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Synthesis, Structure Analysis, and Antitumor Evaluation of 3,6-Dimethyl-1,2,4,5-tetrazine-1,4-dicarboxamide Derivatives

Guo-Wu Rao,* Yan-Mei Guo, and Wei-Xiao Hu[a]

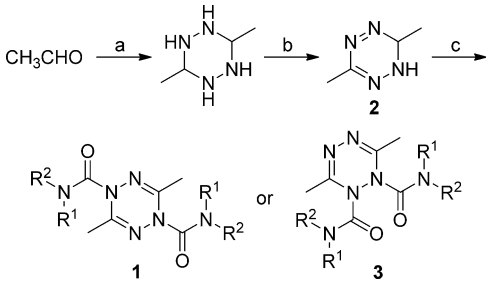
3,6-Dimethyl-1,2,4,5-tetrazine-1,4-dicarboxamide derivatives were synthesized, and their structures were confirmed by single-crystal X-ray diffraction. This reaction yields the 1,4-dicarboxamide derivatives rather than the 1,2-dicarboxamide derivatives. Their in vitro antitumor activities were evaluated against SGC-7901, HO-8910, MCF-7, and A-549 cells. The results showed several compounds to be endowed with cytotoxicity in the low micromolar range. One compound ($IC_{50} = 0.57$ μM) was further evaluated in vivo against an A-549 xenograft in BALB/cA nude mice; it effected 76.4% inhibition of tumor weight through intraperitoneal (i.p.) administration of 40 mg kg^{-1} body weight. Moreover, its acute toxicity was evaluated, and the i.p. LD_{50} value was 325 mg kg^{-1} in mice.

Tetrazine derivatives have good spectral and physical properties,[1] and hold high potential as antiviral and antitumor agents.[2,3] Dihydro-1,2,4,5-tetrazine has four isomers: 1,2-, 1,4-, 1,6-, and 3,6-dihydro-1,2,4,5-tetrazine. There seems to be considerable confusion over the structures of 1,2- and 1,4-dihydro-1,2,4,5-tetrazine isomers, and the same compound is often formulated as both structures. In most cases, the dihydro structure, which would be the initial reaction product, is presented, or the authors have formulated their compounds in the dihydro structure, which appeared to be the most accepted at that time. Some scientists believe that rearrangement can occur between the 1,2- and 1,4-dihydro-1,2,4,5-tetrazine isomers.[3]

3,6-Bis(phenylethynyl)-1,2,4,5-tetramethyl-1,2,4,5-tetrazine had been reported as an antitumor compound.[4] Although no data on antitumor activities were reported, it was the original expression that 1,2,4,5-tetrazine derivatives may possess antitumor activity. We attempted to investigate whether modifications to this original structure could enhance the antitumor activities of this compound class.

Thirteen 1,2,4,5-tetrazine-1,4-dicarboxamide derivatives were synthesized through a one-pot method by combining 3,6-dimethyl-1,6-dihydro-1,2,4,5-tetrazine (**2**) with bis(trichloromethyl) carbonate (BTC) and amine, with 4-dimethylaminopyridine (DMAP) as catalyst. The synthetic route is shown in Scheme 1. The intermediate raw material of compound **2** was prepared according to published methods.[5,6] All compounds were characterized by IR and NMR spectroscopy as well as mass spectrometry, and the results are summarized in Table 1. Their structures were further confirmed by single-crystal X-ray diffraction.

The single-crystal structure of compound **1 d** was determined by X-ray crystallography, and its structure is illustrated in Figure 1. The N2=C2 [1.271(2) Å] and N2^i=C2^i [1.271(2) Å] bonds correspond to typical C=N double-bond lengths, and



Scheme 1. Synthesis of compound series **1**: a) CH_3CHO, $H_2NNH_2{\cdot}H_2O$, EtOH, 5–10 °C, 12 h; b) NaOH, O_2, Pd/C, 10 °C, 35–37 h; c) 1. BTC, DMAP, $CHCl_3$, reflux, 3 h; 2. HNR^1R^2, reflux.

