Synthesis and antitumor activity of *N,N′*-Bis(substitutedphenyl)-3, 6-dialkyl-1, 4-dihydro-*s*- tetrazine-1,4-dicarboxamide

Weixiao Hu*, Haibo Shi, Qing Yuan and Yaquan Sun

College of Pharmaceutical Sciences, Zhejiang University of Technology, Hangzhou, 310014, P. R. China

Eleven compounds of *N,N′*-Bis(substitutedphenyl)-3,6-dialkyl-1,4-dihydro-*s*-tetrazine-1, 4-dicarboxamide were synthesised via reaction of 3,6-dialkyl-1,6-dihydro-*s*-tetrazine and substitutedphenyl isocyanate with yield from 24% to 86%. The antitumor activity of these compounds against P-388 and A-549 *in vitro* was tested. The results show that nine compounds of them showed marked antitumor activity against P-388 and seven compounds possess high antitumor activity against A-549. The IC_{50} of compound **3b** for P-388, Bel-7402, MCF-7 and A-549 are 0.6μM, 0.6μM, 0.5 μM and 0.7μM, respectively. The substitute groups at 3, 6-position in tetrazine rings and in phenyl rings have clearly effected on their antitumor activity.

Keywords: *N*, *N′*- Bis(substitutedphenyl)-3,6-dialkyl-1,4-dihydro-*s*-tetrazine-1, 4-dicarboxamide, synthesis, antitumor activity, structure-activity relationship, IC_{50}

There are several reviews[1,2] indicating that the use of compounds containing the 1,2,4,5-tetrazine skeleton had been claimed for use as pharmaceuticals. Among them, the hexahydro -*s*- tetrazine (Fig. 1) was recommended as an antitumor agent.[3,4] Although there was not any date about antitumor activities to be reported, it is the first time to announce that this kind of compound may possess potential antitumor activity.

Recently our research team found that some *s*-tetrazine derivatives have good antitumor activities, especially 1,4-dihydro-*s*-tetrazine-1,4-dicarboxamides.[5–7] To further investigating how the substitutes located at both 3,6 positions of *s*-tetrazine ring and 3-position of the phenyl ring in the *N*, *N′*-substituted phenyl-3,6-dialkyl-1,4-dihydro-*s*-tetrazine-1,4-dicarboxamide influence their antitumor activities, 11 compounds were designed and synthesised. The chemical structures and synthetic route of the target compounds were shown as **Fig. 2**.

When preparing **2a–2c** from **1a–1c**, the Skorianetz method[8,9] was modified with using of cheaper Pd/C catalyst instead of PtO_2.[10] The **3a–3k** were prepared by the new reaction of **2a–2c** with substituted phenyl isocyanate under 4-dimethyl-aminopyridine (DMAP) as catalyst.

In addition to IR, NMR, MS, and EA, their structures were characterised by X-ray single diffraction. For example, compound **3f** structure was confirmed as Fig. 3.[11] The molecular structure shows that two alkyl groups located at 3,6-positions of *s*-tetrazine ring, and two carboxamide groups were at the 1,4-positions. Therefore, it proved that **3f** was 1,4-dihydro-*s*-tetrazine derivatives and that rearrangement

