Novel synthesis, characterisation and antitumour activity of dimethyl-3,6-di(aryl)-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboxylates

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A series of new dimethyl-3,6-di(aryl)-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboxylates were synthesised through an intermolecular cyclisation reaction of methyl N′-(α-chloro-substituted benzylidene) hydrazinecarboxylate and triethylamine. Their 1,4-dihydro-s-tetrazine structures were characterised by IR, ^{1}H NMR, MS, elemental analysis. Their antitumour activities were evaluated *in vitro* by the SRB method for A-549 cells and the MTT method for P-388 cells. The results show that none of the compounds had a high activity, but the substituents clearly had an effect on their antitumour activity.

Keywords: synthesis, antitumour activity, 1,4-dihydro-s-tetrazine

1,2,4,5-Tetrazine derivatives are of considerable interest because of their unique role in the construction of various nitrogen heterocycles.[1,2]Compounds containing the 1,2,4,5-tetrazine skeleton have been used as pharmaceuticals.[3,4] For example, 3-amino-6-aryl-1,2,4,5-tetrazines showed modest antimalarial activity and some hexahydro-s-tetrazines have useful analgesic and anti-inflammatory activity. The antibacterial and antifungal activities of a series of tetrahydro-s-tetrazines have also been evaluated.

Previously we have shown that some s-tetrazine derivatives have good antitumour activity, especially 1,4-dihydro-s-tetrazine-1,4-dicarboxamide.[5–8] In a further effort to find potential antitumour agents, we have synthesised and evaluated the biological activity of a series of seven 1,2,4,5-tetrazines.[9,10] The chemical structures and synthetic route of the target compounds are shown in Fig. 1.

Results and discussion

In order to prepare **1a**–**g**, a substituted benzaldehyde was reacted with methyl hydrazinecarboxylate using methanol as solvent according to the literature method.[11] When preparing **2a**–**g** from **1a**–**g**, the literature method was modified by using Cl_2 as a chlorinating agent instead of using $SOCl_2$.[12] 2-Methoxy-5-phenyl-1,3,4-oxadiazole (**4**) was synthesised by the intramolecular cyclisation reaction of **2a** in benzene, a solvent of low polarity.[13]

The effect of solvent polarity on the synthesis of the target compounds was investigated (Table 1).It was found that 2-methoxy-5-phenyl-1,3,4-oxadiazole was obtained by an intramolecular cyclisation reaction of methyl N′-(α-chloro-benzylidene)hydrazinecarboxylate and Et_3N using low polarity benzene or toluene as the solvent in –10 °C. A mechanism for the reaction is proposed in Fig. 2. However, an intermolecular cyclisation reaction occurred when the solvent was modified to THF or ethanol which have a higher polarity. The plausible mechanism for this reaction is shown in Fig. 3. Dimethyl-3,6-diphenyl-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboxylates (**3a**) was prepared by using THF as solvent and a reaction temperature of –10 °C. The preparation of the target compounds (**3a**–**g**) including four new compounds is summarised in Table 2.

Compounds **3a**–**g** were evaluated for their antitumour activity *in vitro* by the MTT method for P-388 cells and the SRB methods for A-549 cells. The results are summarised in Table 3. When the concentration of the compound solution is 10^{-6} mol L^{-1}, and the inhibition ratio of the solution to cancer cell growth is more than 50%, the compound is usually considered to be effective. According to this criterion, it can be seen from Table 3 that no compound is effective against both P-388 and

