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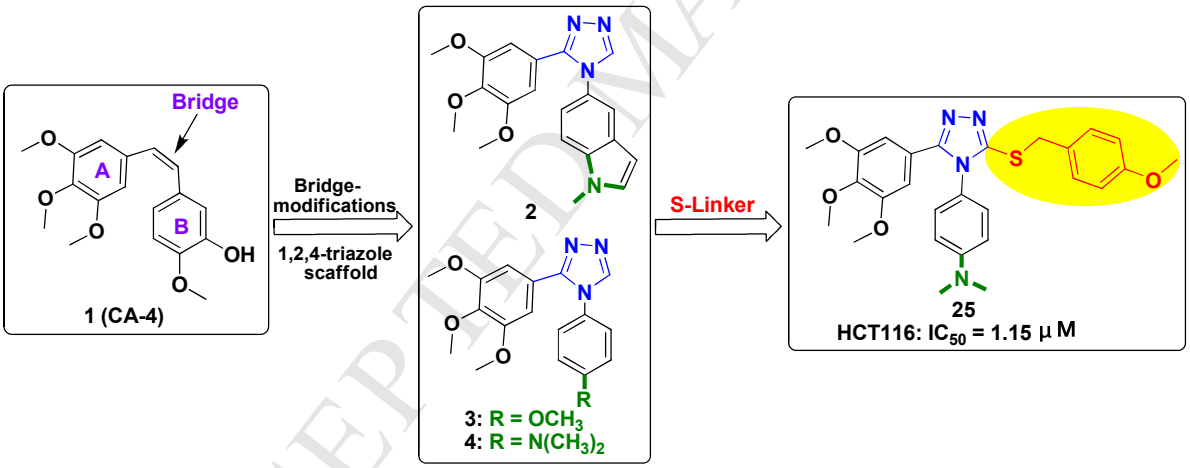

Design, Synthesis, and Biological Evaluation of Novel Alkylsulfanyl-1,2,4-triazoles as Cis-Restricted Combretastatin A-4 Analogues

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ABSTRACT: Thirty-two novel 3-alkylsulfanyl-1,2,4-triazole derivatives, designed as cis-restricted combretastatin A-4 analogues, were synthesized and evaluated for their antiproliferative activities. The results indicated that analogue **20** showed more potent antiproliferative activities against PC-3 cell lines than positive control CA-4. Particularly, the most promising compound **25** displayed 5-fold improvement compared to CA-4 in inhibiting HCT116 cell proliferation with IC_{50} values of 1.15 μM. Further flow-activated cell sorting analysis revealed that compound **20** displayed a significant effect on G_2/M cell-cycle arrest in a dose-dependent manner in PC-3 cells. From this study, analogues **20** and **25** were the most potent anti-cancer agents in this structural class, and were considered lead compounds for further development as anti-cancer drugs.

Keywords: Alkylsulfanyl-1,2,4-triazoles; Synthesis; Antiproliferative activity

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1. Introduction

Natural products are widely used as lead compounds for the discovery of new drugs with novel structures and mechanisms, which is reflected by the fact that increasing commercial drugs are either natural products, or their derivatives [1–5]. Combretastatin A-4 (CA-4, Figure 1) is among the most well-known anticancer agents, originally isolated from the South African bush willow tree, *Combretum caffrum Kuntze* (Combretaceae) [6,7], and its water-soluble prodrug (CA-4P) has now shown promising results in human cancer clinical trials [8]. The discovery of CA-4 has led to a diverse library of antitumor agents designed, due to its simple structure and great anticancer potency [9–16].

The structure-activity relationship (SAR) studies indicated that the presence of a 3,4,5-trimethoxyphenyl ring-A and *cis*-double bond is essential for potent antiproliferative activity [17]. As a result, a significant number of the conformationally restricted analogues, obtained by incorporating the *cis*-olefin bridge into a heterocyclic ring system, have been investigated [18–25]. Among them, 1,2,4-triazole-containing analogues (**2**, **3, 4**) have been reported to possess potent antiproliferative activities comparable to CA-4 [26]. Besides, many biologically active compounds and drugs comprise the S-linkers which were reported to improve important drug-like parameters: decrease lipophilicity, increase water solubility, and serve as good hydrogen bond acceptors [27]. Thus, in view of the previous rationale, we developed an idea that introducing diverse alkylsulfanyl as S-linkers into the C3-position of the analogues **3** and **4** might result in an interesting scaffold structure with potent anticancer activities (Fig. 1). Herein, we described the synthesis and antiproliferative activity of a series of 3-alkylsulfanyl-1,2,4-triazoles as cis-restricted CA-4 analogues **5~36.** To our knowledge, in the thirty-two target analogues, only two compounds **5** and **18** have been reported [28], but their antiproliferative activity has not been tested so far.

*<Insert figure **1** here>*

2. Chemistry

The synthetic route of the 3-alkylsulfanyl-1,2,4-triazole derivatives (**5~36**) was outlined in Scheme **1**. 3,4,5-trimethoxybenzoic acid (**A**) was treated with concentrated sulfuric acid in

anhydrous ethanol at reflux to yield ethyl 3,4,5-trimethoxybenzoate (**B**), which was then converted to hydrazide (**C**) in almost quantitative yield, according to our previously reported approach [29]. The condensation of the hydrazide with the commercially available suitably substituted isothiocyanates resulted in the formation of the intermediates (**D)**, which then underwent intramolecular ring closure to generate the corresponding substituted 4H-1,2,4-triazole-3-thiol ring **5**–**6** in the presence of sodium hydroxide as base in refluxing water, as reported in the literature [30]. Subseqently, analogues **7~36** were easily accessible by S-alkylation directly from intermediates **5~6** with various commercial halogen compounds using K_2CO_3 as base in anhydrous *N*,*N*-dimethylformamide at room temperature. All of the synthesized target compounds were given satisfactory analytical and spectroscopic data, which were in full accordance with their depicted structures.

<*Insert* scheme ***1*** *here*>

3. Pharmacology Results and Discussion

All synthesized analogues, **5~36**, were tested for *in vitro* cytotoxic activity against a panel of human tumor cell lines by MTT method [31]. Cell lines included HT-29 (human colon carcinoma cells), HepG2 (human hepatoma cells), PC-3 (human prostate cancer cell lines), and HCT116 (human colon cancer cell lines). CA-4 was selected as a positive control and the results expressed as IC_{50} (μM) were summarized in Table **1**. Here, the IC_{50} value represents the concentration of one compound resulting in a 50% inhibition in cell growth after a 48 h incubation, and is the average of three independent experiments.

<*Insert* table ***1*** *here*>

For the sake of convenience, 4-methoxy and *N*,*N*-dimethyl analogues were used to represent compounds **5~20** and **21~36**, respectively, throughout the text. Among the 4-methoxy analogues, initially, the importance of substitutions on alkylsulfanyl group was simply explored. As indicted in Table 1, the results displayed that the alkylsulfanyl moiety and its substitutions were critical for

keeping antiproliferative effect and the antiproliferative activities were almost lost when the thiol group was free or replaced by methylthio or ethylthio (**5**, **6** and **7**). Secondly, the free thiol group was subsequently converted to other alkylthios, including benzylthio, acetylthio, and acetamidethio (**8~11**, **12~18** and **19~20**) in order to investigate the influence on the antiproliferative activity. As presented in Table **1**, when changing the thiol group of the 3-position of triazole ring with benzylthio (**8~11**), the cytotoxic activities against HepG2, PC-3 and HCT116 cells were significantly increased by the chain elongation. Meanwhile, the introduction of electron withdrawing groups such as fluoro atom on the benzyl group (**11**), also caused a slight enhancement of the antiproliferative activity. These results suggest that electronic effect of substituents on benzyl group plays a crucial role on antitumor activities. Besides, linker-length of alkylsulfanyl moiety has also profound effects on the antiproliferative activities. Introduction of phenyl acetylthio substitutes on the 3-position of triazole ring (**12~14**) leads to dramatical enhancement of antiproliferative activities against HepG2 cell lines, but naphthyl, cyclopropyl and ethoxyl groups (**16~18**) result in dramatical decrease of the activities. It is worth noting that compound **20**, with *N*-4-chlorophenyl acetamidethio substitute, showed more potent *in vitro* cytotoxic activities against PC-3 with IC_{50} values of 6.29 μM, which represented 3-fold improvement in activity compared to CA-4. Moreover, further flow-activated cell sorting analysis revealed that compound **20** displayed a significant effect on G_2/M cell-cycle arrest in a dose-dependent manner in PC-3 cells.