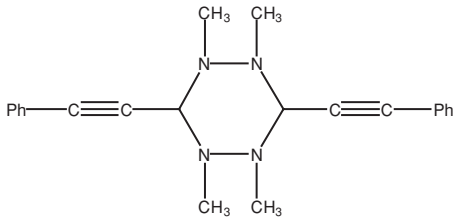

Fig. 1

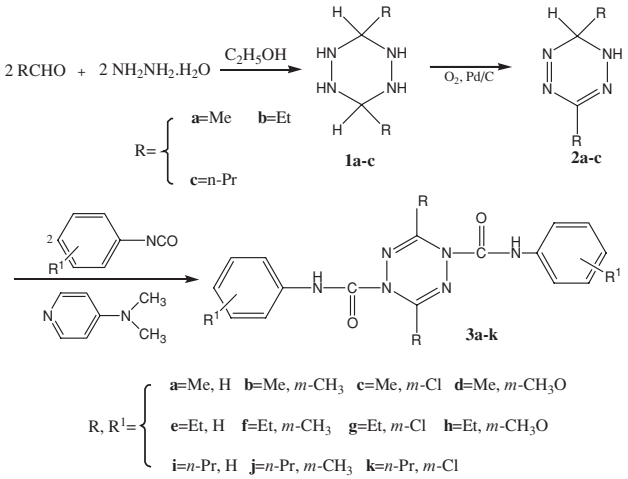

Fig. 2 The synthetic route of the target compounds and their chemical structures.

Table 1 Inhibition of in *vitro* tumor cell growth by tetrazine derivatives

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	79.8	55.0	71.9	0	11.3	80.6	77.8	58.4	23.219.9
3b	82.9	63.2	60.0	62.3	13.2	84.6	79.6	73.8	27.50.8
3c	61.6	16.1	5.4	0	7.4	92.2	42.6	10.9	6.2 19.9
3d	81.2	83.1	82.6	82.5	77.9	88.4	80.8	86.2	81.082.6
3e	91.2	64.1	61.4	1.2	16.4	86.1	80.5	56.6	16.70
3f	92.6	85.1	57.7	8.3	0	84.1	77.5	16.8	4.0 11.1
3g	88.6	70.5	59.7	8.3	0	92.5	78.7	79.4	64.221.6
3h	93.0	85.9	74.9	66.1	31.6	91.3	78.4	77.8	72.950.5
3i	94.3	75.9	66.1	8.2	5.2	72.3	71.5	72.8	16.622.7
3j	84.0	59.4	72.6	10.1	11.1	87.6	78.9	2.3	13.113.4
3k	94.1	65.6	26.4	0	16.5	89.8	78.1	4.0	0 5.9

* Correspondent. E-mail: Huyang@mail.hz.zj.cn

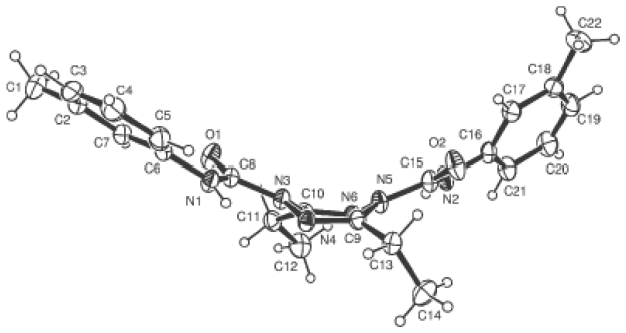

Fig. 3 X-ray structure of 3f.

(from 1,6-dihydro-*s*-tetrazine to 1,4-dihydro-*s*-tetrazine) occurs in the reaction.

Results and discussion

Compounds **3a–k** were evaluated for their antitumor activity *in vitro* by method MTT for P-388 cell and SRB for A-549 cell. The results were summarised in Table 1.

Usually, 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 as to be strongly effective.

According this standard, it can be found from Table 1 that nine compounds which have strong effective to P-388 cell, and seven compounds have strong effective to A-549 cell.

Comparing three series of compounds **3a–3d**, **3e–3h** and **3i–3k** with their antitumor acitivity, it can be found that when the same substituted groups located at 3,6-position of *s*-tetrazine, the substitutes in phenyl rings have much more effect on their activity. electron donor groups, such as $–OCH_3$, $–CH_3$ are of to benefit to the inhibition ration. Comparing three series of compounds **3a, 3e, 3i, 3b, 3f, 3j** and **3d, 3h**. It can be found that when the same carboxamide groups located at 1,4-position in tetrazine rings, the different substitutes (Me, Et, Pr) at 3,6-position have less effect on the activity.

For more accurately to examine antitumor activity, the **3b** was selected to test the IC_{50}. The IC_{50} of **3b** for P-388, Bel-7402, MCF-7 and A-549 are 0.6 μM, 0.6μM, 0.5 μM and 0.7μM, respectively. The results were summarised in Table 2. So the 1, 4-dihydro-*s*-tetrazine is a kind of compound which may have potenial antitumor actities. It is a good lead compound that warrants further investigation.

