová, Helena Mertlíková-Kaiserová, Antonín Holý, Martin Dračínský *

Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo nam. 2, 166 10 Prague, Czech Republic

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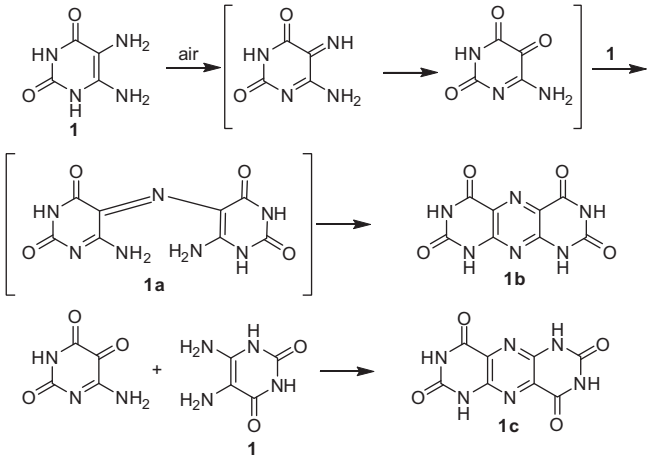
ABSTRACT

The oxidation reactions of 5-aminopyrimidine derivatives in dimethyl sulphoxide (DMSO) were studied. The DMSO solutions of the studied compounds became deeply coloured within a few hours or days. The oxidation products can undergo further condensation reactions with the starting pyrimidines to yield bipyrimidines and/or pyrimidopteridines. The reaction mechanism of the oxidation-condensation reaction was also supported by reactions of the 5-aminopyrimidines with alloxan (2,4,5,6-tetraoxopyrimidine). DMSO is often used as the solvent in in vitro tests of biological activities, but it is also an oxidising agent and may react with solute molecules and significantly affect the quality of the generated biochemical data.

Substituted pyrimidines play a key role in many biochemical processes; for instance, they form the basic building blocks of nucleic acids and are found in high-energy molecules with higher specificity than adenosine-5′-triphospate (ATP). Cytosine-5′-triphosphate (CTP) works as a coenzyme in glycerophospholipid synthesis or in glycosylation reactions of proteins.[1] Synthetically prepared aminopyrimidine derivatives display a wide range of biological activities such as antibacterial,[2] antitumor[3] and antiviral.[4,5] Therefore, the substituted aminopyrimidine structural motif can be found in diverse clinically approved drugs. Interestingly, a substituted aminopyrimidine moiety was also suggested to account for the antioxidant activity of folic acid, and this was hypothesised to play a role in the protective effects of folic acid against cardiovascular, neurological or haematological pathologies.[6]

In our previous work,[7] we studied the antioxidative activities of a series of 5-aminopyrimidines and observed that some of the compounds were unstable in DMSO solutions; within a few hours or days, the solutions coloured and the antioxidative activities decreased. The deleterious effect of DMSO on the performance of certain antioxidant assays owing to its ability to act as a hydroxyl radical scavenger has long been known.[8] However, when working with poorly soluble compounds, DMSO is sometimes impossible to avoid for in vitro screening. The feasibility of the use of DMSO as a solvent in the evaluation of new potential antioxidants has been contradictory. Recent studies have suggested that DMSO is a tolerable vehicle in the antioxidant assays supposing that the relevant controls are provided and that the final DMSO concentration is kept below 5%.[9] However, the stability of the potential antioxidants in DMSO solutions is rarely addressed in the literature in spite of the



Scheme 1. The proposed reaction mechanism of the oxidative self-condensation of compound **1** in water in the presence of air.[10]

* Corresponding author. Tel.: +420 220 183 139; fax: +420 220 183 123.
E-mail address: dracinsky@uochb.cas.cz (M. Dračínský).

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fact that DMSO is a redox-active agent theoretically capable of oxidising the tested compounds.