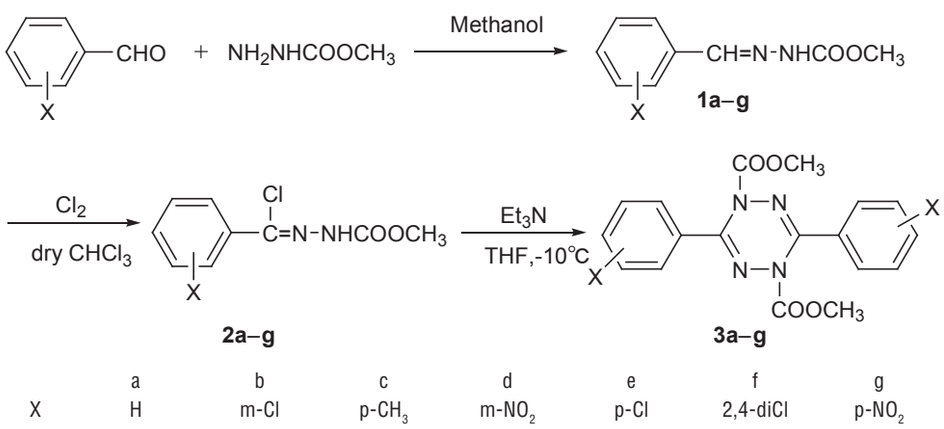

Fig. 1 The synthetic route of the target compounds and their chemical structures.

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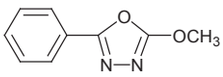
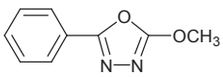
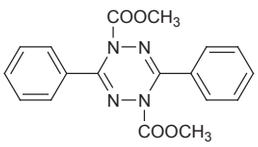
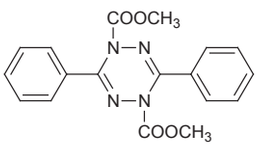
Table 1 The effect of solvent polarity on the synthesis of target compounds

Sovlent	Compound	Reaction temperature/°C	Yield/%	M.p./°C (lit.)	^{1}H NMR
Benzene		–10	95	49–51 (50–51)[14]	4.95 (s, 3H), 7.3–7.9 (m, 5H)[14]
Toluene		–10	73.3	50–52 (50–51)[14]	4.95 (s, 3H), 7.3–7.9 (m, 5H)[14]
THF		–10	90.4	90–92 (90–91)[15]	3.50 (s, 3H), 7.27–7.84 (m, 5H)[15]
Ethanol		–10	82.1	91–92 (90–91)[15]	3.58 (s, 3H), 7.24–7.88 (m, 5H)[15]

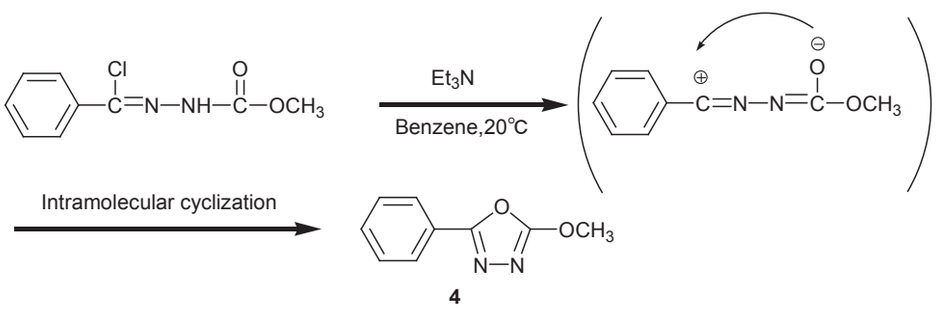


Fig. 2 Intramolecular cyclisation reaction.

