Novel Synthesis and Biological Evaluations of *N,N'*-disubstituted-3,6-bis(substitutedphenyl)-1,2,4,5-tetrazine-1,4-dicarboxamide

Guo-Tong Tong, Lu-Ping Lv*, Ping Tang and Wei-Wei Li
[1] *Linjiang College, Hangzhou Vocational and Technical College, Hangzhou 310018, China.*
mailofllp@126.com*

(Received o February 2017, accepted in revised form 13th October 2017

Summary: A series of novel N,N'-disubstituted-3,6-bis(substitutedphenyl) -1,2,4,5-tetrazine-1,4-dicarboxamides were prepared using the intermolecular cyclization reaction of N-substituted-N'-(α-chloro-substitutedbenzylidene) hydrazinecarboxamide and triethylamine by the modification of solvent polarity. The structures of all the new compounds were characterized by IR, ^{1}H-NMR, MS and elemental analysis. Their cytostatic effects were screened in vitro by the SRB method for A-549 cell and the MTT method for P-388 cell. The results showed that several compounds demonstrate potential antitumor activities against P-388. The substituents have clearly effect on their antitumor activity.

Keywords: Synthesis; Characterize; Antitumor activity; 1,4-Dihydro-s-tetrazine.

Introduction

1,2,4,5-Tetrazine derivatives play a predominant role in constructing diverse aza-containing heterocycles [1, 2]. Hundreds of novel compounds containing the 1,2,4,5-tetrazine skeleton were synthesized and proved to have biological activity in pharmaceuticals [3-7]. For example, 1,2,4-triazolo-1,2,4,5-tetrazines exhibited preferable antitumor activity [8], some hexahydro-s-tetrazines have a high potential for antibacterial activity [9]. A series of tetrahydro-s-tetrazines possessing antiinflammatory and antifungal activities have also been developed [10].

Recently, our group has reported some s-tetrazine derivatives which exhibited preferable antitumor activities, especially, 1,4-dihydro-s-tetrazine-1,4-dicarboxamides [11-14]. It was investigated in A-549 and RERF-LC-MA lung cancer cells, and the underlying molecular mechanism in treating lung cancer was also determined [15]. Our past results have shown that some of s-tetrazine compounds exhibit high activity for A-549 cells and P-388 cells.[16-18] In continuation of our further effort to find promising antitumor agents, we researched the synthesis, structure analysis, biological evaluation of 1,2,4,5-tetrazine compounds and attempted to investigate the structure-property relationship of this compound class [19-20]. In this letter, seven 1,2,4,5-tetrazine derivatives were designed and synthesized. The chemical structures and synthetic route of the target compounds were shown as Fig. 1. Our role was to explore whether these new 1,2,4,5-tetrazine compounds would show an effective inhibition of cell proliferation. Their cytostatic effects were screened in vitro by the SRB method for A-549 cell and the MTT method for P-388 cell to evaluate the antitumor activity of all the newly compounds.

Instruments

Melting points were carried on XRC-1 apparatus and uncorrected. Infrared spectra were recorded from KBr discs on a Nicolex FI-IR-170 instrument. 1H-NMR spectra were run on a Bruker AC400 (400MHZ) spectrometer using TMS as internal standard and CDCl3 as the solvent. Mass spectra were obtained on a HP5989 spectrometer at on ionizing voltage of 70 ev by electron impact. Elemental analyses were performed on a Carlo ERBA-1106 instrument.

Experimental

Synthesis

General procedure for 1,2,4,5-tetrazine. Solvents and reagents were commercially available without further purification. Compounds 1 were synthesized by literature method [21]. The mixture of compound 1 and 40% hydrazine hydrate was heated to reflux for 15 h. A colorless precipitate formed immediately at 0°C, which was filtered, washed with water and dried to give compound 2. The synthesis of compounds 3a-g was carried out by modified literature method [22]. The synthesis of compounds 4a-g was modified with using Cl_2 as chloride agent instead of using $SOCl_2$ according to the literature method [23].

*To whom all correspondence should be addressed.