Within the series of *N*,*N*-dimethyl analogues (**21~32**), the effects of substituents on the antiproliferative activities was strongly correlated with the 4-methoxy analogues. Meanwhile, analogue **25** was an exception, which displayed 5-fold improvement compared to CA-4 in inhibiting HCT116 cell proliferation with IC_{50} values of 1.15 μM.

In order to investigate the mode of action of these compounds on cancer cells, one selected analog **20** was examined for its influence on the cell cycle progression. In this study, PC-3 cells were treated with compound **20** at 3, 6, 12 μM concentrations for 48 h. As shown in Figure **2** and Table **2**, the G2/M peak significantly increased from 15.81% to 20.06% (3 μM), 24.23% (6 μM), and 34.80% (12 μM) after 12 h of treatment. These cell cycle analysis results revealed that the compound arrested the cell cycle at G_2/M phase in a dose-dependent manner, when compared to untreated control cells.

*<Insert figure **2** here>*

<Insert table ***2*** *here>*

Based on the obtained IC_{50} values against the four cancer cell lines, represent fourteen active analogues were evaluated for *in vitro* inhibitory effects on tubulin polymerization at 10 μM concentration and CA-4 was also used as the reference and the results expressed as as a percentage were summarized in Table **2**. Interestingly, to some extent, there is correlation with respect to anti-tubulin activity and cytotoxicity. For example, compound **25** (most active analogues) also displayed the most potent antitubulin activity with an inhibition percentage of 49% at 10μM concentration. Meanwhile, compound **20** also exhibited significant inhibition of tubulin polymerization with a percentage of 31%.

4. Conclusion

In conclusion, by introducing diverse alkylsulfanyl as S-linkers, a series of novel alkylsulfanyl-1,2,4-triazoles as cis-restricted CA-4 analogues exhibiting significantly antiproliferative activities were successfully identified. Analogue **20** showed more potent antiproliferative activities against PC-3 cell lines than positive control CA-4. Particularly, the most promising compound **25** displayed 5-fold improvement compared to CA-4 in inhibiting HCT116 cell proliferation with IC_{50} values of 1.15 μM. More interestingly, the analogue **25** also displayed the most potent antitubulin activity with a percentage of 49% at 10μM concentration. Moreover, further flow-activated cell sorting analysis revealed that compound **20** displayed a significant effect on G_2/M cell-cycle arrest in a dose-dependent manner in PC-3 cells. Further research on the mechanisms of these compounds and modification is underway.

5. Experimental protocols

5.1 Chemistry

Unless otherwise noted, all chemical and biological reagents were purchased from commercial suppliers and used without further purification while solvents were dried in a routine way and redistilled before use. ^{1}H and ^{13}C NMR spectra were recorded on a Mercury-Plus 400 spectrometer

in $CDCl_3$ or DMSO-d_6 solution and chemical shifts were recorded in parts per million (ppm) with TMS as the internal reference. ESI-MS/MS spectra were recorded using an Agilent QTOF 6540 mass spectrometer. The melting points were taken on a Buchi B-545 melting point apparatus and are uncorrected. The yields were not optimized.

*5.1.1 General procedure for the target compounds **5~6***

To a 100 mL round-bottom flask, 3,4,5-trimethoxybenzoic acid (10.6 g, 50 mmol), 1 mL of concentrated sulfuric acid and 30 mL of ethanol were added gradually. The resulting mixture was stirred at reflux for 12 h and was then concentrated on a rotary evaporator. The crude product obtained was poured into 50 ml of water and extracted with ethyl acetate. The combined ethyl acetate extracts were washed with water, dried with anhydrous magnesium sulfate, filtered off by suction and the solvent was evaporated to give crude ethyl 3,4,5-trimethoxybenzoate, which and hydrazine hydrate (7.5 g, 150 mmol, 60%) in 50 mL of ethanol was heated under reflux for 6 h. Excess ethanol was distilled out and the contents were allowed to cool. The solid product obtained was filtered, washed thoroughly with water, and dried to give 3,4,5-trimethoxybenzohydrazide in total yield of 83%, which was used for the next reactions without further purification. A solution of 3,4,5-trimethoxybenzohydrazide (3.39 g, 15 mmol) and appropriate isothiocyanatobenzenes (15 mmol) in ethanol (40 mL) was heated at reflux for 1 h, then cooled at room temperature. The suspension was filtered, and the solid was washed with ethanol and dried to give the intermediate hydrazinecarbothioamide as white solid. Then 1 N NaOH (30 mL) was added to this solid and the mixture was heated at reflux for 1 h. The resulting solution was cooled at room temperature and acidified to pH 5-6 with 1 M HCl. The precipitate was filtered, washed with water, and dried to obtain the title compounds as white solid.

*5.1.1.1 4-(4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazole-3-thiol (**5**).*Yield, 76%; mp 245.8–247.3°C (Lit.[28] 252–253°C); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.57 (s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4'), 3.81 (s, 3H, OCH_3-4"), 6.62 (s, 2H, trimethoxyPh-H), 7.07 (d, *J* = 8.8 Hz, 2H, ArH), 7.30 (d, *J* = 8.8 Hz, 2H, ArH), 14.06 (br, 1H, SH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 55.9, 56.1, 60.5, 106.1, 115.0, 121.3, 127.8, 130.5, 139.3, 150.7, 153.0, 160.1, 169.3. HRMS (ESI) m/z: calcd for $C_{18}H_{19}N_3O_4S$, $[M+H]^+$ 374.11298, found 374.11702.

*5.1.1.2 4-(4-methoxyphenyl)-3-(methylthio)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazole(**6**).*Yield,

93%; mp 100.9–101.2°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.73 (s, 3H, SCH_3), 3.66 (s, 6H, OCH_3-3", -5"), 3.84 (s, 3H, OCH_3-4'), 3.88 (s, 3H, OCH_3-4"), 6.70 (s, 2H, trimethoxyPh-H), 7.03 (d, *J* = 8.8 Hz, 2H, ArH), 7.21 (d, *J* = 8.8 Hz, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 14.8, 56.0, 56.1, 60.5, 105.7, 115.5, 122.3, 126.9, 129.6, 138.8, 153.0, 153.5, 154.6, 160.6. HRMS (ESI) m/z: calcd for $C_{19}H_{21}N_3O_4S$, $[M+H]^+$ 388.12863, found 388.13213.

*5.1.2 General procedure for the target compounds **7~36***

A mixture of 4-substituted-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazole-3-thiols **5** or **6** (1.0 mmol), various commercial halogen compounds (1.0 mmol) and potassium carbonate (K_2CO_3) (0.15 g, 1.1 mmol) in 5.0 mL of anhydrous N,N-dimethylformamide was stirred at room temperature for 1.0–2.0 h. After the reaction was complete according to the TLC detection, the mixture was quenched with water, and then the mixture extracted with ethyl acetate. The organic layer was separated, washed with water and brine, dried over anhydrous magnesium sulfate, filtered, and then concentrated to give the crude product followed by recrystallation from ethanol to afford the target compounds in yields of 62–94%.