Experimental

Phenyl isocyanate, *m*-tolyl isocyanate, *m*-chloro phenyl and *m*-methoxy phenyl isocyanate were prepared from Triphosgene and corresponding anilines. Pd/C was obtained from Aldrich Co. Acetaldehyde was depolymerised from $(CH_3CHO)_3$ according to the published procedure. The synthesis of the 3,6-dialkyl-hexahydro-*s*-tetrazine (**1a–1c**) were carried out as described in the literature.[8,9] 3,6-dialkyl-1,6-dihydro-*s*-tetrazine (**2a–2c**) were synthesised according literature.[8] The others solvents and reagents were commercially available and were distilled prior to use.

Melting points were carried on XRC-1 apparatus and uncorrected. Infrared spectra were recorded from KBr discs on a NicolexFI-IR-170 instrument. ^{1}H NMR spectra were run on a Bruker AC 400 (400MHz) 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.

N,N'-Bis-phenyl-3,6-dimethyl-1,4-dihydro-s-tetrazine-1,4-dicarboxamide (**3a**): A solution of phenyl isocyanate 4.7g (0.04mol) in 30ml of $CHCl_3$ was placed in a 100ml flask equipped with a magnetic stirrer, pressure-equalising dropping fennel, and a thermometer. At 0 °C, the solution of **2a** 1.12g (0.01mol) and *N*, *N'*-dimethylaminepyridine (DMAP) 0.61g (0.005mol) in 30ml of $CHCl_3$ was added with stirring. After the addition is over, the reaction mixture was then allowed to warm to room temperature and subsequently was warmed to reflex for 45h. After removing the solvent, *n–H*exane was added to the residue, and then cooled to 0 °C for 24h, the resulting precipitate was filtered off and recrystallised from EtOH to give 2.0g (58.5%) of **3a** as a white crystalline solid. m.p. 183–184 °C; IR (KBr, cm^{-1}): 3285 s(N–H), 1710 s(–C=O), 1687 s(C=N), 1593 m(Ph), 1529 s(Ph), 1443 s(C–N), 1378 m(CH_3), 1289 m(C–N), 1233 s(-CH_3); ^{1}H NMR ($CDCl_3$): 8.46 (s , 2H,NH), 7.15–7.52 (m, 10H, Ph-H), 2.49(s, 6H, CH_3); Anal. Calcd for $C_{18}H_{18}N_6O_2$: C, 61.70; H, 5.18; N, 23.99. Found: C, 61.76; H, 5.03; N, 23.97.

N, N'-Bis(m-tolyl)-3,6-dimethyl-1,4-dihydro-s-tetrazine-1,4-dicarboxamide (**3b**): Following the method used for **3a,** with 5.2g (0.04mol) of *m*-tolyl isocyanate, 30ml of $CHCl_3$, 1.12g (0.01mol) of **2a**, and 0.61g (0.005mol) of *N,N'*-dimethylaminepyridine (DMAP) in 30ml of $CHCl_3$ to give 3.3g (86.2%) of **3b** as a white crystalline solid. m.p. 138–139 °C. IR (KBr, cm^{-1}): 3300 s(N–H), 1712 s(C=O), 1600 s(Ph), 1530 s(Ph), 1490 m(–CH_3), 1280 s(C–N); ^{1}H NMR ($CDCl_3$): 8.44 (s, -NH, 2H), 6.90–7.44 (m, Ph–H, 8H), 2.48 (s, –CH_3, 6H), 2.36 (s, m–CH_3, 6H); Anal. Calcd for $C_{20}H_{22}N_6O_2$: C, 63.48; H, 5.86; N, 22.21.Found: C, 63.72; H, 6.09; N, 22.57.

