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Antioxidant properties of thio-caffeine derivatives: Identification of the newly synthesized 8-[(pyrrolidin-1-ylcarbonothioyl)sulfanyl] caffeine as antioxidant and highly potent cytoprotective agent

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ABSTRACT

A series of nine thio-caffeine analogues were synthesized and characterised by NMR, FT-IR and MS spectroscopic methods. Molecular structures of four of them were determined using single crystal X-ray diffraction methods. The antioxidant properties of all compounds, at concentration ranges from 0.025 to 0.1 mg/mL, were evaluated by various chemical- and cell-based antioxidant assays. Human erythrocytes were used to examine in vitro haemolytic activity of all compounds and their protective effect against oxidative haemolysis induced by AAPH, one of the commonly used free radical generator. All compounds studied showed no effect on the human erythrocytes membrane structure and permeability with the exception of 8-(phenylsulfanyl)caffeine. Among the nine caffeine thio-analogues tested, the newly synthesized 8-[(pyrrolidin-1-ylcarbonothioyl)sulfanyl]caffeine possessed exceptionally high antioxidant properties. Moreover, it protects human erythrocytes against AAPH-induced oxidative damage as efficiently as the standard antioxidant Trolox. Therefore, 8-[(pyrrolidin-1-ylcarbonothioyl)sulfanyl]caffeine may have a significant cytoprotective potential caused by its antioxidant activity.

Caffeine (**1**), the naturally occurring methylxanthine, is of an unquestionable interest as a leading compound for the development of new derivatives with enhanced activities and/or lower toxicities. It modulates drugs used for curing lung, liver, uterine cervix and breast cancer and enhances gastric secretion and urine production, reduces the risk of developing gallstone disease and also reduces asthma. The anticarcinogenic effect of caffeine has been related to its effect on cell cycle and proliferation.[1] Caffeine easy penetrates through biological membrane[2] and activates the erythrocyte glutathione-*S*-transferase (GST) which is involved in erythrocytes protection.[3] It has been shown that caffeine inhibits eryptosis (suicidal death) of human erythrocytes within the range of its plasma concentration (100 μM)[4] and prevents the accelerated clearance of erythrocytes from circulation and development of anemia. On the other hand, the antiapoptotic effect of caffeine on nucleated cells could be explained by inhibition of the phosphodiesterase and c-AMP production,[5] its inhibitory action on ATM kinase and suppression of p53 phosforylation[6] or suppression of amyloid β-induced caspase-3 activity in neurons.[7] According to Kesavan and co-workers[8–11] caffeine has abilities to scavenge highly reactive free radicals and excited states of oxygen. Antioxidant ability of caffeine is similar to that of the established biological antioxidant glutathione and significantly much higher than that of ascorbic acid. However, other studies demonstrate an absence of antioxidant properties of caffeine using DPPH assay[12–14] or even its pro-oxidant effects.[15,16] Studies have shown that C8-substituted caffeine derivatives are adenosine receptor antagonists,[17–19] acetylcholinesterase inhibitors[20] and monoamine oxidase inhibitors, and that within this group the 8-thiocaffeine analogues belong to a pharmacologically important subclass.[21–24] In general, nearly all organosulfur compounds are considered as antioxidants. Compounds such as allicin, methionine and methylcysteine protect against metal-mediated oxidative DNA damage. Moreover, the results of epidemiological, clinical, in vivo, and in vitro studies have undoubtedly shown the protective effects of sulfur compounds against cellular damage and disease.[25] It therefore came as a surprise to us that although the antioxidant activity of spent coffee extracts rich in caffeine[26] and caffeine alone[27,28] has been extensively examined, similar studies devoted to the thiocaffeine derivatives have not been so farreported. Notably, the