*5.1.2.1 3-(ethylthio)-4-(4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazole (**7**).* Yield, 78%; mp 79.3–80.5°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.47 (t, *J* = 7.2 Hz, 3H, $SCH_2\underline{CH_3}$), 3.35 (dd, J_1 = 7.2 Hz, J_2 = 14.8 Hz, 2H, $S\underline{CH_2}CH_3$), 3.69 (s, 6H, OCH_3-3", -5"), 3.86 (s, 3H, OCH_3-4"), 3.90 (s, 3H, OCH_3-4'), 6.85 (s, 2H, trimethoxyPh-H), 7.08 (d, *J* = 8.8 Hz, 2H, ArH), 7.26 (d, *J* = 8.8Hz, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 15.2, 26.8, 56.0, 56.1, 60.5, 105.7, 115.4, 122.3, 127.0, 129.7, 138.8, 152.5, 153.0, 154.5, 160.5. HRMS (ESI) m/z: calcd for $C_{20}H_{23}N_3O_4S$, $[M+H]^+$ 402.14428, found 402.15155.

*5.1.2.2 3-(benzylthio)-4-(4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazole (**8**).* Yield, 81%; mp 109.4–111.2°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.57 (s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4'), 3.80 (s, 3H, OCH_3-4"), 4.41 (s, 2H, SCH_2), 6.68 (s, 2H, trimethoxyPh-H), 7.07 (d, *J* = 8.4 Hz, 2H, ArH), 7.37 –7.24 (m,7H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 36.5, 56.0, 60.5, 105.7, 115.4, 122.2, 126.8, 127.9, 128.9, 129.4, 129.6, 137.5, 138.8, 152.2, 153.0, 154.6, 160.5. HRMS (ESI) m/z: calcd for $C_{25}H_{25}N_3O_4S$, $[M+H]^+$ 464.15993, found 464.16431.

*5.1.2.3 4-(4-methoxyphenyl)-3-((4-methylbenzyl)thio)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazole (**9**).* Yield, 82%; mp 97.7–98.4°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.33 (s, 3H, Ph-CH_3), 3.65 (s, 6H,

OCH_3-3", -5"), 3.85 (s, 6H, OCH_3-4", -4',), 4.47 (s, 2H, SCH_2), 6.70 (s, 2H, trimethoxyPh-H), 6.97 (d, *J* = 8.8 Hz, 2H, ArH), 7.09 (dd, J_1= 8.4 Hz, J_2 = 14.4 Hz, 4H, ArH), 7.27 (d, *J* = 7.6 Hz,2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 21.1, 36.3, 56.0, 56.1, 60.5, 105.7, 115.4, 122.2, 126.9, 129.3, 129.4, 129.6, 134.4, 137.1, 138.9, 152.2, 153.0, 154.6, 160.5. HRMS (ESI) m/z: calcd for $C_{26}H_{27}N_3O_4S$, $[M+H]^+$ 478.17558, found 478.17993.

5.1.2.4 3-((4-methoxybenzyl)thio)-4-(4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazole (***10***). Yield, 93%; mp120.6–121.8°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.65 (s, 6H, OCH_3-3", -5"), 3.80 (s, 3H, OCH_3-4"), 3.85 (s, 6H, OCH_3-4', $PhCH_2O\underline{CH_3}$), 4.46 (s, 2H, SCH_2), 6.70 (s, 2H, trimethoxyPh-H), 6.83 (d, *J* = 8.4 Hz, 2H, ArH), 6.97 (d, *J* = 8.4 Hz, 2H, ArH), 7.08 (d, *J* = 8.4 Hz, 2H, ArH), 7.30 (d, *J* = 8.8 Hz, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 36.1, 55.5, 56.0, 56.1, 60.5, 105.7, 114.3, 115.4, 122.3, 126.9, 129.2, 129.6, 130.7, 138.9, 152.3, 153.0, 154.5, 159.1, 160.5. HRMS (ESI) m/z: calcd for $C_{26}H_{27}N_3O_5S$, $[M+H]^+$ 494.17050, found 494.17495.

5.1.2.5 3-((4-fluorobenzyl)thio)-4-(4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazole (***11***). Yield, 79%; mp 116.1–117.6°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.57 (s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4'), 3.80 (s, 3H, OCH_3-4"), 4.39 (s, 2H, SCH_2), 6.66 (s, 2H, trimethoxyPh-H), 7.16–7.06 (m, 4H, ArH), 7.27 (d, *J*= 8.8 Hz, 2H, ArH), 7.42 (dd, J_1= 5.4 Hz, J_2= 8.4 Hz, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 35.5, 56.0, 60.5, 105.7, 115.4, 115.5, 115.7, 122.2, 126.8, 129.6, 131.4, 131.5, 133.9, 134.0, 138.9, 152.1, 153.0, 154.6, 160.5, 160.7, 163.1. HRMS (ESI) m/z: calcd for $C_{25}H_{24}FN_3O_4S$, $[M+H]^+$ 482.15051, found 482.15576.

5.1.2.6 2-((4-(4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-3-yl)thio)-1-phenylethanone (***12***). Yield, 85%; mp 125.6–126.3°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.58 (s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4'), 3.83 (s, 3H, OCH_3-4"), 4.92 (s, 2H, SCH_2), 6.66 (s, 2H, trimethoxyPh-H), 7.13 (d, *J* = 8.8Hz, 2H, ArH), 7.39 (d, *J* = 8.8Hz, 2H, ArH), 7.58 (t, *J* = 7.6 Hz,2H, ArH), 7.70 (t, *J* = 7.4 Hz,1H, ArH), 8.03 (t, *J* = 4.0 Hz, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 56.1, 60.6, 105.8, 115.6, 122.1, 126.7, 128.8, 129.3, 129.6, 134.3, 135.7, 138.9, 152.2, 153.1, 154.7, 160.7, 193.6. HRMS (ESI) m/z: calcd for $C_{26}H_{25}N_3O_5S$, $[M+H]^+$ 492.15485, found 492.16234.

5.1.2.7 1-(4-methoxyphenyl)-2-((4-(4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-

*3-yl)thio)ethanone (**13**).* Yield, 88%; mp146.6–147.7°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.58 (s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4'), 3.82 (s, 3H, OCH_3-4"), 3.87 (s, 3H, COPh-O$\underline{CH_3}$), 4.85 (s, 2H, SCH_2), 6.66 (s, 2H, trimethoxyPh-H), 7.10 (dd, J_1 = 9.0 Hz, J_2 = 14.2 Hz, 4H, ArH), 7.39 (d, *J* = 9.2 Hz,2H, ArH), 8.00 (d, *J* = 8.8 Hz,2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 56.1, 60.5, 105.7, 114.5, 115.5, 122.2, 126.8, 128.6, 129.6, 131.2, 138.9, 152.1, 153.0, 154.6, 160.6, 164.0, 191.9. HRMS (ESI) m/z: calcd for $C_{27}H_{27}N_3O_6S$, $[M+H]^+$ 522.16541, found 522.17349.

*5.1.2.8 1-(4-fluorophenyl)-2-((4-(4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-3-yl)thio)ethanone (**14**).* Yield, 86%; mp 178.5–180.2°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.58 (s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4'), 3.82 (s, 3H, OCH_3-4"), 4.89 (s, 2H, SCH_2), 6.66 (s, 2H, trimethoxyPh-H), 7.12 (d, *J* = 8.4 Hz, 2H, ArH), 7.40 (t, *J* = 9.4 Hz, 4H, ArH), 8.12 (t, *J* = 7.0 Hz, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 56.1, 60.5, 105.7, 115.5, 116.2, 116.4, 122.2, 126.8, 129.6, 131.9, 132.0, 132.5, 138.9, 152.0, 153.0, 154.6, 160.6, 164.4, 167.0, 192.3. HRMS (ESI) m/z: calcd for $C_{26}H_{24}FN_3O_5S$, $[M+H]^+$ 510.14542, found 510.15315.