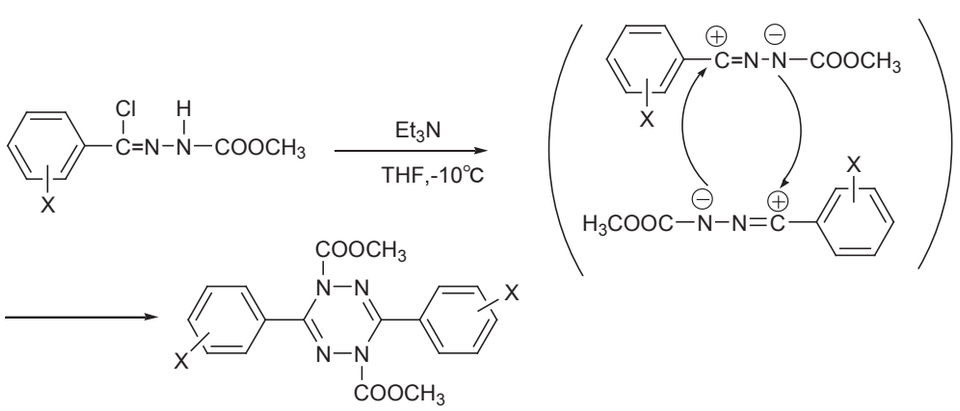


Fig. 3 Intermolecular cyclisation reaction.

Table 2 Synthesis of **3a**–**g**

Entry	X	M.p./°C	Yield/%
3a	H	90–92 (90–91)[15]	90.4
3b[a]	*m*-Cl	200–202	43.7
3c[a]	*p*-CH_3	94–96	75.5
3d[a]	*m*-NO_2	142–145	78.3
3e	*p*-Cl	113–115 (112–113)[15]	86.4
3f	2,4-diCl	114–115 (113–114)[15]	75.8
3g[a]	*p*-NO_2	181–183	75.4

[a]New coupounds.

Table 3 Inhibition of *in vitro* tumour cell growth by the compounds **3a**–**g**

Compd	Rate of inhibition of P-388 C (tetrazine)/mol L^{-1}					Rate of inhibition of A-549 C (tetrazine)/mol L^{-1}				
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
3a	0	15.4	11.9	13.4	12.2	56.2	2.8	6.6	9.8	19.6
3b	0	10.3	12.7	11.0	7.0	55.4	1.9	0.8	7.0	8.5
3c	31.3	17.3	12.6	8.4	9.0	46.6	0	0	0	0
3d	11.0	11.9	23.0	10.1	9.6	57.7	0	0	0	0
3e	17.7	1.1	22.9	13.6	10.4	60.0	2.4	0	0	0
3f	69.3	1.1	11.6	7.6	7.1	57.2	6.7	0.7	3.5	1.3
3g	4.6	0.2	4.7	5.5	6.2	52.9	4.1	4.7	4.8	7.6

Criteria of bioassay evaluation: when the concentration of the compound solution is 10^{-6} mol L^{-1}, the inhibition ration of the solution to cancer cell growth is more than 50%, the compound is considered as to be effective.

A-549 cells. However, in the series **3a**–**g**, when their substituent X is *m*-NO_2 or *p*-Cl and the concentration of the compound solution is 10^{-6} mol L^{-1}, it seems that the groups (*p*-Cl, *m*-NO_2) could produce activity against P-388.

Experimental

Compounds **1a**–**g** were synthesised by the literature method.[11]

The synthesis of compounds **2a**–**g** were carried out by modified literature method.[12]

Compound **4** was synthesised *via* intramolecular cyclisation reaction of **2a** by the literature method.[13] Solvents and reagents were commercially available without further purification. Melting points were determined on a XRC-1 apparatus and are uncorrected. IR spectra were recorded with a KBr discs on a Nicolet FI-IR-170 instrument. ^{1}H NMR spectra were run on a Bruker AC400 (400 MHZ). spectrometer using TMS as internal standard and $CDCl_3$ as the solvent. Mass spectra were obtained on a HP5989A spectrometer at an ionising voltage of 70ev by electron impact. Elemental analyses were performed on a Carlo ERBA-1106 instrument.