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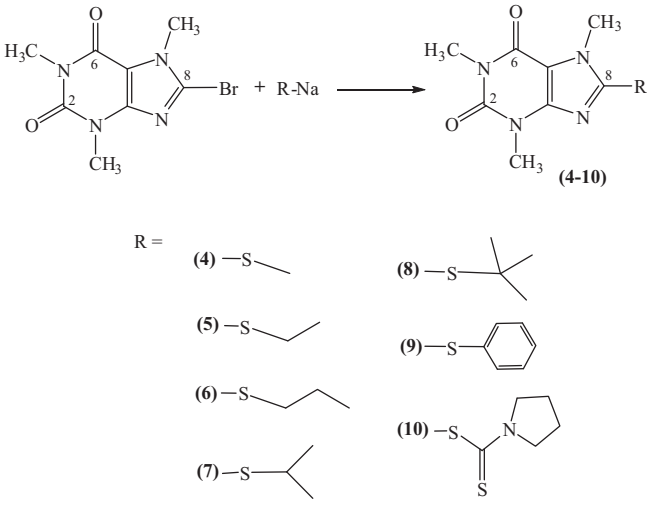
biochemistry of dithiocarbamates is of interest because of their clinical use.[29] Their biological properties include ability to influence oxidative stress, apoptosis and enzyme inhibition.[30–33] Moreover, pyrrolidinedithiocarbamate is widely used as an inhibitor of nuclear factor kappa B(NFkB) and this, or related compounds may have therapeutic potential in inhibiting artheriosclerosis.[34] Relying on the above reports, we expected that there will be some advantage, in terms of biological activity, resulting from a synergism between the biological actions of the caffeine and its sulfur derivatives. We have therefore synthesized and characterised a series of nine structurally diverse thio-caffeine analogues, that included, 6-thiocaffeine, 2,6-dithiocaffeine, 8-thioalkyl derivatives, 8-(phenylsulfanyl)-caffeine and the newly synthesized 8-[(pyrrolidin-1-ylcarbonothioyl)sulfanyl]caffeine, and explored their antioxidant activity as well as their effects on human erythrocytes in vitro. Human red blood cells (RBCs) are widely used in the investigation of antioxidant activity of natural and newly obtained compounds because there are the main targets for free radicals in the circulatory system.[35–38] Although RBCs contain enzymes that are involved in defence against free radicals, namely catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GRd) and glutathione peroxidase (GPx)[39] they are not able to effectively eliminate reactive oxygen species and as a result oxidative haemolysis occurs. The water-soluble free radical generator 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) is commonly used for inducing RBCs membrane injury and oxidative haemolysis in vitro. Thus, in the present study, AAPH was employed to examine the oxidative haemolysis in the presence or absence of the thiocaffeine derivatives as well as the standard antioxidants, namely Trolox and butylated hydroxytoluene (BHT).

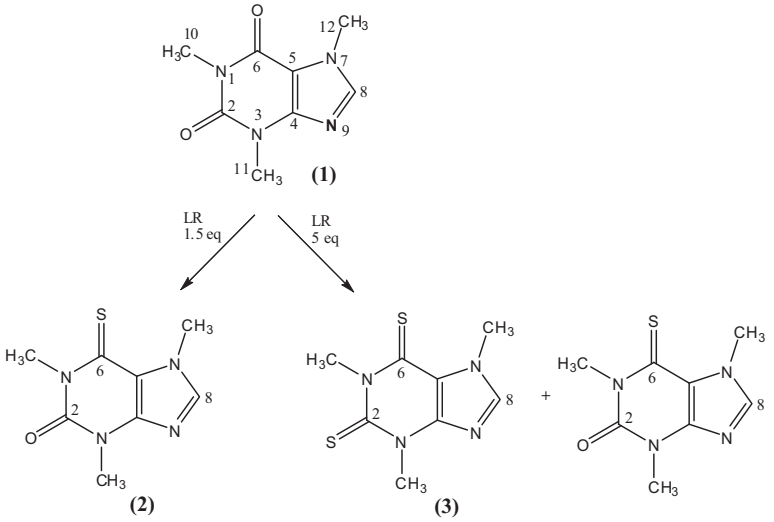
The 6-thiocaffeine (**2**) and 2,6-dithiocaffeine (**3**) were synthesized by reaction of caffeine with Lawesson's reagent in toluene (Scheme 1). Reaction of 8-bromocaffeine with an appropriate sodium thiolate reagent in ethanol solution gives the C8 thiocaffeine analogues (**4**–**10**).[40] The synthetic routes of these target compounds are outlined in Scheme 2. All obtained compounds were structurally (^{1}H NMR, ^{13}C NMR, FTIR, ESI-MS) characterised (see Supplementary materials). For four of them the crystal structures were determined.