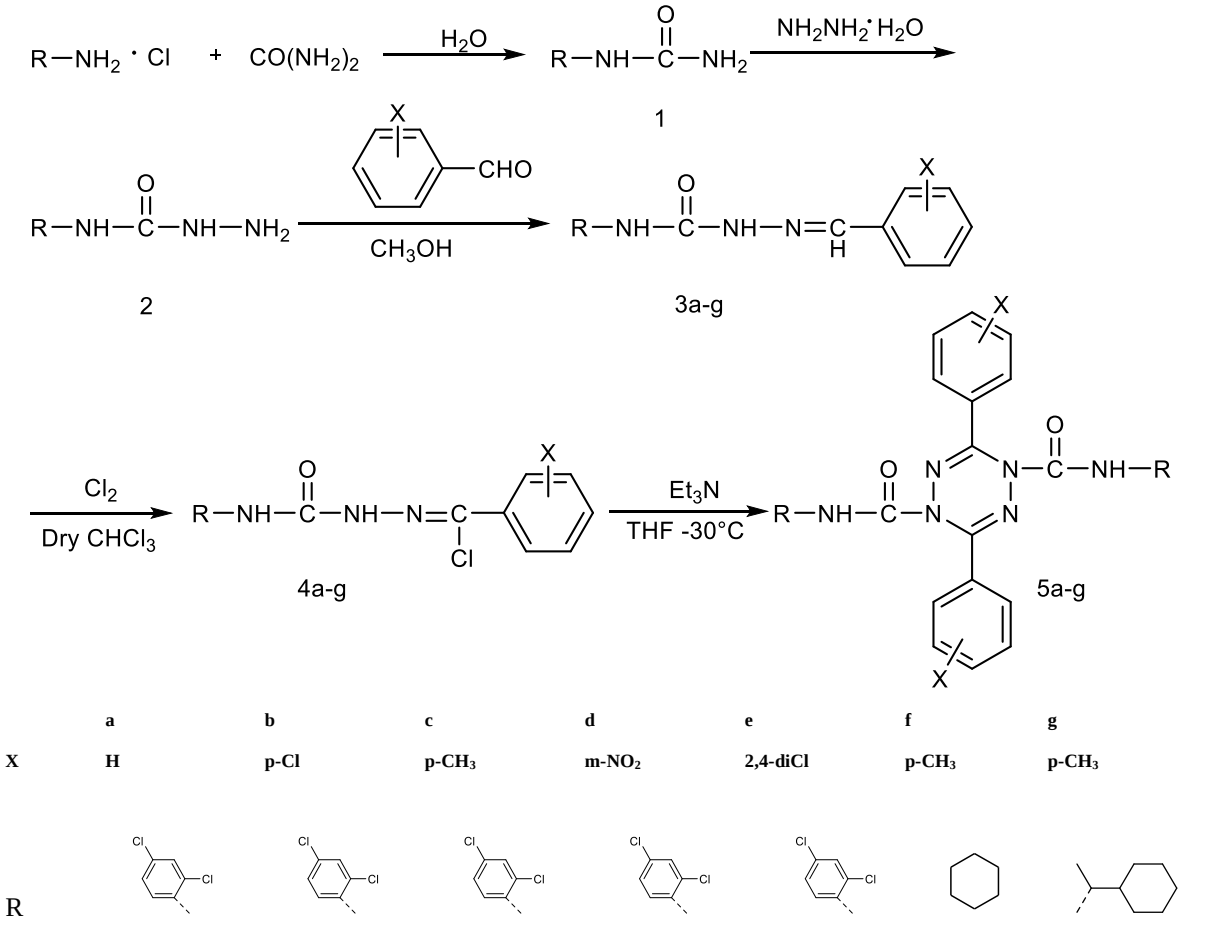


Fig. 1: Synthetic route of the target compounds and their chemical structures.

N,N'-bis(2,4-dichlorophenyl)-3,6-diphenyl-1 ,2,4,5-tetrazine-1,4-dicarboxamide (**5a**). To a mixture of triethylamine (0.60 g, 6.0 mmol) and dried THF (20 ml) at the temperature of -30°C, **4a** (N-phenyl-N'-(α-chloro-2,4-dichlorobenzylidene)hyd razinecarboxamide) (0.80 g, 3.0 mmol) in THF (20 ml) was added dropwise at -30°C. After the addition was complete, white precipitate formed immediately. Then the mixture was heated to 20-25°C and kept stirring at this temperature for 10 h, using TLC (n-hexane: ethyl acetate=1:3) to monitor the reaction. After the reaction, the white precipitate (triethylamine hydrochloride) was filtered. The filtrate was concentrated to give white solid. The solid was recrystallized by absolute ethanol to obtain **5a** (0.60 g, 84.0%) as a white solid. M. p. 142-144°C; IR (KBr,cm^{-1}): 3409m (NH), 1626s (C=O), 1556m (C=N), 1398s (ring); ^{1}H-NMR ($CDCl_3$) δ 8.36 (m, 1H, ArH), 8.00 (m, 2H, ArH), 7.44-7.98 (m, 5H, ArH); ^{13}C NMR (126 MHz, $CDCl_3$) δ 153.89, 153.45, 148.56, 148.34, 138.67, 138.21, 134.58 , 134.12, 133.80 , 133.11 , 132.98 ,132.32 , 129.91, 129.36, 128.43, 128.2 , 127.46, 127.23, 126.33, 126.21, 125.67, 125.26, 124.78, 124.17, 123.95, 123.40, 120.24, 120.03; MS (m/z, %): 305 (M^+-305, 76.09), 270 (16.13), 248 (6.76), 214 (13.82), 166 (7.13), 144 (100), 118 (8.85), 77 (53.61), 63 (4.80); Anal.Calcd.for $C_{28}H_{18}Cl_4N_6O_2$ (MW= 610.02): C, 54.93; H, 2.96; N, 13.73. Found: C, 54.79; H, 3.06; N, 13.58.