*5.1.2.9 1-(3-hydroxy-4-methoxyphenyl)-2-((4-(4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-3-yl)thio)ethanone (**15**).* Yield, 68%; mp176.6–177.5°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.58 (s, 6H, OCH_3-3", -5"), 3.66 (s, 3H, OCH_3-4'), 3.83 (s, 3H, OCH_3-4"), 3.88 (s, 3H, OHPh-OCH_3), 4.83 (s, 2H, SCH_2), 6.67 (s, 2H, trimethoxyPh-H), 7.07 (d, *J* = 8.8 Hz, 1H, ArH), 7.13 (d, *J* = 8.4 Hz, 2H, ArH), 7.40 (d, *J* = 5.2 Hz, 3H, ArH), 7.56 (d, *J* = 8.0 Hz,1H, ArH),9.49 (s, 1H, OH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 56.0, 56.1, 56.2, 60.5, 105.7, 111.7, 115.0, 115.5, 122.1, 122.2, 126.8, 128.7, 129.6, 138.9, 146.9, 152.2, 153.0, 154.6, 160.6, 192.0. HRMS (ESI) m/z: calcd for $C_{27}H_{27}FN_4O_4S$, $[M+H]^+$ 538.1540, found 538.1231.

*5.1.2.10 2-((4-(4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-3-yl)thio)-1-(naphthalen-2-yl)ethanone (**16**).* Yield, 67%; mp 165.4–166.8°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.58 (d, *J* = 3.6 Hz, 6H, OCH_3-3", -5"), 3.65 (d, *J* = 3.6 Hz, 3H, OCH_3-4'), 3.82 (d, *J* = 3.6 Hz,3H, OCH_3-4"), 4.96 (d, *J* = 3.6 Hz, 2H, SCH_2), 6.65 (d, *J* = 3.6 Hz, 2H, trimethoxyPh-H), 7.10 (dd, J_1 = 4.0 Hz, J_2 = 8.4 Hz, 2H, ArH), 7.32 (dd, J_1 = 3.8 Hz, J_2 = 8.6 Hz, 2H, ArH), 7.65 (dd, J_1 = 6.4 Hz, J_2 = 18.8 Hz, 3H, ArH), 8.04 (s, 1H, ArH), 8.25–8.19 (m, 2H, ArH), 8.42 (s, 1H, ArH).^{13}C NMR (100 MHz, DMSO-d_6) δ: 42.6, 56.0, 56.1, 60.5, 105.7, 115.5, 122.1, 125.2, 125.7, 126.7, 127.0, 128.3, 128.9, 129.1, 129.5, 129.9, 133.4, 133.9, 134.2, 138.9, 152.2, 153.0, 154.6, 160.6, 197.1.

HRMS (ESI) m/z: calcd for $C_{30}H_{27}N_3O_5S$, $[M+H]^+$ 542.17050, found 542.17429.

*5.1.2.11 1-cyclopropyl-2-((4-(4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-3-yl) thio)ethanone (**17**).* Yield, 66%; mp 138.5–140.9°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.94 (dd, J_1 = 5.6 Hz, J_2 = 23.6 Hz, 4H, cyclopropyl CH_2), 2.28–2.25 (m, 1H, cyclopropyl CH), 3.58 (s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4'), 3.82 (s, 3H, OCH_3-4"), 4.39 (s, 2H, SCH_2), 6.66 (s, 2H, trimethoxyPh-H), 7.13 (d, J = 8.8 Hz, 2H, ArH), 7.38 (d, J = 8.8 Hz, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 11.4, 19.9, 42.6, 56.0, 56.1, 60.5, 105.7, 115.5, 122.2, 126.8, 129.6, 138.9, 152.1, 153.0, 154.6, 160.6, 203.9. HRMS (ESI) m/z: calcd for $C_{23}H_{25}N_3O_5S$, $[M+H]^+$ 456.15485, found 456.16171.

*5.1.2.12 ethyl2-((4-(4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-3-yl)thio) acetate (**18**).* Yield, 79%; mp104.3–106.0°C (Lit.[28] 103°C); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.20 (t, J= 7.0 Hz, 3H, $CO_2CH_2\underline{CH_3}$), 3.58 (s, 6H, OCH_3-3", -5"), 3.65 (d, J = 1.2 Hz, 3H, OCH_3-4'), 3.82 (s, 3H, OCH_3-4"), 4.08 (s, 2H, SCH_2), 4.13 (dd, J_1 = 7.0 Hz, J_2 = 14.2 Hz, 2H, $CO_2\underline{CH_2}CH_3$), 6.67 (s, 2H, trimethoxyPh-H), 7.13 (d, J = 7.6 Hz, 2H, ArH), 7.38 (d, J = 8.0 Hz, 2H, ArH).^{13}C NMR (100 MHz, DMSO-d_6) δ: 14.4, 34.3, 56.1, 60.5, 61.7, 105.8, 115.5, 122.1, 126.7, 129.6, 138.9, 151.8, 153.0, 154.7, 160.7, 168.5. HRMS (ESI) m/z: calcd for $C_{22}H_{25}N_3O_6S$ $[M+H]^+$: 460.14976, found 460.15305.

*5.1.2.13 N-(4-methoxyphenyl)-2-((4-(4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-3-yl)thio)acetamide (**19**).* Yield, 94%; mp 209.8–210.9°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.66 (s, 6H, OCH_3-3", -5"), 3.79 (s, 3H, OCH_3-4'), 3.86 (s, 3H, OCH_3-4"), 3.89 (s, 3H, NHPh-O$\underline{CH_3}$), 4.18 (s, 2H, SCH_2), 6.72 (s, 2H, trimethoxyPh-H), 6.83 (s, 1H, ArH), 6.83 (s, 1H, ArH), 6.86 (s, 1H, ArH), 7.04 (s, 2H, ArH), 7.06 (s, 1H, ArH), 7.25 (d, J = 3.2 Hz, 1H, ArH), 7.28 (s, 1H, ArH), 7.60 (s, 1H, ArH), 7.63 (s, 1H, ArH), 10.34 (br, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 37.2, 55.6, 56.0, 56.1, 60.5, 105.7, 114.3, 115.5, 121.1, 122.2, 126.8, 129.6, 132.4, 138.9, 152.3, 153.0, 154.6, 155.8, 160.6, 165.4. HRMS (ESI) m/z: calcd for $C_{27}H_{28}N_4O_6S$, $[M+H]^+$ 537.17631, found 537.18231.

*5.1.2.14 N-(4-chlorophenyl)-2-((4-(4-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-3-yl)thio)acetamide (**20**).* Yield, 93%; mp 230.6–232.3°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.58

(s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4'), 3.82 (s, 3H, OCH_3-4"), 4.18 (s, 2H, SCH_2), 6.67 (s, 2H, trimethoxyPh-H), 7.11 (d, J = 8.8 Hz, 2H, ArH), 7.39 (dd, J_1 = 3.8 Hz, J_2 = 8.6 Hz, 4H, ArH), 7.61 (d, J = 8.8 Hz, 2H, ArH), 10.50 (br, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 37.2, 56.0, 56.1, 60.5, 105.7, 115.5, 121.1, 122.1, 126.7, 127.5, 129.1, 129.6, 138.2, 138.9, 152.2, 153.0, 154.7, 160.6, 166.1. HRMS (ESI) m/z: calcd for $C_{26}H_{25}ClN_4O_5S$, $[M+H]^+$ 541.12677, found 541.13289.