The most noticeable differences in the NMR spectra were the downfield shifts of signals corresponding to positions C5, C6 in 6-thio- or C2, C5 and C6 in 2,6-dithiocaffeine, as compared with caffeine. The IR spectrum of **2** showed an absorption band near



Scheme 2. Reaction and conditions: 1 equiv 8-bromocaffeine, 4 equiv sodium thiolate, ethanol, reflux, 2–48 h. Time for completion of the reaction at reflux as indicated by TLC.

1675 cm^{-1} associated to the carbonyl group and absorption band near 1110 cm^{-1} associated to the thiocarbonyl group, whereas in the spectrum of **3** we can observed absorption bands of two thiocarbonyl groups at 1110 and 1070 cm^{-1}. The IR spectrum of compound **5** as representative of the series (**4**–**8**), showed an absorption band at 1319 and 1037 cm^{-1} associated to the thioalkyl group and absorption bands at 1702 and 1656 cm^{-1} associated to the carbonyl groups of the caffeine fragment. The IR spectrum of compound **9** showed an absorption band at 3060 and 1440–1578 cm^{-1} associated to the aromatic ring, whereas in the IR spectrum of **10** the bands in 1400–1100 cm^{-1} region are associated with N—C═S—S stretching vibrations. In the ^{1}H NMR spectra of compounds **4**–**10**, three singlets in the range of 3.37–3.97 ppm indicated the presence of the three methyl groups from caffeine unit. Aromatic protons of compound **9** are present in the range of 7.60–7.30 ppm, while signals at 3.61 and 1.90 ppm present in the spectra of **10** are connected with presence of pyrrolidine ring. The ^{13}C NMR spectrum of compound **9** showed signals at 132–127 ppm corresponding to the phenyl ring. Signal at about



Scheme 1. Reaction and conditions: Caffeine (1), 1.5 or 5 equiv LW (Lawesson's reagent), toluene, reflux at 25 h. Time for completion of the reaction at reflux as indicated by TLC.