N,N'-bis(2,4-dichlorophenyl)-3,6-bis(4-chlo rophenyl)-1,2,4,5-tetrazine-1,4-dicarboxamide (**5b**): Following the method used for **5a**, to a mixture of triethylamine (0.80 g, 8.0 mmol) and THF (25 ml), **4b** (1.37 g, 4.0 mmol) in THF (15 ml) was added dropwise at -30 °C and kept stirring at temperature of 20-25°C for 6 h. The crude material was recrystallized twice from absolute ethanol to obtain 1.11 g pure product **5b**, yield 81.3 %. M. p. 164-168 ; IR (KBr, cm^{-1}): 3402m (NH), 1619s (C=O), 1550m (C=N), 1392s (ring); ^{1}H-NMR ($CDCl_3$) δ 8.17 (m,

1H, ArH), 7.70 (m, 2H, ArH), 7.39-7.49 (m, 4H, ArH); ^{13}C NMR (126 MHz, $CDCl_3$) δ 154.58, 154.32, 147.46, 147.21, 139.78, 139.37, 135.56 , 135.28, 134.43, 134.31, 133.61, 133.26, 130.15, 130.39, 129.91, 129.55, 128.47, 128.12, 127.90 127.67, 126.87, 126.39, 125.54, 125.12, 124.70, 124.22, 121.37,121.08; MS (m/z, %). 339 (M^+-339, 71.79), 304 (13.23), 282 (10.52), 250 (17.90), 202 (15.53), 154 (9.86). Anal.Calcd.for $C_{28}H_{16}Cl_6N_6O_2$ (MW=677.95): C, 49.37; H, 2.37; N, 12.34. Found: C, 49.59; H, 2.24; N, 12.58.

N,N'-bis(2,4-dichlorophenyl)-3,6-di-p-tolyl-1,2,4,5-tetrazine-1,4-dicarboxamide (**5c**): Following the method used for **5a**, To a mixture of triethylamine (0.68 g, 6.8 mmol) and THF (20 ml), **4c** (1.00 g, 3.4 mmol) in THF (20 ml) was added dropwise at -30°C and kept stirring at temperature of 20-25°C for 6 h. The crude material was recrystallized twice from absolute ethanol to obtain 0.70 g pure product **5c**, yield 90.1 %. M. p. 178-180 °C; IR (KBr, cm^{-1}): 3413m (NH), 2930 (CH_3), 1625s (C=O), 1585m (C=N), 1398s (ring); ^{1}H-NMR ($CDCl_3$) δ 8.37 (m, 1H, ArH), 7.87 (m, 2H, ArH), 7.29-7.88 (m, 4H, ArH), 2.43 (s, 3H, CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 153.98, 153.78, 147.69, 147.26, 139.89, 139.21, 134.82, 134.18, 133.83, 133.32, 132.73, 132.06 ,131.03, 131.43, 129.53, 129.21, 128.88, 128.45, 127.56, 127.29, 127.11, 126.92, 125.73, 125.39, 124.88, 124.61, 120.32, 120.09, 21.53 , 21.93; MS (m/z ,%) 319 (M-319, 80.51), 284 (12.54), 228 (10.21), 160 (13.55), 159 (100), 131 (28.82), 117 (38.34), 91 (24.68), 77 (4.97); Anal. Calcd. for $C_{30}H_{22}Cl_4N_6O_2$ (MW=638.06): C, 56.27; H, 3.46; N, 13.12. found: C, 55.94; H, 3.35; N, 12.97.

N,N'-bis(2,4-dichlorophenyl)-3,6-bis(3-nitrophenyl)-1,2,4,5-tetrazine-1,4-dicarboxamide (**5d**): Following the method used for **5a**, To a mixture of triethylamine (0.90 g,9.0 mmol) and THF (35 ml), **4d** (1.74 g, 4.5 mmol) in THF (25 ml) was added dropwise at -30 °C and kept stirring at temperature of 20-25 °C for 8 h. The crude material was recrystallized twice from absolute ethanol to obtain 1.08 g pure product **5d**, yield 68.8 %. M. p. 189-191 °C; IR (KBr, cm^{-1}): 3430m (NH), 1624s (C=O), 1561m (C=N), 1385s (ring); ^{1}H-NMR ($CDCl_3$) δ 8.90 (m, 1H, ArH), 8.17-8.21 (m, 2H, ArH), 7.40-7.73 (m, 4H, ArH), ^{13}C NMR (126 MHz, $CDCl_3$) δ 154.63, 154.21, 148.71,148.12, 147.79 ,147.38, 134.69, 134.28, 133.45, 133.08, 132.43, 132.13, 131.32, 131.16, 129.41, 129.26, 128.81, 128.50, 127.43, 127.09, 126.29, 126.05, 125.77, 125.43, 124.87, 124.31, 119.91, 119.57; MS (m/z, %) 350 (M-350, 80.51), 315 (22.38), 259 (12.52), 191 (15.20), 190 (100), 162(30.23), 148 (42.43), 119 (16.65), 92 (2.21); Anal.Calcd for $C_{28}H_{16}Cl_4N_8O_2$ (MW= 699.99): C, 47.89; H, 2.30; N, 15.96. found: C, 47.74; H, 2.18; N, 15.78.