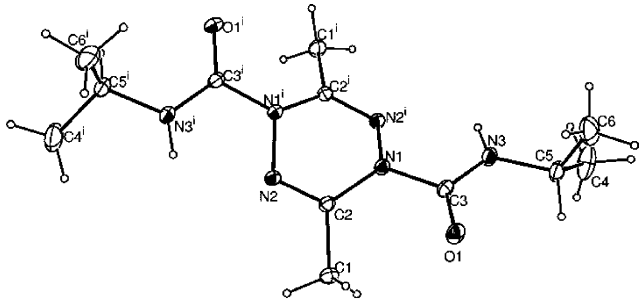


Figure 1. X-ray crystal structure of compound **1 d**.

the C2–N1^i [1.409(3) Å], N1^i–N2^i [1.417(2) Å], C2^i–N1 [1.409(3) Å], and N1–N2 [1.417(2) Å] bond lengths correspond to typical single bonds in compound **1 d**. Therefore, the tetrazine ring is the 1,4-dihydro structure with the N-substituted groups at the 1,4-positions and not the 1,2-positions; the compound is 3,6-dimethyl-N^1,N^4-diisopropyl-1,2,4,5-tetrazine-1,4-dicarboxamide (**1 d**), rather than 3,6-dimethyl-N^1,N^2-diisopropyl-1,2,4,5-tetrazine-1,2-dicarboxamide (**3 d**). Therefore the products of the reaction of compound **2**, BTC, and amine bear the 1,4-dicarboxamide structure rather than that of 1,2-dicarboxamide.

The in vitro antitumor activities of these compounds were evaluated against the growth of human gastric cancer SGC-

[a] Dr. G.-W. Rao, Y.-M. Guo, Prof. Dr. W.-X. Hu
College of Pharmaceutical Science
Zhejiang University of Technology, Hangzhou 310014 (P.R. China)
E-mail: rgw@zjut.edu.cn

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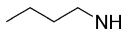
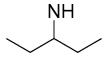
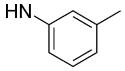
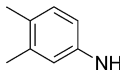
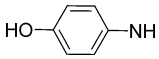
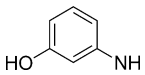
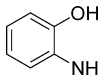
Table 1. Synthesis of compound **1**.

Compd	NR^1R^2	Time [h]	Yield [%]	mp [°C]	^{1}H NMR: δ [ppm][a]
1a		20	11.5	201–202	2.36 (s, 6H), 2.89 (d, J=5.0 Hz, 6H), 6.47 (s, 2H)
1b		15	2.8	154–155	1.20 (t, J=7.0 Hz, 6H), 2.36 (s, 6H), 3.29–3.35 (m, 4H), 6.48 (s, 2H)
1c		38	21.3	120–122	0.96 (t, J=7.5 Hz, 6H), 1.57-1.61 (m, 4H), 2.37 (s, 6H), 3.25 (q, J=7.5, 6.3 Hz, 4H), 6.52 (d, J=5.5 Hz, 2H)
1d		13	35.1	169–170	1.21 (d, J=6.5 Hz, 12H), 2.36 (s, 6H), 3.95–3.99 (m, 2H), 6.29 (d, J=8.0 Hz, 2H)
1e		40	46.1	112–113	0.95 (t, J=7.3 Hz, 6H), 1.36–1.40 (m, CH_2), 1.55 (t, J=7.3 Hz, 4H), 2.36 (s, 6H), 3.26–3.31 (m, 4H), 6.50 (s, 2H)
1f		18	16.8	134–135	0.95 (d, J=7.0 Hz, 12H), 1.79–1.87 (m, 2H), 2.37 (s, 6H), 3.12 (t, J=6.8 Hz, 4H), 6.59 (t, J=5.8 Hz, 2H)
1g		48	1.6	109–111	0.93 (t, J=7.5 Hz, 6H), 1.18 (d, J=5.5 Hz, 6H), 1.49–1.57 (m, 4H), 2.36 (s, 6H), 3.76–3.82 (m, 2H), 6.27 (d, J=8.5 Hz, 2H)
1h		53	7.4	134–135	0.92 (t, J=7.5 Hz, 12H), 1.42–1.49 (m, 4H), 1.55–1.64 (m, 4H), 2.36 (s, 6H), 3.63–3.68 (m, 2H), 6.23 (d, J=9.0 Hz, 2H)
1i		51	9.0	146–147[7]	2.35 (s, 6H), 2.47 (s, 6H), 6.92–7.35 (m, 8H), 8.41 (s, 2H)
1j		56	25.6	195–197	2.25 (s, 6H), 2.28 (s, 6H), 2.49 (s, 6H), 7.11 (d, J=8.1 Hz, 2H), 7.23 (dd, J=2.2, 5.9 Hz, 2H), 7.31 (d, J=1.9 Hz, 2H), 8.37 (s, 2H)
1k		38	26.7	256–257	2.38 (s, 6H), 6.69–6.73 (m, 4H), 7.32–7.35 (m, 4H), 9.06 (s, 2H), 9.26 (s, 2H)
1l		46	15.7	236–238	2.40 (s, 6H), 6.48–6.50 (m, 2H), 6.97–6.99 (m, 2H), 7.09 (t, J=8.0 Hz 2H), 7.17 (t, J=2.3 Hz, 2H), 9.14 (s, 2H), 9.40 (s, 2H)
1m		51	5.0	246–248	2.42 (s, 6H), 6.80–6.83 (m, 2H), 6.89–6.95 (m, 4H), 7.98 (dd, J=1.2, 6.8 Hz, 2H), 9.02 (s, 2H), 10.16 (s, 2H)