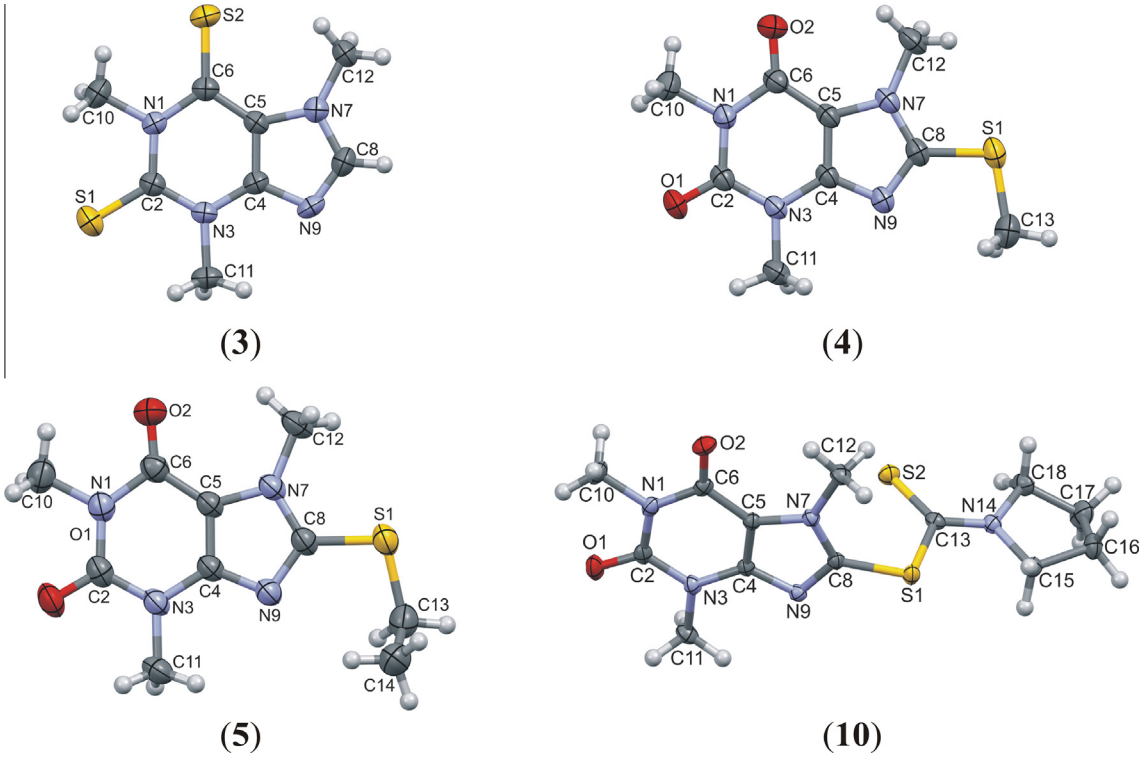


Figure 1. Sulfur analogue of caffeine **3** and C8 sulfur-substituted caffeines **4**, **5** and **10** as present in crystals at 295 K (**3**, **4** and **5**) and at 150 K (**10**). Atomic displacement parameters are drawn at the 40% probability level. Hydrogen atoms are represented as spheres of arbitrary size. Only one of two alternative positions of the methyl hydrogen atoms in **3** is displayed.