N,N'-bis(2,4-dichlorophenyl)-3,6-bis(2,4-dichlorophenyl)-1,2,4,5-tetrazine-1,4-dicarboxamide (**5e**): Following the method used for **5a**, To a mixture of triethylamine (0.70 g,7.0 mmol) and THF (30 ml), **4e** (1.43 g, 3.5 mmol) in THF (15 ml) was added dropwise at -30 °C and kept stirring at temperature of 20-25 °C for 3 h. The crude material was recrystallized twice from absolute ethanol to obtain 1.12 g pure product **5e**, yield 86.1 %; M. p. 151-153°C; IR (KBr, cm^{-1}): 3435m (NH), 1625s (C=O), 1576m (C=N), 1381s (ring); ^{1}H-NMR ($CDCl_3$) δ 8.27 (m, 1H, ArH), 7.85 (m, 1H, ArH), 7.27-7.73 (m, 4H, ArH); ^{13}C NMR (126 MHz, $CDCl_3$) δ 153.84, 153.21, 147.76, 147.21, 138.82, 138.41, 135.57, 135.15, 133.67, 133.08, 132.78, 132.43, 131.59, 131.06, 130.69, 130.23, 128.67, 128.29, 127.92, 127.56, 126.49, 126.19, 125.69, 125.25, 124.62, 124.22, 120.61, 120.32; MS (m/z , %) 373 (M-373, 67.31), 338 (21.34), 282 (13.13), 214 (12.65), 213 (100), 185 (38.42), 169 (31.64), 91 (20.38); Anal.Calcd for $C_{28}H_{14}Cl_8N_6O_2$ (MW=745.87): C, 44.84; H, 1.88; N, 11.20. found: C, 44.64; H, 1.80; N, 11.07.

N,N'-dicyclohexyl-3,6-di-p-tolyl-1,2,4,5-tetrazine-1,4-dicarboxamide (**5f**): Following the method used for **5a**, To a mixture of triethylamine (0.50 g, 5.0 mmol) and THF (20 ml), **4f** (0.73 g, 2.5 mmol) in THF (10 ml) was added dropwise at -30°C and kept stirring at temperature of 20-25°C for 3.5 h. The crude material was recrystallized twice from absolute ethanol to obtain 0.37 g pure product **5f**, yield 57.2 %. M. p. 170-172 °C; IR (KBr, cm^{-1}): 3439m (NH), 2930m (CH_3),1621s (C=O), 1583m (C=N), 1381s (ring); ^{1}H-NMR ($CDCl_3$) δ 7.25-7.79 (m, 8H, ArH), 5.30 (M, 2H, NH), 3.62 (s, 2H, CH), 2.41 (s, 6H, CH_3), 1.25-2.13 (m, 20H, CH_2); ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.49, 166.18, 147.86, 147.21, 140.39, 140.18, 134.96, 134.53, 133.64, 133.45, 132.36,132.11, 131.43, 131.10, 126.90, 126.61, 54.65, 54.15, 33.36, 33.21, 33.87, 33.31, 25.56, 25.26, 24.48, 24.18, 24.80, 24.23, 21.11, 21.23; MS (m/z, %) 258 (M-258, 100.00), 257 (5.44), 176 (21.42), 133 (8.57); Anal.Calcd for $C_{30}H_{38}N_6O_2$ (MW=745.87): C, 70.01; H, 7.44; N, 16.33. found: C, 70.21;H, 7.49; N, 16.05.

N,N'-bis(1-cyclohexylethyl)-3,6-di-p-tolyl-1, 2,4,5-tetrazine-1,4-dicarboxamide (**5g**): Following the method used for **5a**, To a mixture of triethylamine (1.01 g, 1.0 mmol) and THF (15 ml), **4g** (1.54 g, 5.0 mmol) in THF (15 ml) was added dropwise at -30°C and kept stirring at temperature of 20-25°C for 4.5 h. The crude material was recrystallized twice from absolute ethanol to obtain 1.05 g pure product **5g**, yield 77.5 %. M. p .112-114°C; IR (KBr, cm^{-1}): 3433m (NH), 2932m (CH_3), 1625s (C=O), 1586m (C=N), 1385s (ring); ^{1}H-NMR ($CDCl_3$) δ 7.29-7.70 (m, 8H, ArH), 6.01 (M, 2H, NH), 3.10 (m, 2H, CH), 2.42 (s, 6H, CH_3), 2.10 (m, 2H, CH), 1.35-2.03 (m, 20H, CH_2), 1.26 (m, 6H, CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.80, 166.32, 147.98, 147.76, 139.86, 139.53, 134.68, 134.23, 133.39, 133.13, 132.43, 132.01, 131.45, 131.11, 127.57, 127.26, 65.76, 65.41, 46.67, 46.23, 31.66, 31.25, 30.99, 30.75, 26.48, 26.12, 25.87, 25.44, 25.21, 24.90, 21.61, 21.23, 16.11, 15.80; MS (m/z, %) 285 (M-285, 100.00), 284 (21.34), 203 (25.32), 133 (18.45); Anal.Calcd for $C_{34}H_{46}N_6O_2$ (MW=570.37): C, 71.55; H, 8.12; N, 14.72. found: C, 71.40; H, 8.02; N, 14.57.