Dimethyl-3,6-diphenyl-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboxylate (**3a**): Triethylamine (2.04 g, 20.0 mmol) and dried THF (30 mL) were mixed and cooled to –10 °C. Compound **2a** (methyl N′-(α-chloro-benzylidene)hydrazinecarboxylate) (2.8 g, 13.2 mmol) in THF (25 mL) was added dropwise into the reaction vessel with stirring. After the addition was complete, the mixture was heated to 20–25 °C and kept stirring at this temperature for 3 h, using TLC (n-hexane : ethyl acetate = 7 : 3). After the reaction, the precipitate (triethylamine hydrochloride) was filtered. The filtrate was concentrated to remove the solvent. The residue was triturated with absolute ethanol (6 mL) to afford a white precipitate. The solid was filtered and recrystallised from absolute ethanol/water to obtain 2.1 g product, yield 90%. M.p. 90–92 °C. IR (KBr, cm^{-1}): 2974s (CH_3), 1772m (C=O), 1585m (C=N), 1356s (ring).^{1}H NMR ($CDCl_3$) ppm: 7.24–7.84 (m, 5H, ArH), 3.50 (s, 3H). MS (*m/z*, %).176 (M-176, 100), 149 (4.0), 104 (40.4), 77 (65.7), 63 (6.3), 51 (30.5), 43 (3.8).Anal. calcd for $C_{18}H_{14}Cl_2N_4O_4$ (MW = 352.36): C, 61.36; H, 4.58; N, 15.90; found: C, 61.30; H, 4.86; N, 16.24%.

Dimethyl-3,6-di(2-chlorophenyl)-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboxylates (**3b**): Following the method used for **3a** with triethylamine (4.46 g, 44.0 mmol) in 30 mL of THF and 7.0 g (28.3 mmol) of **2b** in 20 mL of THF and recrystallisation from absolute ethanol/water twice gave **3b** (2.6 g, 43.7%) as white crystals. M.p. 91–93 °C. IR (KBr, cm^{-1}): 2983s (CH_3), 1782m (C=O), 1634m (C=N), 1336s (ring), 1064 (Ar–Cl).^{1}H NMR ($CDCl_3$) δ (ppm) 7.27–7.81 (m, 4H, ArH), 3.55 (s, 3H). MS (*m/z*, %): 421 (M^+, 2.7), 210 (100), 166 (57.1), 139 (36.6), 111 (4.2), 75 (14.3), 63 (5.9), 50 (8.9), 43 (20.8). Anal. calcd for $C_{18}H_{14}Cl_2N_4O_4$ (MW=421.23): C, 51.32; H, 3.35; N, 13.30; found: C, 51.45; H, 3.50; N, 13.64%.

Dimethyl-3,6-ditolyl-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboxylate (**3c**): Following the method used for **3a**, with triethylamine (0.9 g, 8.5 mmol) in 50 mL of THF, and 2.8 g (13.2 mmol) of **2c** in 25 mL THF and recrystallisation from absolute ethanol/water twice gave **3c** (1.5 g, 75.5%) as white crystals. M.p. 94–96 °C. IR (KBr, cm^{-1}): 2980s (CH_3), 1767m (C=O), 1593s (C=N), 1386m (ring).^{1}H NMR ($CDCl_3$) δ (ppm) 7.27–7.84 (m,4H, ArH),4.23 (s,3H, CH_3), 2.41 (s,3H, CH_3).MS: *m/z* (%) 190 (M-190, 100),145 (3.6),120 (4.4),104 (2.2),77 (10.1),63 (3.8),51 (2.9). Anal. calcd for $C_{20}H_{18}N_4O_4$ (MW = 380.40): C, 63.15; H, 5.30; N, 14.73; found: C, 63.21; H, 5.29; N, 14.86%.