[a] 400 or 500 MHz, $CDCl_3$ or [D_6]DMSO.

7901, human ovarian cancer HO-8910, human breast cancer MCF-7, and human lung cancer A-549 cell lines by SRB and MTT assays. Cisplatin (DDP) was introduced as a positive control in the assay of MCF-7 and A-549 cells. The results are summarized in Table 2 and show that three compounds—**1e**, **1i**, and **1l**—are highly effective against cancer cell lines. IC_{50} values for compounds **1e** and **1l** against SGC-7901 cells are 1.34 and 3.81 μM, respectively. IC_{50} values for compound **1i** against the SGC-7901, HO-8910, MCF-7, and A-549 cell lines are 0.72, 0.90, 0.45, and 0.57 μM, respectively. Compound **1i** and **1l** display similar cytotoxic activities against the tested cancer cells.

Table 2. Anticancer activities against SGC-7901, HO-8910, MCF-7, and A-549 cell lines in vitro.

Compd	IC_{50} [μM][a]			
	SGC-7901	HO-8910	MCF-7	A-549
1a	NT	NT	>100	>100
1b	>100	>100	>100	>100
1c	>100	>100	>100	>100
1d	>100	>100	>100	>100
1e	1.34	>100	279.34	68.47
1f	>100	>100	>100	>100
1g	>100	>100	>100	>100
1h	>100	>100	>100	>100
1i	0.72	0.90	0.45	0.57
1j	NT	NT	>100	117.21
1k	>100	>100	NT	NT
1l	3.81	6.56	7.96	5.55
1m	NT	NT	>100	57.28
DDP	NT	NT	27.15	17.74

[a] NT: not tested.

Compound **1i** was profiled for its acute toxicity to Kunming male and female mice by intraperitoneal (i.p.) administration, and the results are listed in Table 3. All mice given a single i.p. injection of compound **1i** at a dose of 262.0 mg kg^{-1} body weight showed no clinical signs or loss of body weight, and the mortality rate was zero. At 295.0 mg kg^{-1}, the mortality rate was 40% (4/10) and various clinical signs such as loosening of hair, inanimation, and loss of locomotor activity were observed post-injection and disappeared slowly on live mice.

Table 3. Acute toxicity of compound **1i** on Kunming mice.[a]

Dose [mg kg^{-1}]	Males[b]	Females[b]	Clinical signs	Mortality	LD_{50} [mg kg^{-1}]
262.0	5	5	Normal	0	325
295.0	5	5	[c]	4	
328.0	5	5	[c]	8	
410.0	5	5	[c]	8	
512.0	5	5	[c]	9	

[a] 14 days post-single-i.p. administration. [b] Number of mice tested in the group. [c] Loosening of hair; inanimation; loss of locomotor activity; atrophy of spleen.