Biological activity assays

A-549 cells (1 x 10^4 cells per well) were plated in 96-well plates. After 4 h of incubation, 1 µl of the drugs was added into the well in which each cell well contained 99 µl of the cell dilutions. The final concentration was between 0.01 µM and 100 µM. The cells were then incubated for 24 h at 37°C. After this period, 20 µl of culture medium containing (4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTT) was added into the wells and incubated at different concentrations (0.01 µM to 100 µM) for 1 h.

P-388 cells were stained for 30 minutes in 0.4%(wt/vol) Sulforhodamine B (SRB) dissolved in 1% acetic acid. At the end of the staining , SRB was removed and cultures were quickly rinsed four times with 1% acetic acid to remove unbound dye.The acetic acid was poured directly into the culture wells from a beaker. Residual washing solution was removed by sharply flicking plates over a sink,which ensured the complete removal of rinsing solution. After being rinsed, the cultures were dried in air until no moisture was left. Bound dye was solubilized with unbuffered Tris base (pH 10.5) at different concentrations (0.01 µM to 100 µM) for 1 h on a gyratory shaker.

Then the absorbencies of above samples were performed at 490 nm using a microplate reader. The value of inhibition ratios was calculated as the percentage reduction in absorbance versus untreated control cultures. The IC_{50} values of the screened 1,2,4,5-tetrazine derivatives were calculated according to the inhibition ratios.

Results and Discussion

Synthesis

Aniline hydrochloride, cyclohexanamine hydrochloride or 1-cyclohexylethan-1-amine hydrochloride was reacted with urea using water as solvent to give compound **1** according to the literature method [18]. The mixture of compound **1** and 40% hydrazine hydrate was heated to obtain compound **2.** When preparing compound **3a-3g**, substituted benzaldehyde was reacted with compound **2** using methanol as solvent according to the literature method [19]. When preparing compound **4a-g** from **3a-g**, the literature method was modified with using Cl_2 as chloride agent instead of using $SOCl_2$ [20].

Comparsion experiment was carried out to investigate the effect of solvent polarity on the synthesis of target compounds (Table-1). It was found that intramolecular cyclisation reaction of N-((2,4-dichlorophenyl)carbamoyl)benzohydrazonoyl chloride and Et_3N happened using low polarity benzene or toluene as the solvent at the temperature of -30°C, which gave N-(2,4-dichlorophenyl)-5-phenyl-1,3,4-oxadiazol-2-amine. The mechanism of the intramolecular cyclisation reaction could be proposed as Fig. 2. To our surprise, intermolecular cyclization reaction occurred when using higher polarity THF as the solvent. The plausible mechanism of the reaction could be deduced as Fig. 3. N,N'-bis(2,4-dichlorophenyl)-3,6-diphenyl-1,2,4,5-tetrazine-1,4-dicarboxamide (**5a**) was synthesized by using THF as solvent at reacting temperature of -30°C. The preparation of target compounds (**5a-g)** including seven new 1,2,4,5-tetrazine derivatives is shown in Table-2.

Table-1: The Effect of solvent polarity on the synthesis of target compounds.

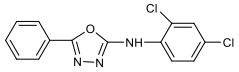
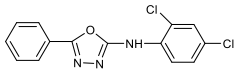
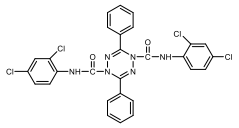
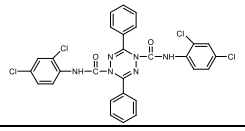
solvent	compound	Reaction Temperature /°C	Yield %	M. p. / °C	^{1}H-NMR
Benzene		**-30**	**88.1**	**122-124**	**8.21 (m, 1H), 8.10 (m, 2H), 7.34-7.88 (m, 5H)**
Toluene		**-30**	**78.3**	**122-124**	**8.22 (m,1H), 8.13 (m, 2H), 7.35-7.85 (m, 5H)**
THF		**-30**	**84.0**	**142-144**	**8.36 (m, 1H), 8.00 (m, 2H), 7.44-7.98 (m, 5H).**
Ethanol		**-30**	**78.2**	**141-143**	**8.32 (m, 1H), 7.93 (m, 2H),7.40-7.95(m, 5H)**

Table-2: Structure of compounds **5a-g.**

Entry	X	R	M. p. (°C)	Yield(%)
5a*	**H**		**142-144**	**84.0**
5b*	***p*-Cl**		**164-168**	**81.3**
5c*	***p*-CH$_3$**		**178-180**	**90.1**
5d*	**m-NO$_2$**		**189-191**	**68.8**
5e*	**2,4-diCl**		**151-153**	**86.1**
5f*	***p*-CH$_3$**		**170-172**	**57.2**
5g*	***p*-CH$_3$**		**112-114**	**77.5**

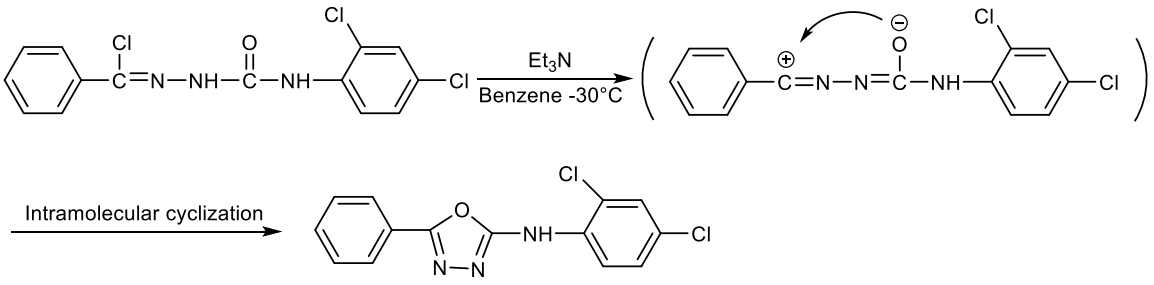


Fig. 2: Mechanism of intramolecular cyclization reaction.