N,N'-Bis(m-chlorophenyl)-3,6-dimethyl-1,4-dihydro-s-tetrazine-1,4-dicarboxamide (**3c**): Following the method used for **3a,** with 6.2g (0.04mol) of *m*-chlorophenyl isocyanate, 30ml of $CHCl_3$, 1.12g (0.01mol) of **2a**, and 0.61g (0.005mol) of N,N'-dimethylaminepyridine (DMAP) in 30ml of $CHCl_3$ to give 3.1g (74.6%) of **3c** as a white crystalline solid. m.p. 210–211 °C. IR (KBr, cm^{-1}): 3345 s(N–H), 1718 s(C=O), 1598 s(Ph), 1520 s(Ph), 1450 m(–CH_3), 1284 s(C–N); ^{1}H NMR ($CDCl_3$): 8.49 (s, –NH, 2H), 7.10–7.65 (m, Ph–H, 8H), 2.49 (s, –CH_3, 6H); Anal. Calcd for $C_{18}H_{16}Cl_2N_6O_2$: C, 51.56; H, 3.85; N, 20.04. Found: C, 51.75; H, 4.08; N, 20.39.

N, N'-Bis(m-methoxyphenyl)-3,6-dimethyl-1,4-dihydro-s-tetrazine-1,4-dicarboxamide (**3d**): Following the method used for **3a,** with 6.0g (0.04mol) of *m*-methoxyphenyl isocyanate, 30ml of $CHCl_3$, 1.12g (0.01mol) of **2a**, and 0.61g (0.005mol) of *N*, *N'*-dimethylaminepyridine (DMAP) in 30ml of $CHCl_3$ to give 2.3g (56.1%) of **3c** as a white crystalline solid. m.p. 136–137 °C. IR (KBr, cm^{-1}): 3350 s(N–H), 1708 s(C=O), 1600 s(Ph), 1525 s(Ph), 1457 m(–OCH_3), 1300 m(C–N), 1247 s(C–N); ^{1}H NMR ($CDCl_3$): 9.06 (s, –NH, 2H), 6.95–8.17 (m, Ph-H, 8H), 3.92 (s, –OCH_3, 6H), 2.50 (s, –CH_3, 6H); Anal. Calcd for $C_{20}H_{22}N_6O_4$: C, 58.53; H,5.40; N, 20.48. Found: C, 58.55; H, 5.34; N, 20.65.

N, N'-Bis-phenyl-3,6-diethyl-1,4-dihydro-s-tetrazine-1,4-dicarboxamide (**3e**): Following the method used for **3a,** with 4.7g (0.04mol) of phenyl isocyanate, 30ml of $CHCl_3$, 1.4g (0.01mol) of **2b**, and 0.61g (0.005mol) of *N,N'*-dimethylaminepyridine (DMAP) in 30ml of $CHCl_3$ for 72h to give 2.0g (52.9%) of **3e** as a white crystalline solid. m.p. 131–133 °C. IR (KBr, cm^{-1}): 3340 s(N–H), 2963 s(CH_3), 2928 s(CH_2), 1698 s(C=O), 1593 s(Ph), 1505 s(Ph), 1443 m(–CH_3), 1317 s(C–N), 1222 m(C–N); ^{1}H NMR (DMSO): 9.25 (s, –NH, 2H), 7.10–7.60 (m, Ph–H, 10H), 2.89 (q, –CH_2–, 4H), 1.15 (t, CH_3, 6H); Anal. Calcd for $C_{20}H_{22}N_6O_2$: C, 63.48; H, 5.86; N, 22.21. Found: C, 63.40; H, 5.88; N, 22.38.

Table 2 Determine the IC_{50} of compound **3b**

Cancer cell	Rate of inhibition/%IC_{50}(μM)																
	Concentration (μM)																
	0	5.0	3.125	2.5	1.563	1.25	1.0	0.781	0.625	0.5	0.399		0.313	0.25	0.195	0.125	0.0625
P-388	-	-	97.3	-	91.1	-	-	83.1	-	-	55.4	-	-	2.3	-	-	0.6
Bel-7402	-	87.8	-	89.0	-	77.8	-	-	54.2	-	-	27.4	-	-	-	-	0.6
MCF-7	-	-	-	-	-	-	71.2	-	-	64.3	-	-	35.7	-	1.1	0	0.5
A-549	-	-	-	-	-	-	93.6	-	-	36.6	-	-	0	-	0	0	0.7