Dead mice had obvious atrophy of the spleen, and living mice had no clear lesions of internal organs. At 328.0 mg kg^{-1}, the same clinical signs were observed, and eight of the treated mice died within nine days post-injection. At the high dose of 512.0 mg kg^{-1}, identical clinical signs were observed, and nine of the treated mice were dead within seven days post-injection. From the mortality data of all tested animals, the i.p. LD_{50} value (325 mg kg^{-1}) for compound **1 i** was calculated according to the method of Austen and Brocklehurst.[8]

Based on these antitumor data in vitro and acute toxicity data, we further evaluated the in vivo antitumor activity of compound **1 i** against A-549 xenografts in BALB/cA nude mice in comparison with docetaxel as positive control. Compound **1 i** was administered at three doses (10, 20, and 40 mg kg^{-1} i.p.) and docetaxel at 12 mg kg^{-1} i.p.; the results are documented in Figures 2–4 and Table 4. Figure 2 shows the effects of compound **1 i** on tumor growth in nude mice presented as tumor volume (V_{tum}) over time. A statistically significant dose-dependent decrease in V_{tum} was observed with increasing doses of compound **1 i**. With respect to tumor weight, the results presented in Table 4 show that compound **1 i**, administered at 40 mg kg^{-1} i.p., exhibited excellent efficacy against A-549 xenografts in BALB/cA nude mice by 76.4%, slightly lower than docetaxel (87.4%) administered at 12 mg kg^{-1}. Regarding body weight and toxicity, there was no statistical difference between average total body weights in mice treated with compound **1 i** and control, whereas significant body weight loss was continuously observed beginning at day 4 after treating with docetaxel (Figure 3). The results are presented as the picture of the curative effect of compound **1 i** against A-549 xenografts in BALB/cA nude mice (Figure 4). The present study indicated that compound **1 i** could be effective in the treatment of human lung cancer.

In conclusion, compound **1 i** has relatively low toxicity and good efficacy in vivo. All these results revealed that 3,6-dimethyl-1,2,4,5-tetrazine-1,4-dicarboxamide derivatives may have potential antitumor activities.

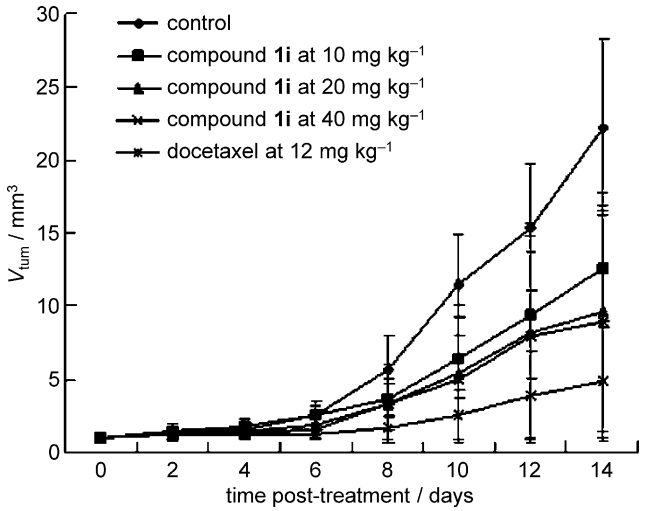


Figure 2. Effects of compound **1 i** on tumor growth in BALB/cA nude mice. The results are presented as tumor volume (V_{tum}) over time.

Table 4. Antitumor efficacy of compound **1 i** against A-549 xenografts in nude mice.[a]

Compd	Dose [mg kg^{-1}]	No. mice[b]	Tumor weight [g]	Tumor weight inhibition [%][c]
Control	–	12	1.82	–
1 i	10	6	0.92	49.5[d]
1 i	20	6	0.53	70.9[d]
1 i	40	6	0.43	76.4[d]
Docetaxel	12	6	0.23	87.4[d]

[a] During 14 days post-xenograft; compounds administered i.p. [b] All females. [c] Percentage of tumor-weight inhibition versus control. [d] $p < 0.01$ versus vehicle-treated control group.