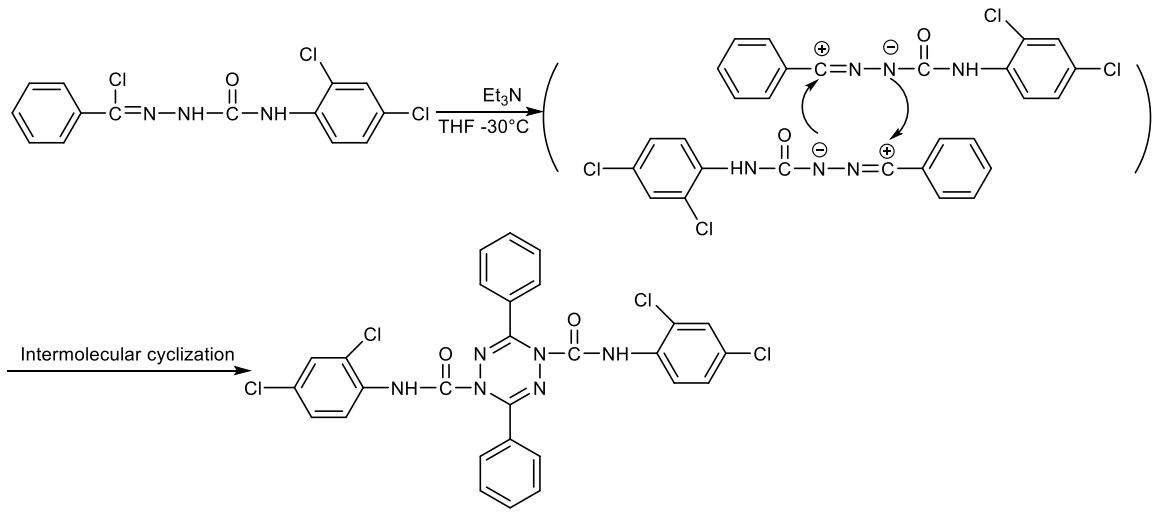


Fig. 3: Mechanism of intermolecular cyclisation reaction.

Biological Evaluation

The antitumor activity of the target compounds **4a-g** and **5a-g** were evaluated in vitro by method MTT for P-388 cell and SRB for A-549 cell and the results were summarized in Table-3. Usually, when the concentration of the compound solution is 10^{-5} mol/l or 10^{-6} mol/l, the growth inhibition rate of the solution to cancer cell is more than 85% or more than 50% respectively, the compound is considered to be strong effective. According to this standard, this series of s-tetrazine derivatives and their precursors show little antitumor activities against both P-388 cell and A-549 cell. The antitumor activities of compounds **4c**, **4f**, **5a**, **5b**, **5c**, **5f** and **5g** against P-388 cells at 10^{-4}mol/l were 54.8%, 58.1%, 81.7.%, 85.4%, 82.3%, 78.7% and 59.6% respectively. However, almost all of this series of s-tetrazine derivatives and its precursors exhibited appreciable inhibition rate against A-549 cells at 10^{-4}mol/l, with inhibition rates more than 50%. To our surprise, comparing the target compounds **5a-g**, when their substituted X was p-Cl or p-CH_3 and the concentration of the compound solution was 10^{-6}mol/l, it seemed that the groups (p-Cl, p-CH_3) could be favourable to the antitumor activity for P-388.

Furthermore, IC_{50} assays of compounds **5a**, **5b**, **5c**, **5d**, **5f** and **5g** along with the control drug 5-Fluorouracil and Aclarubicin were carried out to evaluate their anti-human lung cancer and anti-human lymphoblastic leukemia activity. Based on the value in Table-4, It was found that the order of the activity against P-388 cells was **5b** (X=*p*-Cl, R=2,4-dichlorophenyl) >**5c** (X=*p*-CH_3, R=2,4-dichlorophenyl) >**5a** (X=*H*, R=2,4-dichlorophenyl) >**5f** (X=*p*-CH_3, R=cyclohexyl). Compound **5b** exhibited the best activity with IC_{50} values of 4.45μM. Compounds **5a**, **5b**, **5c** and **5f** showed higher sensitivity against P-388 cells compared with A-549 cell. However, all these compounds presented lower activity compared with control drug Aclarubicin (0.31 μM) and 5-Fluorouracil (3.15 μM).