We hypothesise that DMSO could oxidise 5-aminopyrimidines similarly to other oxidation agents (e.g. air,[10] potassium ferricyanide[11] or mercury oxide[12]). The oxidative self-condensation of 5,6-diaminopyrimidines has been known for a long time.[10] The proposed reaction mechanism[10] is depicted in Scheme 1. The initial step of the reaction is the oxidation of the starting pyrimidine derivative (**1**) to the pyrimidine-quinone-imine, which may undergo hydrolysis under the reaction conditions to give quinone. Subsequent condensation of quinone-imine or the quinone with the 5-aminogroup of unchanged **1** can yield the bipyrimidine intermediate **1a**, which can undergo third-ring closure by direct loss of ammonia or by preliminary hydrolysis followed by dehydration; the final product of the reaction is the pyrimidopteridine product **1b** (bis-alloxazine). Alternatively, the condensation of quinone-imine or the quinone with the 6-aminogroup of unchanged **1** would lead to pyrimidopteridine **1c**.[10]

The pyrimidopteridine products of the reaction are highly insoluble and deeply coloured. Several pyrimidopteridine derivatives were prepared as potential coronary vasodilators, but their pharmacological evaluation did not prove their clinical usefulness.[13] On the other hand, it is possible to use these pyrimidopteridines as electron-transfer particles, which work as low reduction potential flavin mimics.[14,15]

The aim of this work was to study the decomposition of 5-aminopyrimidines in DMSO solutions, to identify the products and to propose the reaction mechanism of the reactions.

The studied compounds were dissolved in DMSO and the solutions were stirred at room temperature. Immediately after dissolution, the solutions were colourless. Typically, deep colour changes were apparent within a few hours. The progress of the reactions was monitored by UV/VIS and NMR spectroscopy.

Compound **1** was transformed into a mixture of compounds **1b** and **1c** after stirring overnight in DMSO. The products are the same as in the previously described air oxidation of **1**.[10]

The solution of compound **2** changed its colour to deep purple overnight. After two months, the purple colour changed to light yellow and a yellow precipitate appeared in the reaction mixture (see Fig. S2 in SI). In the UV/VIS spectra, we observed that compound **2** was first transformed into a purple intermediate product **2a** (with absorption maxima at 347 and 550 nm) and within a few more days it was consequently converted into a yellow product **2b** (with an absorption maximum at 397 nm), which is stable (see Fig. 1). The isosbestic point at 365 nm provides the evidence for the successive reactions taking place (**2**→**2a**→**2b**).

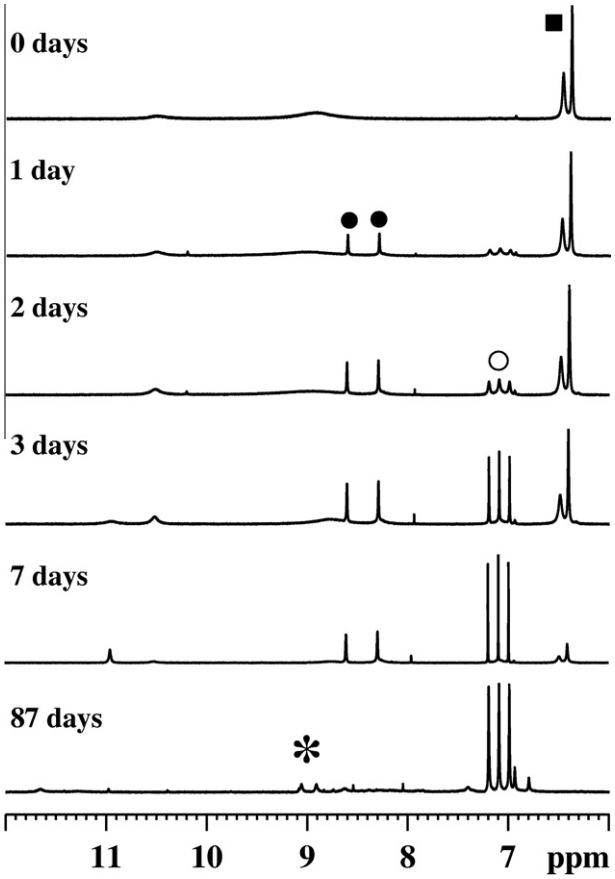


Figure 2. The time evolution of the ^{1}H NMR spectra of compound **2** in DMSO. The starting compound **2** (■) reacts in DMSO to generate the intermediate product **2a** (●). Simultaneously, the ammonium cation occurs (○). Consequently, **2a** is converted to the final product **2b** (*).