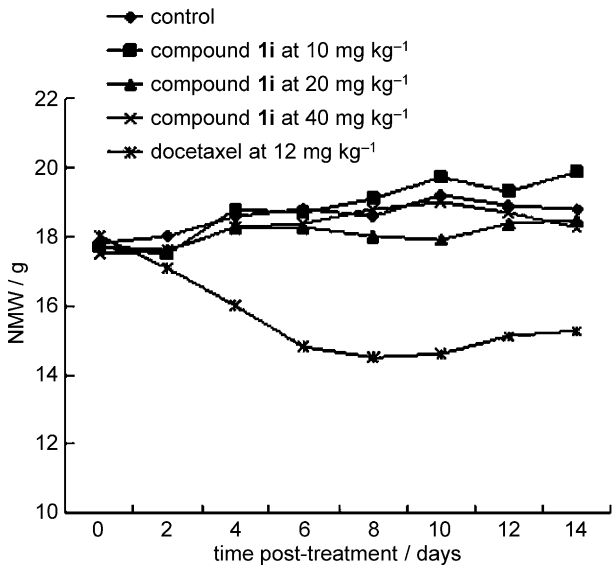


Figure 3. Effects of compound **1 i** on tumor growth in BALB/cA nude mice. The results are presented as nude mice weight (NMW) over time.

Experimental Section

Synthesis of compound 2: Fresh CH_3CHO (51 mL) was placed in a 250 mL flask equipped with a mechanical stirrer. At −2 °C, 80% $H_2NNH_2{\cdot}H_2O$ (55 mL) and EtOH (14 mL) were then added dropwise with stirring. The reaction mixture was then maintained at 5–10 °C for 12 h. The mixture was then filtered off with suction, and the white product of 3,6-dimethyl-hexahydro-1,2,4,5-tetrazine was obtained. 3,6-Dimethylhexahydro-1,2,4,5-tetrazine was placed in a 500 mL four-inner flask equipped with a mechanical stirrer. A solution of NaOH (374 mL, 3.3%) and 5% Pd/C (1.80 g) was added with stirring, and O_2 gas was passed through the mixture at 10 °C for 35–37 h. The reaction mixture was filtered off with suction, saturated with NH_4Cl, extracted with CH_2Cl_2, and dried with Na_2SO_4. After removal of the drying agent, the yellow solution was concentrated carefully at atmospheric pressure to dryness to afford compound **2** as a bright-yellow solid (17.39 g, 34.2%, according to CH_3CHO); mp: 107–108 °C (Ref. [5]: 107–108 °C).

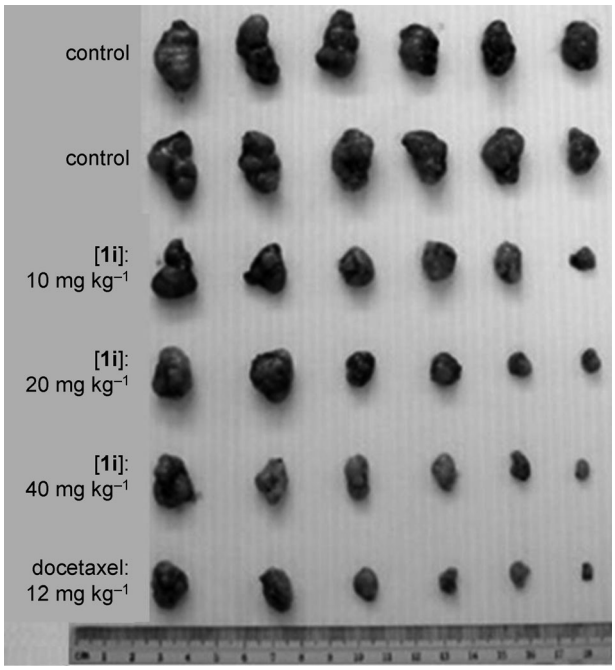


Figure 4. The curative effect of compound **1 i** against A-549 lung xenografts in BALB/cA nude mice. Shown are the tumors from six mice per dosing of compound **1 i** or docetaxel at day 14 after treatment; the control set comprised 12 mice. Animal studies for evaluation of in vivo antitumor activity were carried out according to guidelines of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