Table-3: Inhibition rate of in vitro tumor cell growth by compounds **4a-g** and **5a-g.**

Compd.	Rate of inhibition of P-388 C(tetrazine)/(mol/l)					Rate of inhibition of A-549 C(tetrazine)/(mol/l)				
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
4a	**10.3**	**8.4**	**5.7**	**4.7**	**4.7**	**86.3**	**12.8**	**9.3**	**0.6**	**0**
4b	**15.5**	**13.7**	**6.1**	**5.5**	**0**	**49.3**	**6.7**	**6.2**	**3.6**	**0**
4c	**54.8**	**20.1**	**10.3**	**6.7**	**6.4**	**76.6**	**0**	**0**	**0**	**0**
4d	**45.8**	**19.7**	**13.8**	**8.2**	**6.4**	**50.0**	**4.4**	**0**	**0**	**0**
4e	**14.0**	**7.5**	**4.9**	**4.2**	**0**	**80.1**	**19.0**	**3.4**	**2.4**	**0**
4f	**58.1**	**10.3**	**8.4**	**6.6**	**5.3**	**67.5**	**17.8**	**8.7**	**4.4**	**2.3**
4g	**44.7**	**32.2**	**8.7**	**7.5**	**6.8**	**72.5**	**16.3**	**14.9**	**6.6**	**2.8**
5a	**81.7**	**54.6**	**13.3**	**7.1**	**5.7**	**79.3**	**37.1**	**23.4**	**11.2**	**5.6**
5b	**85.4**	**58.1.**	**24.4**	**15.2**	**4.7**	**75.3**	**23.4**	**12.3**	**6.8**	**6.5**
5c	**82.3**	**46.4**	**43.3**	**16.3**	**6.4**	**63.6**	**34.8**	**12.1**	**4.5**	**2.4**
5d	**32.2**	**11.3**	**8.7**	**6.1**	**4.3**	**76.3**	**34.1**	**14.6**	**0**	**0**
5e	**16.5**	**7.0**	**0**	**0**	**0**	**50.2**	**7.5**	**0**	**0**	**0**
5f	**78.7**	**23.3**	**7.9**	**0**	**0**	**69.9**	**27.5**	**6.9**	**4.3**	**2.3**
5g	**59.6**	**13.9**	**6.7**	**8.5**	**5.3**	**62.9**	**12.1**	**9.1**	**0**	**0**

*Criteria of bioassay evulation: when the concentration of the compound solution is 10^{-6}mol/l, the inhibition ration of the solution to cancer cell growth is

more than 50%, the compound is considered to be effective.

Table-4: IC_{50} values of selected compounds against P-388 and A-549.

Compounds	IC_{50}± SD (μM)	
	P-388	A-549
5a	9.61±0.63	11.62±1.08
5b	4.45±0.34	42.43±3.21
5c	4.78±0.38	40.75±3.20
5d	>100	22.06±1.87
5f	26.91±0.88	49.38±5.21
5g	>100	45.81±3.43
Aclarubicin	0.31±0.05	0.27±0.03
5-Fluorouracil	22.6±1.27	3.15±0.22

Conclusion

In conclusion, we have developed a novel synthesis of 1,2,4,5-tetrazine derivatives bearing 1,4-dicarboxamide. Antitumor activities against A-549 and P-388 cell lines of target compounds and their precursors were evaluated in vitro and exhibited different cytotoxic activity, compound **5b** exhibited the best activity with IC_{50} values of 4.45 μM. Compounds **5a**, **5b**, **5c** and **5f** show higher sensitivity against P-388 cells compared with A-549 cell. Development of synthetic method and biological evaluations of 1,2,4,5-tetrazine derivatives possessing antitumor activity will be the continuing work within our group.

Acknowledgments

We are indebted to Higher Dducation Classroom Teaching Reform Project of Zhejiang Province (No.kg2015694), Education Science Planning Project in Zhejiang Province for financial support (No. 2017SCG031) ,Hangzhou agricultural scientific research project (20160432B25, 20180432B35) and National center for drug screening, shanghai, china for evaluation of antitumor activities.