5.1.2.15 4-(4-(dimethylamino)phenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazole-3-thiol ***(21)***. Yield, 79%; mp 224.5–225.8°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.94 (s, 6H, 2×NCH_3), 3.57 (s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4"), 6.64 (s, 2H, trimethoxyPh-H), 6.80 (d, J = 8.8 Hz, 2H, ArH), 7.13 (d, J= 8.8 Hz, 2H, ArH), 14.00 (br,1H, SH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 40.6, 56.1, 60.5, 106.0, 112.6, 121.4, 123.3, 129.6, 139.2, 150.7, 151.7, 153.0, 169.5. HRMS (ESI) m/z: calcd for $C_{19}H_{22}N_4O_3S$, $[M+H]^+$ 387.14462, found 387.15019.

5.1.2.16 N,N-dimethyl-4-(3-(methylthio)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-4-yl)ani line ***(22)***. Yield, 81%; mp 184.6–187.5°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.75 (s, 3H, SCH_3), 3.05 (s, 6H, 2×NCH_3), 3.69 (s, 6H, OCH_3-3", -5"), 3.86 (s, 3H, OCH_3-4"), 6.79 (d, J= 9.2 Hz, 2H, ArH), 6.87 (s, 2H, trimethoxyPh-H), 7.13 (d, J= 9.2 Hz, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 14.7, 56.1, 60.5, 105.6, 112.8, 122.1, 122.5, 128.7, 138.7, 151.4, 153.0, 153.9, 154.6. HRMS (ESI) m/z: calcd for $C_{20}H_{24}N_4O_3S$, $[M+H]^+$ 401.16027, found 401.16721.

5.1.2.17 4-(3-(ethylthio)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-4-yl)-N,N-dimethylaniline ***(23)***. Yield, 70%; mp164.1–165.5°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.33 (t, J = 7.2 Hz, 3H, $SCH_2\underline{CH_3}$), 2.96 (s, 6H, 2×NCH_3), 3.13 (dd, J_1= 7.2 Hz, J_2= 14.8 Hz, 2H, $S\underline{CH_2}CH_3$), 3.58 (s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4"), 6.71 (s, 2H, trimethoxyPh-H), 6.80 (d, J = 9.2 Hz, 2H, ArH), 7.18 (d, J = 8.8 Hz, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 15.2, 26.6, 56.0, 60.5, 105.6, 112.8, 122.0, 122.4, 128.7, 138.7, 151.4, 153.0, 154.6. HRMS (ESI) m/z: calcd for $C_{21}H_{26}N_4O_3S$, $[M+H]^+$ 415.17592, found 415.18366.

5.1.2.18 4-(3-(benzylthio)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-4-yl)-N,N-dimethylaniline ***(24)***. Yield, 67%; mp 168.1–170.1°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.01 (s, 6H, 2×NCH_3), 3.66 (s, 6H, OCH_3-3", -5"), 3.84 (s, 3H, OCH_3-4"), 4.50 (s, 2H, SCH_2), 6.70 (d, J = 8.8 Hz, 2H, trimethoxyPh-H), 6.77 (s, 2H, ArH), 6.98 (d, J = 8.8 Hz, 2H, ArH), 7.26 (s, 1H, ArH), 7.31 (d, J =

6.8 Hz, 2H, ArH), 7.39 (d, *J* = 6.8 Hz, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 36.3, 56.1, 60.5, 105.6, 112.7, 122.1, 122.4, 127.8, 128.7, 128.8, 129.4, 137.6, 138.8, 151.4, 152.6, 153.0, 154.6. HRMS (ESI) m/z: calcd for $C_{26}H_{28}N_4O_3S$, $[M+H]^+$ 477.19157, found 477.19620.

*5.1.2.19 4-(3-((4-methoxybenzyl)thio)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-4-yl)-N,N-dimethylaniline (**25**).* Yield, 77%; mp 170.1–171.1°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.01 (s, 6H, 2×NCH_3), 3.66 (s, 6H, OCH_3-3", -5"), 3.80 (s, 3H, OCH_3-4"), 3.84 (s,3H, CH_2Ph-O$\underline{CH_3}$), 4.46 (s, 2H, SCH_2), 6.71 (d, *J* = 8.4 Hz, 2H, ArH), 6.77 (s, 2H, trimethoxyPh-H), 6.83 (d, *J* = 8.0 Hz, 2H, ArH), 7.00 (d, *J*= 8.8 Hz, 2H, ArH), 7.32 (d, *J* = 8.4 Hz, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 35.8, 55.5, 56.0, 60.5, 105.6, 112.7, 114.2, 122.1, 122.5, 128.7, 129.3, 130.7, 138.8, 151.3, 152.7, 153.0, 154.5, 159.0. HRMS (ESI) m/z: calcd for $C_{27}H_{30}N_4O_4S$, $[M+H]^+$ 507.20213, found 507.20448.

*5.1.2.20 N,N-dimethyl-4-(3-((4-methylbenzyl)thio)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-4-yl)aniline (**26**).* Yield, 68%; mp 177.6–179.1°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.27 (s, 3H, CH_3), 2.94 (s, 6H, 2×NCH_3), 3.57 (s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4"), 4.35 (s, 2H, SCH_2), 6.70 (s, 2H, trimethoxyPh-H), 6.78 (d, *J*= 9.2 Hz, 2H, ArH), 7.10 (t, *J* = 9.4 Hz, 4H, ArH), 7.25 (d, *J* = 8.0 Hz, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 21.1, 36.0, 56.0,60.5, 105.5, 112.7, 121.9, 122.3, 128.7, 129.3, 129.4, 134.3, 137.1, 138.8, 151.4, 152.8, 153.0, 154.6. HRMS (ESI) m/z: calcd for $C_{27}H_{30}N_4O_3S$, $[M+H]^+$ 491.20722, found 491.21129.

*5.1.2.21 4-(3-((4-fluorobenzyl)thio)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-4-yl)-N,N-dimethylaniline (**27**).* Yield, 72%; mp 170.1–172.6°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.94 (s, 6H, 2×NCH_3), 3.57 (s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4"), 4.39 (s, 2H, SCH_2), 6.70 (s, 2H, trimethoxyPh-H), 6.78 (d, *J* = 8.8 Hz, 2H, ArH), 7.16–7.09 (m, 4H, ArH), 7.43 (dd, J_1 = 5.6 Hz, J_2 = 8.4 Hz, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 35.3, 56.0, 60.5, 105.6, 112.8, 115.5, 115.7, 122.0, 122.4, 128.7, 131.4, 131.5, 134.0, 134.1, 138.8, 151.4, 152.5, 153.0, 154.6, 160.6, 163.1. HRMS (ESI) m/z: calcd for $C_{26}H_{27}FN_4O_3S$, $[M+H]^+$ 495.18214, found 495.18709.

*5.1.2.22 2-((4-(4-(dimethylamino)phenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-3-yl)thio)-1-phenylethanone (**28**).* Yield, 81%; mp160.8–162.8°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.96 (s, 6H, 2×NCH_3), 3.58 (s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4"), 4.91 (s, 2H, SCH_2), 6.70 (s, 2H,

trimethoxyPh-H), 6.83 (d, *J* = 8.8 Hz, 2H, ArH), 7.21 (d, *J* = 8.8 Hz, 2H, ArH), 7.58 (t, *J* = 7.8 Hz,2H, ArH), 7.70 (t, *J* = 7.4 Hz,1H, ArH), 8.04 (d, *J* = 7.6Hz, 2H, ArH).^{13}C NMR (100 MHz, DMSO-d_6) δ: 56.1, 60.5, 105.6, 112.8, 122.0, 122.4, 128.7, 128.8, 129.2, 134.1, 135.8, 138.8, 151.4, 152.5, 153.0, 154.7, 193.7. HRMS (ESI) m/z: calcd for $C_{27}H_{28}N_4O_4S$, $[M+H]^+$ 505.18648, found 505.19214.