The kinetics of these transformations were also monitored by ^{1}H NMR spectroscopy. For illustration, the time-response changes in the ^{1}H NMR spectra of compound **2** are displayed in Figure 2. The NMR signal of the starting compound (■; 6.35 ppm) practically disappears from the reaction mixture by day 7. The signal of the purple intermediate product **2a** (●; 8.30 and 8.60 ppm) appears within only one day of incubation. On day 2, the signal of an ammonium cation is detected (○; 7.09 ppm, J = 51 Hz). In a few months, it is possible to observe the NMR signal of the final prod-

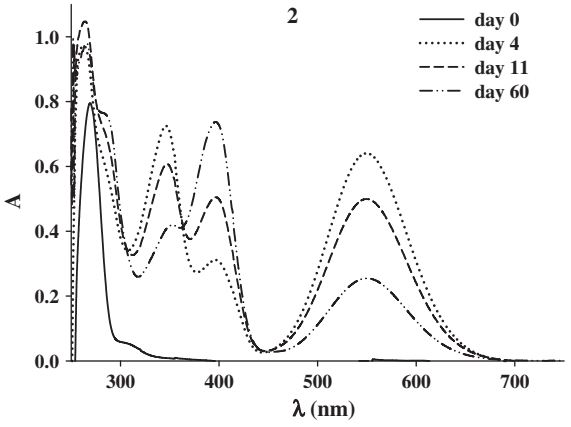


Figure 1. The changes in the absorption spectra of a 10 mM solution of compound **2** in DMSO over a period of two months at room temperature. For the UV/VIS spectra measurement, the solutions were diluted to 0.2 mM concentration.

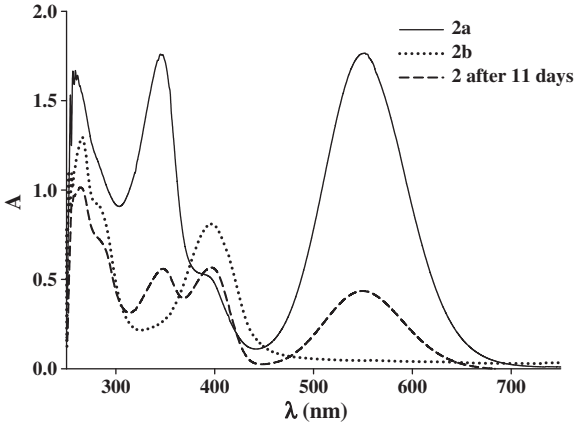


Figure 3. The absorption spectra characterising the individual products (**2a**, **2b**) isolated from the DMSO solution of compound **2** compared to the DMSO solution of compound **2** standing at room temperature for 11 days.

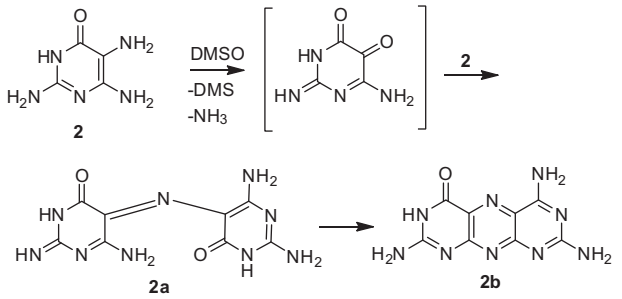
uct of a successive reaction of **2a**, the yellow **2b** (*, 8.9 and 9.0 ppm). The low intensity of these signals is caused by the low solubility of **2b** in DMSO.

We isolated the two products of the reaction (**2a** and **2b**). The absorption spectra of the isolated products are shown in Figure 3. The absorption maxima of the individual products (**2a** and **2b**) are in agreement with the maxima observed in the reaction mixture after 11 days at room temperature, that is at 347 and 550 nm for **2a** and 397 nm for **2b**.