Dimethyl-3,6-di(m-nitro-phenyl)-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboxylate (**3d**): Following the method used for **3a**, with triethylamine (0.8 g, 7.9 mmol) in 50 mL of THF and 1.2 g (5.2 mmol) of **2d** in 25 mL of THF and recrystallisation from absolute ethanol/water gave **3d** (0.9 g, 78.3%) as white crystals. M.p. 142–144 °C. IR (KBr, cm^{-1}): 2975s (CH_3), 1796m (C=O), 1523m (C=N), 1352s (ring).^{1}H NMR ($CDCl_3$δppm): 7.69–8.7 (m,4H, ArH),4.19 (s,3H).MS: *m/z* (%) 221 (M-221, 100), 176 (3.6), 150 (18.4), 117 (11.2), 89 (2.0), 77 (5.3), 63 (2.0), 58 (18.1). Anal. calcd for $C_{18}H_{14}N_6O_8$ (MW = 442.34): C, 48.87; H, 3.19; N, 19.00; found: C, 48.98; H, 3.21; N, 19.17%.

Dimethyl-3,6-di(p-chloro-phenyl)-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboxylate (**3e**): Following the method used for **3a**, with triethylamine (2.0 g, 20.0 mmol) in 25 mL of THF, and 3.0 g (12.1 mmol) of **2e** in 75 mL THF and recrystallisation from absolute ethanol twice gave **3e** (2.2 g, 86.4%) as white crystals. M.p. 112–114 °C. IR (KBr, cm^{-1}): 2970 (CH_3), 1760 (C=O), 1610m (C=N), 1336s (ring). ^{1}H NMR ($CDCl_3$) δ (ppm) 7.45–7.89 (m,4H, ArH),4.25 (s,3H). MS (*m/z, %*): 210 M-210, 100), 168 (7.2), 140 (6.9), 112 (5.0), 102 (3.9), 75 (13.4), 59 (4.0). Anal. calcd for $C_{18}H_{14}Cl_2N_4O_4$ (MW = 421.23): C, 51.32; H, 3.35; N, 13.30; found: C, 51.37; H, 3.21; N, 13.23%.

Dimethyl-3,6-di(2,4-dichloro-phenyl)-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboxylate (**3f**): Following the method used for **3a**, with triethylamine (1.6 g, 17.2 mmol) in 25 mL of THF, and 3.0 g (10.7 mmol) of **2f** in 75 mL THF and recrystallisation from absolute ethanol twice gave **3f** (2.6 g, 75.8%) as white crystals. M.p. 114–115 °C. IR (KBr, cm^{-1}): 2978s (CH_3), 1774m (C=O), 1599m (C=N), 1370 (ring).^{1}H NMR ($CDCl_3$δppm): 7.38–7.86 (m, 3H, ArH),4.26 (s,3H). MS *m/z* (%): 244 (M-244, 100), 201 (10.9), 175 (33.4), 159 (11.1), 136 (12.3), 75 (10.5), 59 (23.3). Anal. calcd for $C_{18}H_{12}Cl_4N_4O_4$ (MW = 490.12): C, 44.11; H, 2.47; N, 11.43; found: C, 44.13; H, 2.46; N, 11.65%.

Dimethyl-3,6-di(4-nitro-phenyl)-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboxylate (**3g**): Following the method used for **3a**, with 0.8 g (7.9 mmol) of triethylamine in 20 mL of THF, and 0.3 g (1.2 mmol) of **2g** in 10 mL THF and recrystallisation from absolute ethanol/ethyl acetate twice gave **3g** (0.2 g, 75.4%) as yellow crystals. M.p. 181–183 °C. IR (KBr, cm^{-1}): 983s (CH_3), 1778 (C=O), 1613m (C=N), 1370s (ring).^{1}H NMR ($CDCl_3$δppm) 7.69–8.77 (m,4H, ArH), 4.29 (s,3H).MS *m/z* (%): 221 (M-221, 100), 191 (2.6), 177 (1.4), 151 (1.4), 120 (3.5), 92 (2.5), 75 (4.9), 59 (22.3), 50 (1.5). Anal. calcd for $C_{18}H_{14}N_6O_8$ (MW = 442.34): C, 48.87; H, 3.19; N, 19.00; found: C, 48.84; H, 3.09; N, 19.05%.

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