*5.1.2.23 2-((4-(4-(dimethylamino)phenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-3-yl)thio)-1-(4-methoxyphenyl)ethanone (**29**)*. Yield, 86%; mp 145.2–147.8°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.96 (s, 6H, 2×NCH_3), 3.58 (s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4"), 3.87 (s, 3H, COPh-OCH_3), 4.84 (s, 2H, SCH_2), 6.70 (s, 2H, trimethoxyPh-H), 6.82 (d, *J* = 9.2 Hz, 2H, ArH), 7.09 (d, *J* = 8.8 Hz,2H, ArH), 7.20 (d, *J* = 8.8 Hz,2H, ArH), 8.01 (d, *J* = 8.8 Hz, 2H, ArH).^{13}C NMR (100 MHz, DMSO-d_6) δ: 561, 60.5, 105.6, 112.8, 114.5, 122.0, 122.4, 128.6, 128.7, 131.2, 138.8, 151.4, 152.5, 153.0, 154.6, 164.0, 192.0. HRMS (ESI) m/z: calcd for $C_{28}H_{30}N_4O_5S$, $[M+H]^+$ 535.19705, found 535.20162.

*5.1.2.24 2-((4-(4-(dimethylamino)phenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-3-yl)thio)-1-(4-fluorophenyl)ethanone (**30**)*. Yield, 64%; mp 172.6–174.1°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.96 (s, 6H, 2×NCH_3), 3.58 (s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4"), 4.88 (s, 2H, SCH_2), 6.69 (s, 2H, trimethoxyPh-H), 6.82 (d, *J* = 8.8 Hz, 2H, ArH), 7.21 (d, *J* = 8.8 Hz, 2H, ArH), 7.40 (t, *J* = 8.6 Hz,2H, ArH), 8.12 (t, *J* = 7.0 Hz,2H, ArH).^{13}C NMR (100 MHz, DMSO-d_6) δ: 56.0, 60.5, 105.6, 112.8, 116.2, 116.4, 121.8, 122.2, 128.7, 131.8, 131.9, 132.5, 138.8, 151.4, 152.5, 153.0, 154.7, 164.4, 167.0, 192.3. HRMS (ESI) m/z: calcd for $C_{27}H_{27}FN_4O_4S$, $[M+H]^+$ 523.17706, found 523.18156.

*5.1.2.25 2-((4-(4-(dimethylamino)phenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-3-yl)thio)-1-(3-hydroxy-4-methoxyphenyl)ethanone (**31**)*. Yield, 66%; mp 173.6–174.7°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.96 (s, 6H, 2×NCH_3), 3.58 (s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4"), 3.87 (s, 3H, HOPh-O$\underline{CH_3}$), 4.81 (s, 2H, SCH_2), 6.70 (s, 2H, trimethoxyPh-H), 6.83 (d, *J* = 8.4 Hz, 2H, ArH), 7.06 (d, *J* = 8.8 Hz, 1H, ArH), 7.21 (d, *J* = 8.8 Hz, 2H, ArH), 7.39 (s, 1H, ArH),7.57 (d, *J* = 8.4 Hz, 1H, ArH), 9.50 (s, 1H, OH).^{13}C NMR (100 MHz, DMSO-d_6) δ: 56.1, 56.2, 60.5, 111.7, 112.8, 115.0, 122.0, 122.1, 122.4, 128.7, 138.8, 146.9, 151.4, 152.6, 153.0, 154.6, 192.1. HRMS (ESI) m/z: calcd for $C_{27}H_{27}FN_4O_4S$, $[M+H]^+$ 551.1920, found 551.1902.

*5.1.2.26 2-((4-(4-(dimethylamino)phenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-3-yl)thio)-1-(naphthalen-2-yl)ethanone (**32**).* Yield, 62%; mp159.6–160.3°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.96 (s, 6H, 2×NCH_3), 3.58 (s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4"), 4.95 (s, 2H, SCH_2), 6.69 (s, 2H, trimethoxyPh-H), 6.80 (d, J = 8.4 Hz, 2H, ArH), 7.14 (d, J = 8.4 Hz, 2H, ArH), 7.65 (dd, J_1 = 7.0 Hz, J_2 = 14.6 Hz, 3H, ArH), 8.04 (d, J = 7.2 Hz, 1H, ArH), 8.22 (dd, J_1 = 7.6 Hz, J_2 = 17.6 Hz, 2H, ArH), 8.42 (d, J = 8.4 Hz, 1H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 42.4, 56.1, 60.5, 105.6, 112.8, 121.9, 122.3, 125.2, 125.7, 127.0, 128.3, 128.6, 128.9, 129.1, 129.7, 130.0, 133.4, 133.9, 134.3, 138.8, 151.4, 152.6, 153.0, 154.6, 197.2. HRMS (ESI) m/z: calcd for $C_{31}H_{30}N_4O_4S$, $[M+H]^+$ 555.20213, found 555.20602.

*5.1.2.27 1-cyclopropyl-2-((4-(4-(dimethylamino)phenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-3-yl)thio)ethanone (**33**).* Yield, 64%; mp152.6–154.1°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.94 (dd, J_1 = 5.2 Hz, J_2 = 22.4 Hz, 4H, 2×CH_2), 2.27 (dd, J_1 = 5.2 Hz, J_2 = 22.4 Hz, 1H, CH), 2.96 (s, 6H, 2×NCH_3), 3.58 (s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4"), 4.38 (s, 2H, SCH_2), 6.70 (s, 2H, trimethoxyPh-H), 6.82 (d, J = 8.8 Hz, 2H, ArH), 7.20 (d, J = 8.4 Hz, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 11.4, 19.9, 42.4, 56.1, 60.5, 105.6, 112.8, 121.9, 122.4, 128.7, 138.8, 151.4, 152.4, 153.0, 154.6, 204.0. HRMS (ESI) m/z: calcd for $C_{24}H_{28}N_4O_4S$, $[M+ H]^+$ 469.18648, found 469.19303.

*5.1.2.28 ethyl2-((4-(4-(dimethylamino)phenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-3-yl) thio)acetate (**34**).* Yield, 79%; mp 124.5–125.4°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.20 (t, J = 7.2 Hz, 3H, $CO_2CH_2\underline{CH_3}$), 2.96 (s, 6H, 2×NCH_3), 3.58 (s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4"), 4.07 (s, 2H, SCH_2), 4.13 (dd, J_1 = 7.2 Hz, J_2 = 14.0 Hz, 2H, $CO_2\underline{CH_2}CH_3$), 6.70 (s, 2H, trimethoxyPh-H), 6.83 (d, J = 9.2 Hz, 2H, ArH), 7.20 (d, J = 9.2 Hz, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 14.4, 34.1, 56.1,60.5, 61.7, 105.6, 112.8, 121.8, 122.3, 128.7, 138.8, 151.4, 152.2, 153.0, 154.7, 168.6. HRMS (ESI) m/z: calcd for $C_{23}H_{28}N_4O_5S$, $[M+H]^+$ 473.18140, found 473.18882.

*5.1.2.29 2-((4-(4-(dimethylamino)phenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-3-yl)thio)-N-(4-methoxyphenyl)acetamide (**35**).* Yield, 92%; mp 193.9–195.3°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.96 (s, 6H, 2×NCH_3), 3.58 (s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4"), 3.72 (s, 3H,

NHPh-O$\underline{CH_3}$), 4.14 (s, 2H, SCH_2), 6.71 (s, 2H, trimethoxyPh-H), 6.81 (d, *J* = 9.2 Hz, 2H, ArH), 6.90 (d, *J* = 8.8 Hz, 2H, ArH), 7.21 (d, *J* = 8.8 Hz, 2H, ArH), 7.49 (d, *J* = 8.8Hz, 2H, ArH), 10.22 (br, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 37.0, 55.6, 56.1, 60.5, 105.6, 112.8, 114.3, 121.0, 121.9, 122.4, 128.7, 132.4, 138.8, 151.4, 152.8, 153.0, 154.6, 155.8, 165.5. HRMS (ESI) m/z: calcd for $C_{28}H_{31}N_5O_5S$, $[M+H]^+$ 550.20794, found 550.21313.