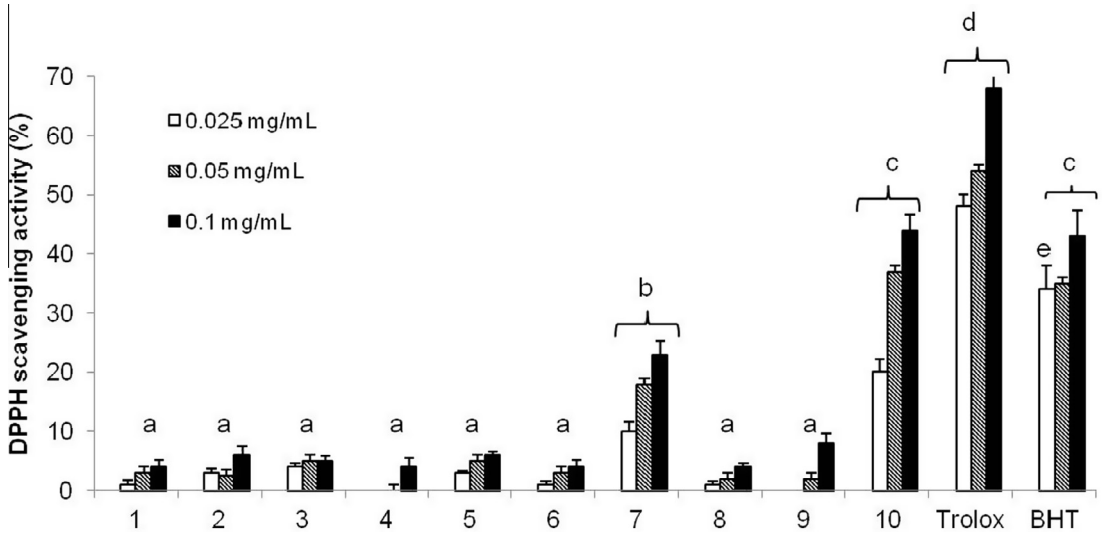


Figure 2. DPPH free radical scavenging activity of compounds tested and standard antioxidants Trolox and BHT at different concentrations. Results are presented as average ± SD ($n = 3$). Different letters indicate samples that were significantly different ($p < 0.05$). The numbers are respectively: caffeine (**1**); 6-thiocaffeine (**2**); 2,6-dithiocaffeine (**3**); 8-(methylthio)caffeine (**4**); 8-(ethylthio)caffeine (**5**); 8-(propylthio)caffeine (**6**); 8-(isopropylthio)caffeine (**7**); 8-(tertbutylthio)caffeine (**8**); 8-(phenylsulfanyl)caffeine (**9**); 8-[(pyrrolidin-1-yl-carbonothioyl)sulfanyl]caffeine (**10**).

185 ppm are connected with thiocarbonyl group of **10**. The signals corresponding to caffeine carbonyl groups of **4**–**10** appearing near 150 and 154 ppm are assigned to C2 and C6 respectively. In the ESI-MS spectra of compounds **2**, **3** and **9** molecular ions $[M+1]^+$ are observed, whereas for compounds **4**–**6**, **8** and **10** sodium adducts $[M+Na]^+$ are present. The exception is compound **7** for which the loss of $(CH_3)_2$ fragment from the $[M+Na]^+$ ion gives signals at $m/z = 261$.

The structural features of the molecules as present in crystals are illustrated in Figure 1, prepared using the Mercury program.[41] The xanthine ring and the C—S bonds in **4**, **5** and **10** are coplanar, while the terminal methyl group in **5** and the (pyrrolidin-1-ylcarbonothioyl)sulfanyl moiety in **10** are inclined at nearly right angles with respect to the xanthine molecular plane. Unlike caffeine, which crystallizes as a monohydrate, but also occurs in two anhydrous, extensively disordered forms,[42] all X-ray investigated

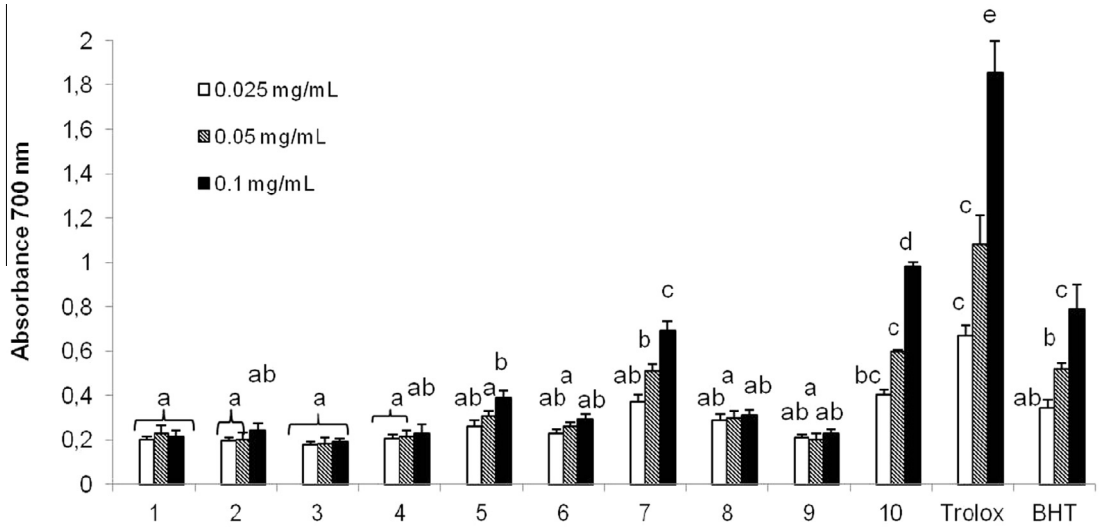


Figure 3. Fe^{3+}–Fe^{2+} reductive potential of compounds tested, and standard antioxidants Trolox and BHT at different concentrations. Results are presented as average ± SD (n = 3). Different letters indicate samples that were significantly different (p <0.05). Other indications as in Figure 2.