N,N′-Bis(m-toly)l-3,6-diethyl-1,4-dihydro-s-tetrazine-1,4-dicarboxamide **(3f)**: Following the method used for **3a,** with 5.2g (0.04mol) of *m*-tolyl isocyanate, 30ml of $CHCl_3$, 1.4g (0.01mol) of **2b**, and 1.2g (0.01mol) of *N*,*N*′-dimethylaminepyridine (DMAP) in 30ml of $CHCl_3$ for 48h to give 1.0g (24.6%) of **3f** as a white crystalline solid. m.p. 90–91 °C. IR (KBr, cm^{-1}): 3248 s(N–H), 2970 s(CH_3), 2918 s(CH_2), 1708 s(C=O), 1600 s(Ph), 1458 m(–CH_3), 1344 s(C–N), 1259 m(C–N); ^{1}H NMR (DMSO): 8.45 (s, –NH–, 2H), 6.95–7.35 (m, Ph-H, 8H), 2.97 (q, –CH_2–, 4H), 2.36 (s, m–CH_3, 6H), 1.24 (t, CH_3, 6H); Anal. Calcd for $C_{22}H_{26}N_6O_2$: C, 65.01; H, 6.45; N, 20.67. Found: C, 65.02; H, 6.46; N, 20.55.

N, N′-Bis(m-chlorophenyl)-3,6-diethyl-1,4-dihydro-s-tetrazine-1,4-dicarboxamide **(3g)**: Following the method used for **3a,** with 4.6g (0.03mol) of *m*-chlorophenyl isocyanate, 30ml of $CHCl_3$, 0.7g (0.005mol) of **2b**, and 0.61g (0.005mol) of *N*,*N*′-dimethylaminepyridine (DMAP) in 30ml of $CHCl_3$ for 60h to give 1.0g (44.7%) of **3g** as a white crystalline solid. m.p. 120–121 °C. IR (KBr, cm^{-1}): 3375 s(N–H), 2973 m(CH_3), 2941 m(CH_2), 1706 s(C=O), 1627 s(Ph), 1584 s(Ph), 1513 s(Ph), 1416 m(–CH_3), 1315 s(C–N), 1239 m(C–N); ^{1}H NMR (DMSO): 9.43 (s, –NH, 2H), 7.15–7.78 (m, Ph–H, 8H), 2.89 (q, –CH_2–,4H), 1.15 (t, m–CH_3,6H); Anal. Calcd for $C_{20}H_{20}Cl_2N_6O_2$: C, 53.70; H, 4.51; N, 18.79. Found: C, 53.56; H, 4.53; N, 18.80.

N, N′-Bis(m-methoxyphenyl)-3,6-diethyl-1,4-dihydro-s-tetrazine-1,4-dicarboxamide **(3h)**: Following the method used for **3a,** with 3.6g (0.024mol) of *m*-methoxyphenyl isocyanate, 30ml of $CHCl_3$, 0.56g (0.004mol) of **2b**, and 0.5g (0.004mol) of *N*,*N*′-dimethylaminepyridine (DMAP) in 30ml of $CHCl_3$ for 45h to give 0.8g (45.6%) of **3h** as a white crystalline solid. m.p. 104–106 °C. IR (KBr, cm^{-1}): 3364 s(N–H), 2973 m(CH_3), 2941 m(CH_2), 1708 s(C=O), 1590 s(Ph), 1517 s(Ph), 1456 m(–OCH_3), 1436 m(–CH_3), 1314 s(C–N), 1235 m(C–N); ^{1}H NMR (DMSO): 9.23 (s, –NH, 2H), 6.68–7.27 (m, Ph–H, 8H), 2.88 (q, –CH_2-,4H), 3.75 (s, –OCH_3, 6H), 1.15 (t, CH_3, 6H); Anal. Calcd for $C_{22}H_{26}N_6O_4$: C, 60.26; H, 5.98; N, 19.17. Found: C, 60.40; H, 5.84; N, 19.07.