Synthesis of compound 1 d: BTC (3.96 g, 13.3 mmol) was dissolved in $CHCl_3$ (10 mL) with magnetic stirring, and a solution of compound **2** (1.12 g, 10.0 mmol) and DMAP (0.50 g, 4.1 mmol) in $CHCl_3$ (20 mL) was added dropwise with stirring at −12 °C. The mixture was then stirred at reflux for 3 h. After the blowing of N_2 gas, a solution of *i*PrNH$_2$ (2.41 g, 40.8 mmol) and $CHCl_3$ (10 mL) was added dropwise with stirring. The mixture was stirred at reflux for 13 h. The organic phase was washed with H_2O, dried ($MgSO_4$), filtered, and concentrated in vacuo. The resulting residue was a red solid of the crude product (1.39 g, 49.3%). The residue was recrystallized from EtOH to give compound **1 d** as a white solid (0.99 g, 35.1%). R_f = 0.88 (petroleum ether/EtOAc, 1:1); mp: 169–170 °C; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 150.9, 148.8, 42.1, 22.9, 18.2 ppm; IR (KBr): $\tilde{\nu}$ = 3317, 1685, 1634, 1428, 1384, 1132, 1077, 974 cm^{-1}; HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{12}H_{23}N_6O_2$: 283.1882, found: 283.1883, $[M+Na]^+$, calcd for $C_{12}H_{22}N_6NaO_2$: 305.1702, found: 305.1703; HPLC: t_R = 6.52 min (>99%). Compounds of **1 a**–**n** were synthesized in the same manner.

Crystal data for compound 1 d: A colorless block of dimensions 0.35×0.33×0.28 mm^3 was used for data collection with a Bruker SMART CCD area-detector diffractometer with graphite monochromated MoK_α radiation (λ = 0.71073 Å). The structure was solved by direct method procedures as implemented in the SHELXS97[9] program. The positions of all non-hydrogen atoms were included in the full-matrix least-squares refinement using the SHELXL97[9] program. Hydrogen atoms were added at calculated positions and refined using a riding model; they were given isotropic displacement parameters equal to 1.2 (or 1.5 for methyl H atoms) times the equivalent isotropic displacement parameters of their parent atoms, and C–H distances were restrained to 0.96 Å for methyl H atoms and 0.98 Å for methine H atoms, while N–H distances were set at 0.86 Å. $C_{12}H_{22}N_6O_2$, M_r = 282.36 Da, tetragonal, a = 9.759(3), b = 9.759(3), c = 16.604(9) Å, U = 1581.4(10) Å^3, T = 298(2) K, space group: P 43 21 2, Z = 4, ρ_{calcd} = 1.186 g cm^{-3}, μ(MoK_α) = 0.085 mm^{-1}, 12003 reflections measured, 1476 unique (R_{int} = 0.0209) which were used in all calculations. Fine R_1 = 0.0477, *wR* (F^2) = 0.1535 (all data). All crystallographic details for compound **1 d** have been deposited with the Cambridge Crystallographic Data Centre: CCDC-855337 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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Keywords: antitumor agents · cytotoxicity · tetrazines · X-ray diffraction

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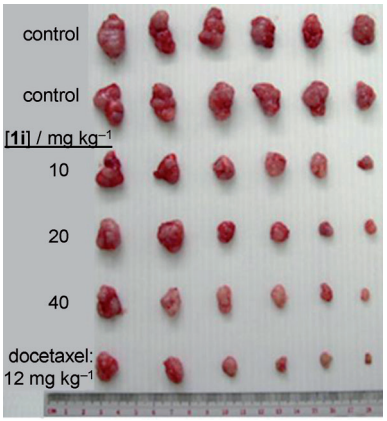
COMMUNICATIONS

Tumor downsizing: A series of tetrazine derivatives were synthesized and characterized. Their antitumor activity in vitro showed several compounds to be endowed with cytotoxicity in the low micromolar range. The activity of one compound in vivo and its acute toxicity were further evaluated. The results revealed that it has relatively low toxicity and good efficacy.



G.-W. Rao, Y.-M. Guo, W.-X. Hu*

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Synthesis, Structure Analysis, and Antitumor Evaluation of 3,6-Dimethyl-1,2,4,5-tetrazine-1,4-dicarboxamide Derivatives