The high-resolution mass spectra (HRMS) revealed the compositions of the products **2a** and **2b** to be $C_8H_{10}N_9O_2$ and $C_8H_8N_9O$, respectively. The hydrolysis of compound **2b** with sodium nitrite and dilute hydrochloric acid yielded compound **1b** of molecular formula $C_8H_4N_6O_4$, the UV/VIS spectrum of which was identical with the previously published spectrum of bis-alloxazine.[10] The structures of compounds **2a** and **2b** together with the proposed mechanism of the oxidation reaction are depicted in Scheme 2. First, compound **2** is oxidised by DMSO to the pyrimidine-quinone-imine while an ammonium cation (which was observed in the ^{1}H NMR spectra) is released. The quinone-imine can subsequently condense with the 5-aminogroup of another molecule **2**, after which the intermediate **2a** can undergo a new ring closure to give **2b** as the final product of the reaction. In the ^{13}C NMR spectrum of compound **2a**, we observed four signals, which is in agreement with the proposed structure of **2a**, where the iminogroup of one ring can be in fast tautomer exchange equilibrium with the amino group of the second ring. Unfortunately, the very low solubility of pyrimidopteridines did not allow us to acquire the ^{13}C NMR spectrum of compound **2b**. Interestingly, we did not observe the formation of a condensation product analogical to the compound **1c**.

We performed the reaction also in dried DMSO under an inert atmosphere, and the reaction rates were unchanged. When the reaction was performed in larger quantities, the smell of dimethyl sulphide (DMS) was apparent. These observations are in agreement with the proposed mechanism. Interestingly, when the DMSO solution was alkalised with a drop of NaOH solution, we were not able to detect the purple intermediate **2a** and the reaction to **2b** was completed in 24 h. Conversely, when a drop of HCl was added to the DMSO solution, the reaction was slowed down.

The solution of tetraaminopyrimidine **3** in DMSO changed from colourless to orange overnight. The oxidation products of compound **3**, two constitutional isomers **3b** (yellow) and **3c** (red), were isolated and their absorption spectra were compared with the reaction mixture in DMSO (see Fig. S3 in SI). The molecular formula $C_8H_8N_{10}$ was determined by HRMS for both compounds **3b** and **3c**. The 'open form' similar to the intermediate **2a** was not detected in this case because the second condensation reaction is probably too fast. The reason for the different reactivity might be in different acido-basic properties of the two compounds. After hydrolysis of the amino groups of both isomers with sodium nitrite in dilute hydrochloric acid, we obtained compounds **1b** and **1c**, respectively (see Scheme 3). Compound **1c** was also prepared (for the comparison of spectral properties) by a known oxidation reaction of 5-aminouracil with potassium ferricyanide.[11]



Scheme 2. The proposed mechanism of compound **2** oxidation and self-condensation.

Another studied compound was 5-aminobarbituric acid (**4**). The reaction was observed by UV/VIS spectroscopy and ^{1}H NMR spectroscopy. In the DMSO solution, the ammonium cation released (observed by ^{1}H NMR) and the 'open form' **4a** (i.e. a metalochromic indicator murexide, see Scheme 3) was detected, but no subsequent intramolecular condensation was observed. This is not surprising, because no aminogroup is present in **4a**. Product **4a** has its absorption maximum at 532 nm and by comparison with the commercially available sample of murexide, its structure was unambiguously confirmed.

The DMSO oxidation of 5,6-diamino-4-oxo-2-thiopyrimidine (**5**) was also studied. The changes in the UV/VIS spectra and the presence of ammonia cation observed in ^{1}H NMR suggest that similar oxidation and condensation reactions take place. However, a complex mixture of products appeared in the solution and the isolation of the products failed. Some unidentified subsequent reactions involving the reactive SH group (e.g. oxidation of the SH group) probably took place. The DMSO solution of 2,5-diamino-4,6-dimethoxypyrimidine (**6**) slowly changed its colour to orange. However, the only change we were able to detect in the ^{1}H NMR spectra was the hydrolysis of one of the methoxy groups (signals of methanol appeared in the spectra together with an amidic proton and one methoxy group). Apparently, the hydrolysed product may undergo further reactions (hydrolysis of the other methoxy group, oxidation and condensation), which leads to colour changes, but the subsequent reactions were so slow that the products were below the NMR detection limit even after two months. The DMSO solution of 2,5-diamino-4,6-dichloropyrimidine (**7**) slowly changed its colour to dark red, which was further converted into yellow. However, both the NMR and MS spectra revealed that a complicated mixture of products was formed. The chlorine atoms might have been hydrolysed and/or substituted, because the typical isotopic pattern of chlorinated compounds was not observed in the MS spectra.