N, N′-Bis-phenyl-3,6-dipropyl-1,4-dihydro-s-tetrazine-1,4-dicarboxamide **(3i)**: Following the method used for **3a,** with 4.7g (0.04mol) of phenyl isocyanate, 30ml of $CHCl_3$, 1.68g (0.01mol) of **2c**, and 1.2g (0.01mol) of *N*,*N*′-dimethylaminepyridine (DMAP) in 30ml of $CHCl_3$ for 62h to give 2.0g (47.7%) of **3i** as a white crystalline solid. m.p. 128–130 °C. IR(KBr, cm^{-1}): 3360 s(N–H), 2964 m(CH_3), 2936 s(CH_2), 1708 s(C=O), 1593 s(Ph), 1521 s(Ph), 1466 m(CH_2), 1305 s(C–N), 1218 m(C–N); ^{1}H NMR (DMSO): 8.45(s, -NH, 2H), 7.13–7.50(m, Ph-H, 10H), 2.94(t, -CH_2-, 4H), 1.67 (m, –CH_2–, 4H), 1.00 (t, CH_3, 6H); Anal. Calcd for $C_{22}H_{26}N_6O_2$: C, 65.01; H, 6.45; N, 20.67. Found: C, 65.03; H, 6.66; N, 20.75.

N,N′-Bis(m-tolyl)-3,6-dipropyl-1,4-dihydro-s-tetrazine-1,4-dicarboxamide **(3j)**: Following the method used for **3a,** with 5.2g (0.04mol) of *m*-tolyl isocyanate, 30ml of $CHCl_3$, 1.68g (0.01mol) of **2c**, and 0.7g (0.006mol) of *N*,*N*′-dimethylaminepyridine (DMAP) in 30ml of $CHCl_3$ for 45h to give 1.5g (34.5%) of **3j** as a white crystalline solid. m.p. 91–93 °C. IR (KBr, cm^{-1}): 3362 s(N–H), 2961 s(CH_3), 2934 s(CH_2), 1708 s(C=O), 1611 s(Ph), 1594 s(Ph), 152 s(Ph)7, 1455 s(CH_3), 1301 s (C–N), 1254 m(C–N); ^{1}H NMR (DMSO): 8.45 (s, –NH, 2H), 6.92–7.45 (m, Ph-H, 8H), 2.89 (t, –CH_2–, 4H), 2.30 (s, m–CH_3, 6H), 1.67 (m, –CH_2-, 4H), 0.92 (t, CH_3, 6H); Anal. Calcd for $C_{24}H_{30}N_6O_2$: C, 66.34; H, 6.96; N, 19.34. Found: C, 66.17; H, 7.04; N, 19.44.

N, N′-Bis(m-chlorophenyl)-3,6- dipropyl -1,4-dihydro-s-tetrazine-1,4-dicarboxamide **(3k)**: Following the method used for **3a,** with 6.1g (0.04mol) of *m*-chlorophenyl isocyanate, 30ml of $CHCl_3$, 1.68g (0.01mol) of **2c**, and 1.2g (0.01mol) of *N*,*N*′-dimethylaminepyridine (DMAP) in 30ml of $CHCl_3$ for 50h to give 1.3g (27.3%) of **3k** as a white crystalline solid. m.p. 120–121 °C. IR (KBr, cm^{-1}): 3362 s(N–H), 2961 s(CH_3), 2934 s(CH_2), 1708 s(C=O), 1611 s(Ph), 1594 s(Ph), 1527 s(Ph), 1454 s(CH_3), 1301 s(C–N), 1254 m(C–N); ^{1}H NMR (DMSO): 9.48 (s, –NH,2H), 7.14–7.79 (m, Ph–H, 8H), 2.89 (t, –CH_2–, 4H), 1.57 (m, –CH_2–, 4H), 0.90 (t, CH_3, 6H); Anal. Calcd for $C_{22}H_{24}Cl_2N_6O_2$: C, 55.59; H, 5.09; N, 17.68. Found: C, 55.47; H, 5.08; N, 17.63.

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