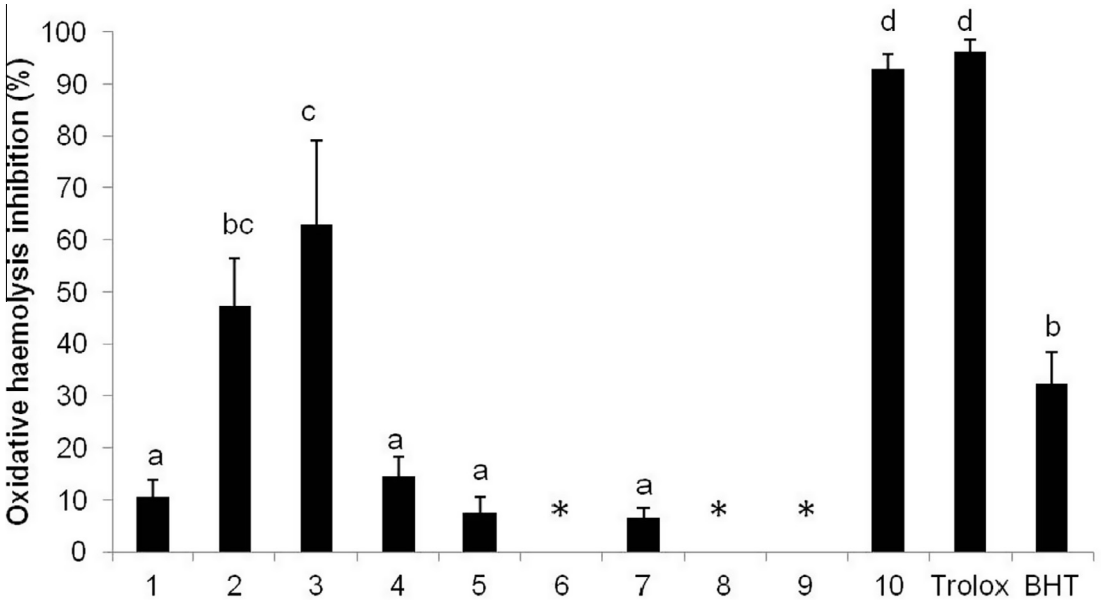


Figure 4. In vitro protective effects of compounds tested and standard antioxidants Trolox and BHT against 60 mM AAPH-induced haemolysis after 20 min pre-incubation with RBCs. Results are presented as means ± SD (n = 4). *—no haemolysis inhibition detected. Different letters indicate samples that were significantly different (p <0.05). Other indications as in Figure 2.

compounds reported in this Letter form anhydrous crystals. A hydrophilic centre at the imidazole nitrogen atom, utilised in the hydrated caffeine crystals as H-bond acceptor from the included water molecule, seems not to play a structure determining role in the crystals of the dithioanalogue **3** as well as in the remaining C8-substituted derivatives. More detailed description of X-ray structures is provided in Supplementary material.

As can be seen in Figure 2, compound **10** showed the strongest DPPH radical scavenging activity in the concentration-dependent manner. The scavenging effect of **10** at the highest concentration (0.1 mg/mL) was equal to 67% of the activity of standard Trolox and was identical to standard BHT. Compound **7** exhibited 34% activity of Trolox and 50% activity of BHT. Other thiocaffeines showed weak DPPH scavenging activity (less than 10%), comparable to that of caffeine.