References

1. N. Saracoglu, Recent advances and applications in 1,2,4,5-tetrazine chemistry，*Tetrahedron*, **63**, 4199 (2007).
2. M. J. Haddadin and E. H. Ghazvini-Zhadeh, A novel method for the synthesis of 3,5-disubstituted-(NH)-1,2,4-triazoles from 3,6-diaryl-1,2,4,5-tetrazines，*Tetrahedron Lett.*, **51**, 1654 (2010).
3. H. Neunhoeffer, Frankfurt, *Comprehensive Heterocyclic Chemistry*, A.R. Katritzky, ed., Vol.3, p. 531 (1984).
4. J. Sauer, Oxford, *Comprehensive Heterocyclic Chemistry*, A. J. Boulton, ed., Elsevier, Vol.6, pp.901-953 (1996).
5. H. I. Falfushynska; L. L. Gnatyshyna and O. B. Stoliar, Population-related molecular responses on the effect of pesticides in Carassius auratus gibelio, *Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol*., **155**, 396 (2012).
6. D. Nhu, S. Duffy, V. M. Avery, A. Hughes and J. B. Baell, Antimalarial 3-arylamino-6-benzylamino-1,2,4,5-tetrazines, *Bioorg. Med. Chem. Lett.*, **20**, 4496 (2010).
7. V. K. Pandey, 3,6-Dibenzoyl-1,4-bis(5-alkoxy-1,3,4-thiadiazolyl)-1,4-dihydro-1,2,4,5-tetrazines as potential antiviral agents, *Indian Drugs*, **23**, 500 (1986).
8. F. Xu, Z. Z. Yang, J. R. Jiang, W. G. Pan, X. L. Yang, J. Y. Wu, Y. Zhu, J. Wang, Q. Y. Shou and H. G. Wu, Synthesis, antitumor evaluation and molecular docking studies of 1,2,4-triazolo[4,3-b]-1,2,4,5tetrazine derivatives , *Bioorg. Med. Chem. Lett*., **26**, 3042 (2016).
9. S. Tabassum, M. Parveen, A. Ali, M. Alam, A. Ahmad, A. U. Khan and R. A. Khan, Synthesis of aryl-1,2,4,5-tetrazinane-3-thiones, in vitro DNA binding studies, nuclease activity and its antimicrobial activity, *J. Mol. Struct.*, **1020**, 33 (2012).
10. P. Sharma, A. Kumar, V. Sahu and J. Singh, Diels-Alder reaction strategy to synthesize 1,2,3,6-tetrahydro-1,2,4,5-tetrazines and exploration of their anti-inflammatory potential, *Arkivoc*, **12**, 218 (2008).
11. W. X. Hu, H. B. Shi, Q. Yuan and Y. Q. Sun, Synthesis and antitumor activity of N,N'-bis(substitutedphenyl)-3,6-dialkyl-1,4-dihydro-s-tetrazine-1,4-dicarboxamide, *J. Chem. Res*., **2**, 91 (2005).
12. W. X. Hu, G. W. Rao and Y. Q. Sun, Synthesis and antitumor activity of s-tetrazine derivatives, *Bioorg. Med. Chem. Lett*., **14**, 1177 (2004).
13. W. X. Hu, Y. Q. Sun and Q. Yuan, Catalytical rearrangement of 1,6-dihydro-S-tetrazine with isocyanate , *Chem. J. Chin. univ*., **23**, 1877 (2002).

14. Z. B. Cai, M. Zhou, W. X. Hu and Z. Y. Yang, Synthesis and antineoplastic activity of N,N'-disubstitutedphenyl-3,6-dimethyl-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboamide derivatives, *Acta pharmaceut. Sin.*, **35**, 793 (2000).
15. X. F. Shen, Z. Wu, S. F. Chen, Y. Chen, J. Xia, Y. P. Lv and Y. L. Zhou, Induction of G2/M phase arrest and apoptosis by ZGDHU-1 in A549 and RERF-LC-MA lung cancer cells, *Oncol. Lett.*, **12**, 989 (2016).
16. G. W. Rao, and W. X. Hu, Synthesis, structure analysis, and antitumor activity of 3,6-disubstituted-1,4-dihydro-1,2,4,5-tetrazine derivatives，*Bioorg. Med. Chem. Lett*., **16**, 3702 (2006).
17. G. W. Rao, C. Wang, J. Wang, Z. G. Zhao and W. X. Hu, Synthesis, structure analysis, antitumor evaluation and 3D-QSAR studies of 3,6-disubstituted-dihydro-1,2,4,5-tetrazine derivatives, *Bioorg. Med. Chem. Lett*., **23**, 6474 (2013).
18. L. P. Lv, X. F. Zhou, H. B. Shi, J. R. Gao, W. X. Hu, Novel synthesis, characterisation and antitumour activity of dimethyl-3,6-di(aryl) -1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboxylates, *J. Chem. Res*., **6**,368 (2014).
19. G. W. Rao, Y. M. Guo and W. X. Hu, Synthesis, structure analysis and antitumor evaluation of 3,6-dimethyl-1,2,4,5-tetrazine-1,4-dicarboxamide Derivatives, *Chem. Med. Chem.*, **7**, 973 (2012).
20. G. W. Rao and W. X. Hu, Synthesis, structure analysis and antitumor activity of 3,6-disubstituted-1,4-dihydro-1,2,4,5-tetrazine derivatives, *Bioorg. Med. Chem. Lett*., **16**, 3702 (2006).
21. M. R. Shah, M. Arfan, H. Amine, Z. Hussain, M. I. Qadir, M. I. Choudhary, D. Vanderveer, Mesaik, S. Samreen, A. Jabeen I. Khan, Synthesis of new bergenin derivatives as potent inhibitors of inflammatory mediators NO and TNF-α. *Bioorg. Med. Chem. Lett.* **22**, 2744 (2012).
22. L. P. Lv, W. P. Yu, W. B. Yu, X. F. Zhou and X. C.Hu, (E)-Methyl N '-[1-(4-methoxyphenyl)ethylidene]hydrazinecarboxylate, *Acta Crystallogr E*, **64**, o1676 (2008).
23. S. Ito, Y. Tanaka and A. Kakehi, Synthesis of 2,5-diaryltetrazoles from N-phenylsulfonylbenzhydrazidoyl chlorides and arylhydrazines, *Bull. Chem. Soc. Jpn.*, **49**, 762 (1976).