*5.1.2.30 N-(4-chlorophenyl)-2-((4-(4-(dimethylamino)phenyl)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-3-yl)thio)acetamide (**36**)*. Yield, 88%; mp 181.7–182.9°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.96 (s, 6H, 2×NCH_3), 3.58 (s, 6H, OCH_3-3", -5"), 3.65 (s, 3H, OCH_3-4"), 4.17 (s, 2H, SCH_2), 6.70 (s, 2H, trimethoxyPh-H), 6.81 (d, *J* = 8.8 Hz, 2H, ArH), 7.21 (d, *J* = 8.8 Hz, 2H, ArH), 7.38 (d, *J* = 8.8 Hz, 2H, ArH), 7.61 (d, *J* = 8.8 Hz, 2H, ArH), 10.50 (br, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 37.0, 56.1, 60.5, 105.6, 112.8, 121.1, 121.8, 122.2, 127.5, 128.7, 129.1, 138.1, 138.8, 151.4, 152.7, 153.0, 154.7, 166.2. HRMS (ESI) m/z: calcd for $C_{27}H_{28}ClN_5O_4S$, $[M+H]^+$ 554.15841, found 554.16218.

5.2 Pharmacology evaluation

5.2.1 Antitumor activity

The antitumor activities of compounds **5**–**36** were evaluated with HT-29, HepG2, PC-3, and HCT116 cell lines by the standard MTT assay *in vitro*. The cancer cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were splitted at 70-80% confluence, about twice a week by trypsinization.

Exponentially growing cells were plated in 96-well plates (5000 cells/well) and incubated at 37 °C for 24 h for attachment. Test compounds were prepared by dissolving in dimethyl sulfoxide (DMSO) at 20 mM and diluted with the medium into a series of concentrations. The culture medium was then changed, and cells grew in medium with the test compounds. DMSO (0.1%) was used as negative control. Cells were incubated at 37 ℃ for 48 h. Then the medium was replaced with MTT solution (5 mg/mL, 100 μL) followed by incubation for another 4 h. The medium was then aspirated and formazan crystals were dissolved in DMSO (150 μL) for about 10 min. The absorbance at 570 nm (Abs) of the suspension was measured by an enzyme-linked immunosorbent assay (ELISA) reader. The inhibition percentage was calculated using the following formula: % inhibition = $(Abs_{control} - Abs_{compound})/Abs_{control} \times 100\%$. The IC_{50} values of the test compounds and

CA-4 were measured by treating cells with drugs of various concentrations, and analyzed by use of the prism statistical package (GraphPad Software, San Diego, CA, U.S.A.).

5.2.2 Flow-activating cell sorting analysis (FACS)

The effect of compound **20** on cell cycle phase distribution of human prostate cancer PC-3 was assessed using flow cytometry. When the cells grew to about 70% confluence in 60 mm dishs over night, they were treated with compound **20** at given concentrations (3, 6, 12 μM). After 48h, cells were harvested by trypsinization, washed with PBS, and fixed in 75% ice cold (4 °C) ethanol overnight. Then, they were washed with PBS, incubated with RNase (10 μg/mL final concentration) at 37 °C for 30 min, stained with propidium iodide (50 μg/mL final concentration), and analyzed by flow cytometry (Beckman Coulter).

5.2.3 In vitro tubulin polymerization assay

Pig brain microtubule protein was isolated by employing three cycles of temperature-dependent assembly/disassembly according to method described by Shelanski, et al [32]. Homogeneous tubulin was prepared from microtubule protein by phosphocellulose (P11) chromatography as has been described previously [33]. The purified proteins were stored in aliquots at -70 °C.

Microtubule polymerization of tubulin protein, in solutions containing different concentrations of compounds in PEM buffer (100 mM PIPES, 1 mM $MgCl_2$, and 1 mM EGTA), 1 mM GTP, and 5% glycerol, was monitored at 37 °C by using light scattering at 340 nm with a SPECTRA MAX 190 (MD) spectrophotometer. Plateau absorbance values were used for calculations. CA-4 was used as standard inhibitor of tubulin polymerisation, while DMSO was used as negative control. The percent inhibition values for selected compounds were compared to the value of CA-4 and measured the same day under the same conditions.

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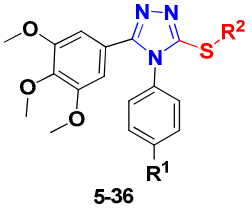
Figure captions

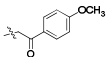
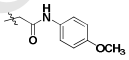
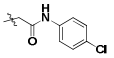
Fig. 1. Structures of CA-4 and some 1,2,4-triazole-based CA-4 analogues.

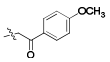
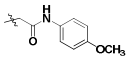
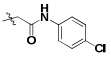
Fig. 2. Effect of compound **20** on cell cycle and apoptosis in PC-3 cells. Flow cytometry analysis of PC-3 cells treated with **20** for 48 h. (A) Control; (B) **20**, 3 μM; (C) **20**, 6 μM; (D) **20**, 12 μM.

Table 1. Cytotoxic activities of compounds 5~36 against human tumor cells.



5-36

Comp.	R^1	R^2	*In vitro* cytotoxicity IC_{50}(μM) [a]				Tubulin polymerization (% inhibition) [b]
			HT-29	**HepG2**	**PC-3**	**HCT116**	
5	CH_3O	H	>60	57.50	>60	>60	NT [c]
6	CH_3O	CH_3	>60	>60	>60	>60	NT
7	CH_3O	C_2H_5	>60	>60	57.70	>60	NT
8	CH_3O	$C_6H_5CH_2$	>60	>60	34.10	42.37	16
9	CH_3O	4-$CH_3C_6H_4CH_2$	>60	31.59	56.50	33.30	15
10	CH_3O	4-$CH_3OC_6H_4CH_2$	>60	41.10	40.31	49.64	29
11	CH_3O	4-$FC_6H_4CH_2$	50.70	34.97	34.97	38.74	NT
12	CH_3O		>60	16.21	>60	>60	NT
13	CH_3O		>60	18.12	>60	>60	30
14	CH_3O		42.50	10.74	58.25	>60	12
15	CH_3O		>60	>60	>60	>60	NT
16	CH_3O		53.40	>60	>60	>60	NT
17	CH_3O		>60	>60	37.20	>60	36
18	CH_3O		>60	>60	>60	>60	NT
19	CH_3O		45.25	>60	25.83	>60	10
20	CH_3O		58.02	>60	6.29	>60	31
21	$N(CH_3)_2$	H	>60	>60	>60	>60	NT
22	$N(CH_3)_2$	CH_3	>60	>60	>60	>60	NT
23	$N(CH_3)_2$	C_2H_5	>60	>60	46.18	>60	NT
24	$N(CH_3)_2$	$C_6H_5CH_2$	>60	>60	55.82	54.04	NT

25	$N(CH_3)_2$	4-$CH_3OC_6H_4CH_2$	>60	>60	>60	1.15	49
26	$N(CH_3)_2$	4-$CH_3C_6H_4CH_2$	27.45	>60	37.32	>60	21
27	$N(CH_3)_2$	4-$FC_6H_4CH_2$	44.30	26.40	46.45	>60	NT
28	$N(CH_3)_2$		45.15	18.90	38.60	42.53	16
29	$N(CH_3)_2$		63.96	18.77	22.00	>60	38
30	$N(CH_3)_2$		78.05	>60	42.30	31.08	NT
31	$N(CH_3)_2$		>60	>100	>100	>100	NT
32	$N(CH_3)_2$		>60	>60	18.06	54.48	26
33	$N(CH_3)_2$		>60	>60	45.92	>60	NT
34	$N(CH_3)_2$		28.80	>60	>60	>60	NT
35	$N(CH_3)_2$		51.37	>60	56.07	>60	NT
36	$N(CH_3)_2$		>60	>60	>60	39.26	17
	CA-4		1.96	5.29	22.03	6.10	80

[a] IC_{50} values are presented as mean values of three independent experiments done in quadruplicates. Coefficients of variation were <10%. [b] Compounds were tested at a final concentration of 10μM. [C] NT: not tested.