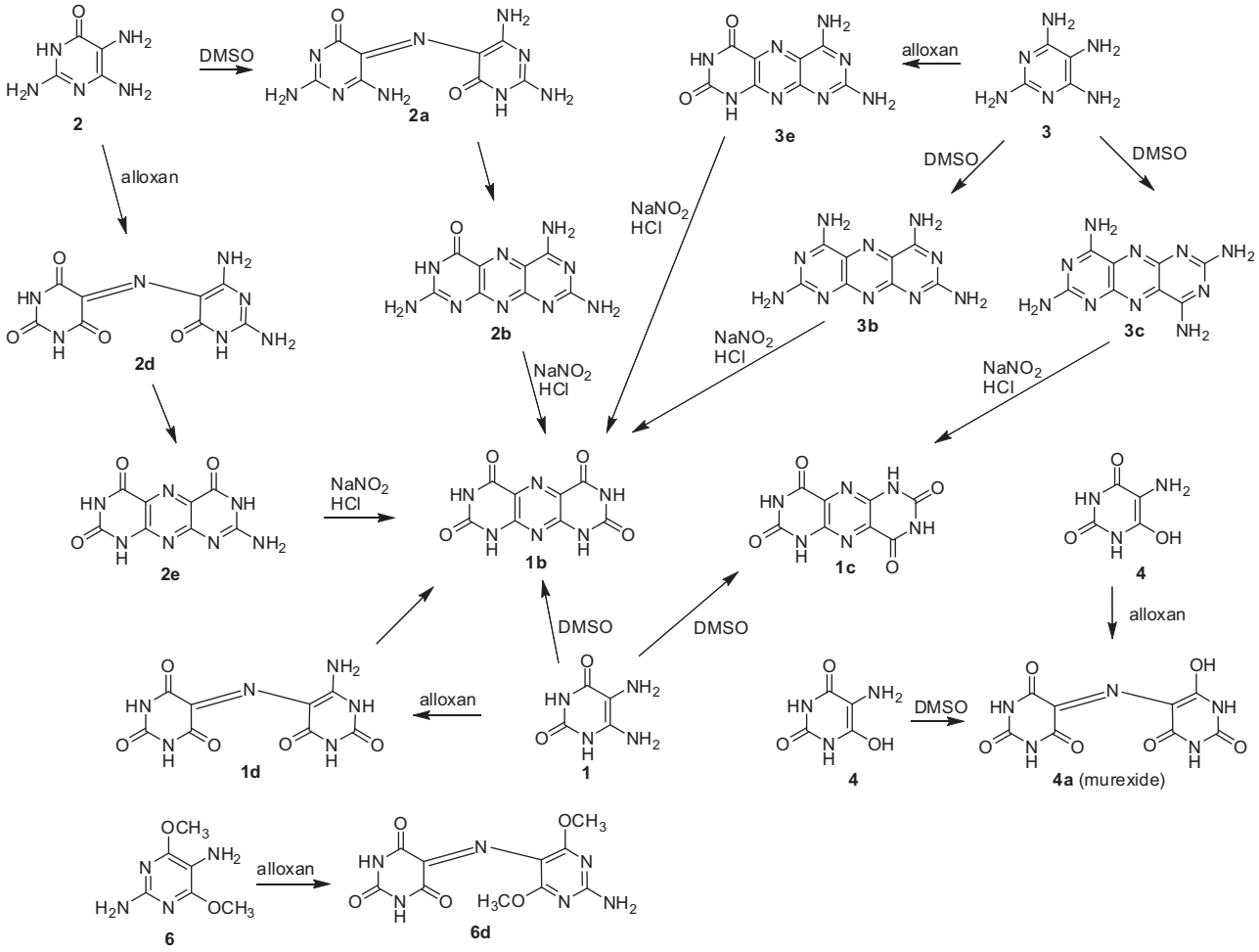
To support further the proposed reaction mechanism and the structures of the condensation products, we performed condensation reactions of compounds **1**, **2**, **3**, **4** and **6** with alloxan (2,4,5,6-tetraoxopyrimidine). The reactions were performed in DMSO by mixing equimolar amounts of alloxan with a substituted 5-aminopyrimidine. Compound **1** gave bipyrimidine **1d**, which gave pyrimidopteridine **1b** after heating to 120 °C. Similarly, we obtained bipyrimidine **2d** and pyrimidopteridine **2e** from compound **2**. The product **2e** was hydrolysed with sodium nitrite to **1b**.