The reducing capacity of a compound may act as an important indicator of its potential antioxidant activity. The method is based on the reduction of (Fe^{3+})-ferricyanide complex to ferrous form due to the presence of antioxidant. As shown in Figure 3, compound **10** exhibits the dose-dependent reducing power. At the highest concentration activity of **10** was equal to 65% of Trolox activity and was significantly higher than standard BHT. Similarly to results obtained with DPPH assay, reducing activity of compound **7** (38% of Trolox activity) was significantly higher than that of other derivatives and identical to BHT.

Caffeine as well as its thioanalogues have no capacity to ferrous ions chelate (data not shown).

The haemolytic activity of compounds **1**–**10** was estimated against human erythrocytes in vitro. Neither caffeine nor its thioanalogues were able to induce alterations in the erythrocyte

shape and their membrane permeability up to a concentration of 0.1 mg/mL. 8-(Phenylsulfanyl)caffeine (**9**) known as good MAO-B activity inhibitor,[22] was an exception (Table S1, Supplementary materials). Exposure of human erythrocytes to compound **9** induced haemolysis equal to 57.17% ± 7.32 at 0.1 mg/mL and 26.91% ± 11.9 at 0.05 mg/mL, respectively. No significant haemolysis (less than 5%) was observed at concentration of **9** equal 0.025 mg/mL. No significant RBCs shape changes were observed after incubation (60 min) with caffeine and its thio-analogues up to concentration 0.1 mg/mL. Compound **9** with confirmed membrane-permeabilizing activity, was an exception again. Optical microscopic evaluation of the erythrocytes morphology after 60 min incubation with **9** showed mostly the presence of echinocytes and spherocytes (Table S2, Supplementary materials), with some RBCs-ghosts between them. It is known that echinocytic transformation undergoes when molecules of compound incorporate into egzoplazmatic layer of the erythrocyte membrane,[43] therefore it can be concluded that **9** incorporates into the outer layer of a membrane and in consequence induce alterations in its molecular structure. The statistically significant decrease (p <0.05) of RBCs osmotic resistance (0.488% NaCl) under **9** treatment (*versus* control cells resistance equal to 0.451% NaCl) confirm its partitioning into the RBCs membrane (Table S2, Supplementary materials).

As shown in Figure 4, analogue **10** was a potent agent in RBC protecting against free radicals induced haemolysis, as effective as Trolox. Interestingly, 6-thiocaffeine (**2**) and 2,6-dithiocaffeine (**3**) did not exhibit an antioxidant potential in both DPPH and reducing power assay, but significantly inhibited haemolysis induced by AAPH. The protective activity of caffeine (**1**) and compounds **4**, **5** and **7** was similar and significantly lower (equal to 10%). Compounds **6**, **8** and **9** were not active in RBCs protection against free radicals damage.

To conclude, our results demonstrate that insertion of the (pyrrolidin-1-ylcarbonothioyl)sulfanyl moiety into the caffeine molecule leads to compound **10** that shows antioxidant potential significantly high to explain its cytoprotective effect. This finding substantiates classification of 8-[(pyrrolidin-1-ylcarbonothioyl)-sulfanyl]caffeine for further studies aimed at the use of this compound as an effective antioxidant for preventing oxidative-stress induced diseases.

Supplementary data

CCDC-1477518–1477521 contain the supplementary crystallographic data for **3**, **4**, **5** and **10**, respectively. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.06.091.

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40. Representative synthesis: 8-(Methylthio)caffeine (**4**). A mixture of 8-bromocaffeine (1 mmol) and sodium methanethiolate (4 mmol) in ethanol was heated under reflux for 2 h and when cooled to room temperature. The solution was evaporated until the product started to precipitate. The resulting precipitate was filtered off and recrystallized from ethanol. Yield 70%: mp 183–185 ^{0}C. ESI-MS: m/z 263 $[M+Na]^{+}$. ^{1}H NMR δ 3.84 (s, 3H), 3.57 (s, 3H), 3.40 (s, 3H), 2.72 (s, 3H); ^{13}C NMR δ154.52, 151.76, 151.24, 148.44, 108.65, 32.14, 29.71, 27.75, 14.85.
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