620

625

Table 2. Effect of compound 20 on cell cycle distribution in PC-3 cells.

Concentration	Sub-G_1(%)	G_0/G_1(%)	S(%)	G_2/M(%)
0μM	0.29	51.58	31.7	15.81
3μM	0.43	47.98	30.76	20.06
6μM	0.62	46.05	31.71	24.23
12μM	0.37	37.76	27.08	34.80

630

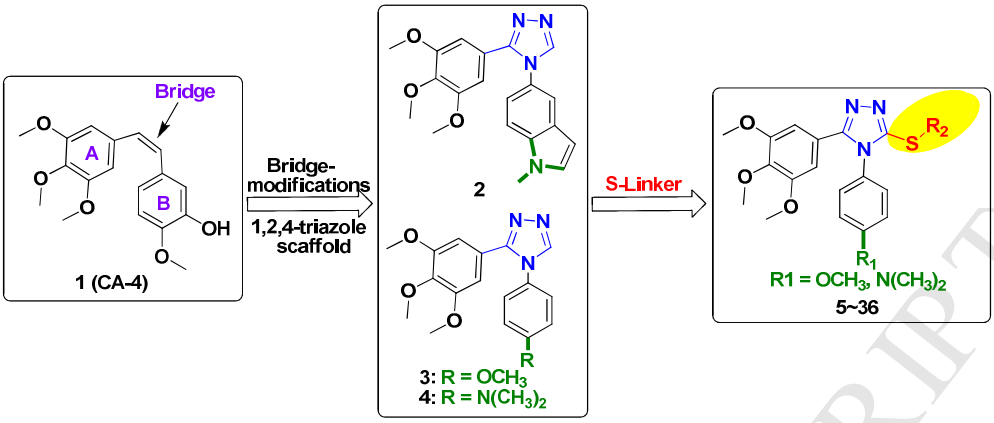
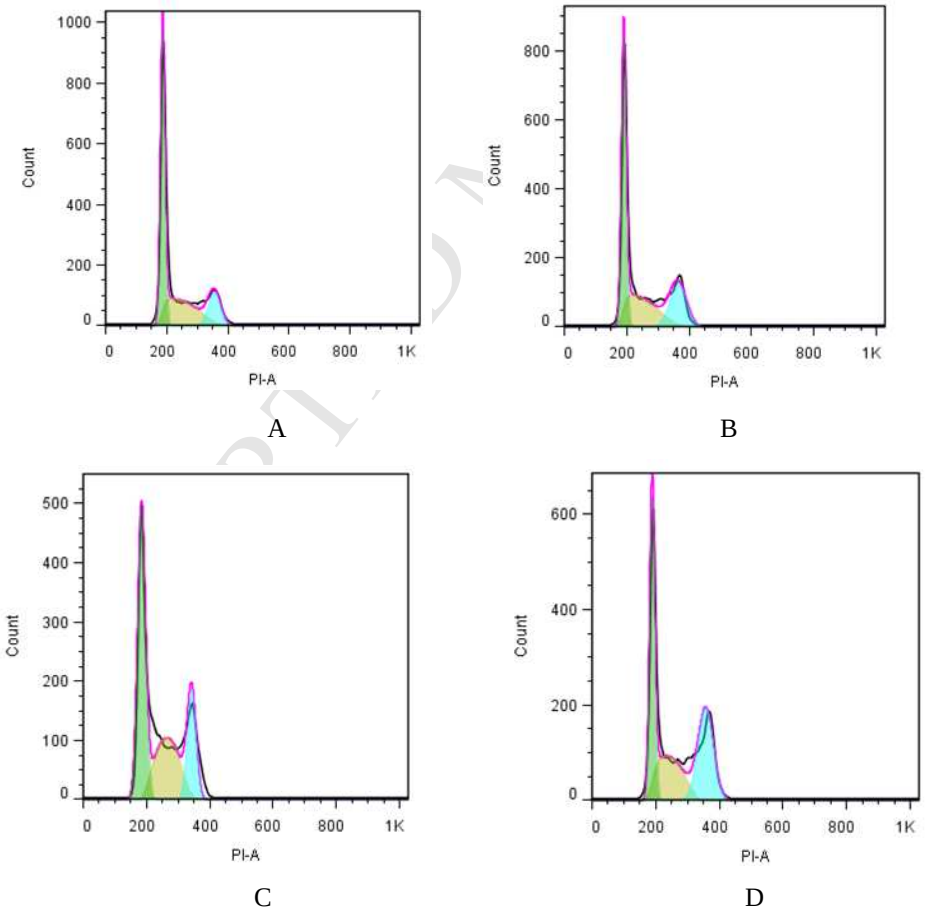


Figure 1. Structures of CA-4 and some 1,2,4-triazole-based CA-4 analogues.

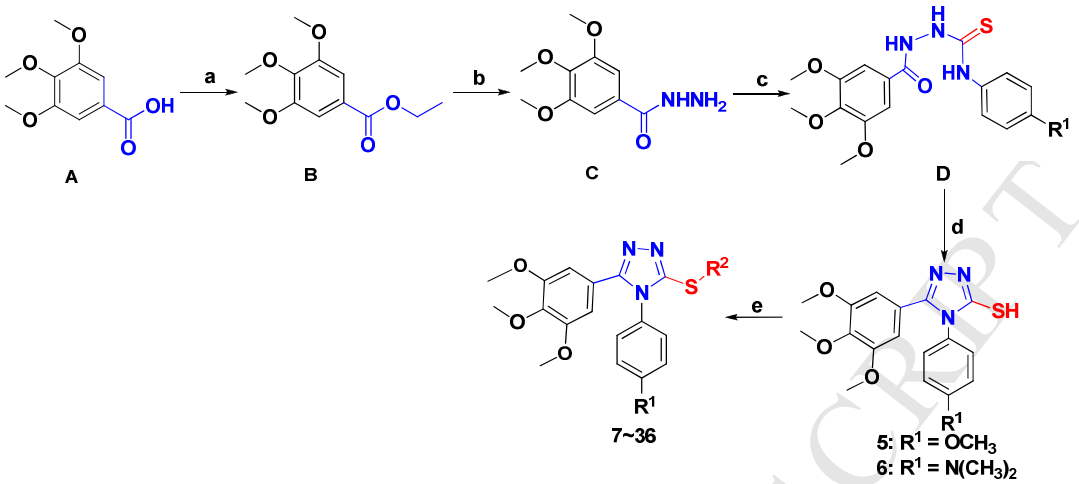
635



640

Figure 2. Effect of compound **20** on cell cycle and apoptosis in PC-3 cells. Flow cytometry analysis of PC-3 cells treated with **20** for 48 h. (A) Control; (B) **20**, 3 μM; (C) **20**, 6 μM; (D) **20**, 12 μM.

645



Scheme 1. Synthesis of the target compounds 5~36. Reagents and conditions: (a) Con. H_2SO_4, ethanol, reflux; (b) $NH_2NH_2 \cdot H_2O$, ethanol, reflux; (c) isothiocyanate, ethanol, reflux; (d) 1M NaOH, reflux; (e) K_2CO_3, RX, DMF,rt.

Highlights

• 32 novel 1,2,4-triazole derivatives were designed and synthesized as cis-restricted combretastatin A-4 analogues. • Antiproliferative activity of these compounds was evaluated. • Analogues **20** and **25** exhibited much stronger antitumor activity than CA-4. • Flow-activated cell sorting analysis suggested that compound **20** mainly arrested PC-3 cells in G_2/M stage.