We were not able to isolate the bipyrimidine derivative from the reaction mixture of compound **3** with alloxan; the only product of the reaction was pyrimidopteridine **3e**, which was also hydrolysed with sodium nitrite to **1b**. 5-Aminobarbituric acid (**4**) gave murexide (**4a**) after reaction with alloxan, and compound **6** condensed with alloxan to a bipyrimidine derivative **6d**.

We have also studied a series of disubstituted 5-aminopyrimidines (with hydrogen atom in position 2 or 6). They were oxidised more slowly than the trisubstituted derivatives **1**–**6**. In the UV/VIS spectra of these compounds, we observed similar changes like for compounds **1**–**6**. However, we were not able to isolate the oxidation products for the majority of the disubstituted 5-aminopyrimidines in sufficient quantity for proper characterisation. The full discussion of these compounds is given in the SI.

For comparison, we dissolved 2,6-diamino-4-oxopyrimidine (i.e. compound **2** without the 5-aminogroup) in DMSO and we did not observe any changes in the UV/VIS or NMR spectra. Obviously, the 5-aminogroup is crucial for the oxidation reaction. Neither did we observe any reaction of this compound with alloxan.

All of the reactions were monitored by UV/VIS spectroscopy; the spectra are shown in the SI. The isolated products were charac-



Scheme 3. The observed oxidation-condensation reactions of 5-aminopyrimidines in DMSO.

terised by UV/VIS spectra and high-resolution mass spectra. The UV/VIS spectra are shown in the SI; the absorption maxima are summarised in Table 1. The wavelength of the absorption maxima is dependent on the number of oxo- and amino-substituents, for example in the series of compounds **1b**, **2e**, **3e**, **2b** and **3b** the number of aminogroups goes from zero to four and the absorption maximum goes from 360 to 424 nm.

Unfortunately, the pyrimidopteridine products of the reactions are very poorly soluble and we were not able to acquire ^{13}C NMR spectra for the majority of them. In the ^{1}H NMR spectra, the signals of the exchangeable protons (NH and OH) were very broad, probably because of fast tautomer interconversions. The positions of the proton signals were also very dependent on the conditions of the measurement; minor changes of temperature or pH probably cause shifts in the tautomer equilibria. In some cases, we did not obtain identical ^{1}H NMR spectra for the same products obtained from two different reactions. Therefore, we do not believe that the ^{1}H NMR spectra are particularly useful for the characterisation of this type of compound.

Table 1
The absorption maxima of the prepared compounds

Compound	λ_{max} (nm)
1b	360
1c	404
1d	515
2a	550
2b	398
2d	538
2e	377
3b	424
3c	492
3e	380
4a	532
6d	544

The DMSO solutions of the substituted 5-aminopyrimidines exhibited signs of instability during storage (colour changes). The antioxidative activity of the coloured samples was clearly lower than that of the freshly prepared solutions.[7] In this study, we have shown that 5-aminopyrimidines can undergo oxidation and subsequent condensation reactions in DMSO. Bipyrimidines and pyrimidopteridines are formed during the reactions. Importantly, the rate of the decomposition of 5-aminopyrimidines in DMSO appears to correlate with their antioxidative activity.[7] The trisubstituted 5-aminopyrimidines (**1**–**3** and **5**) were found to be more active in the antioxidant assays and were also faster transformed to their oxidation products in DMSO. On the other hand, the antioxidative activities of the disubstituted 5-aminopyrimidines were much lower or not detected at all and they were also more stable in DMSO. We observed that the more electron-donor substituents are attached to the pyrimidine skeleton, the faster the DMSO oxidation of 5-aminopyrimidines is.

Here, we would like to point out that the antioxidative activities of various antioxidants (reducing agents) can be negatively affected by reaction with DMSO (an oxidising agent). We can speculate that some very strong antioxidants will turn totally inactive

after their dissolution in DMSO. This fact may also complicate a direct comparison of the antioxidant activity data on certain groups of compounds between different laboratories. Furthermore, many other biochemical assays have colourimetric end points and highly coloured impurities (such as those observed in this study) may affect the results significantly. Therefore, the storage of tested compounds in DMSO solutions should be avoided and freshly prepared solutions are desirable for biochemical screening.

Acknowledgment

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.08.065.

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