Synthesis of esters and amides of 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxylic acid

Shi-Jie Zhang[a], Yi-Chun Ma[b], Wei Zhou[a], Guo-Wu Rao[a], Liang-Liang Chen[a] and Wei-Xiao Hu[a]*

[a]*College of Pharmaceutical Science, Zhejiang University of Technology, Hangzhou, 310032, P. R. China*

[b]*College of Modern Science and Technology, China Jiliang University, Hangzhou, 310018, P. R. China*

Alkyl esters and alkyl amides of 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxylic acid were synthesised and characterised by IR, ^{1}H NMR, EI-MS and elemental analysis. The structure of 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxylic was confirmed by X-ray diffraction analysis. Some selected compounds were evaluated against P-388 and A-549 cancer cell lines, but showed poor inhibitory activities.

Keywords: tetrazine, crystal structure, transesterification, amidation, anticancer

1,2,4,5-Tetrazines have been widely used in organic synthetic chemistry and medicinal chemistry.[1] Since Eremeev first reported that 1,2,4,5-tetramethyl-3,6-bis(phenylethynyl)-1,2,4,5-tetrazine possessed potential anticancer activities,[2] 1,2,4,5-tetrazine derivatives have been found to have a wide range of herbicidal, insecticidal, antiviral and anticancer properties.[3,4,5] Our group have synthesised 3,6-aryl substituted 1,2,4,5-tetrazine derivatives and they are generally effective against P-388 and A-549 cancer cell lines.[5-7] However, there are no reports of the anticancer activities of the 3,6-dicarboxylates of 1,4-dihydro-1,2,4,5-tetrazine nor of the synthesis of 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxamide. We now describe the synthesis of some esters and amides of 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxylic acid and the evaluation of their anticancer activities.

Results and discussion

Boger's method for the synthesis of unbranched dialkyl esters of dihydro-1,2,4,5-tetrazine-3,6-dicarboxylic acid (Scheme 1) was examined.[8] Dihydro-1,2,4,5-tetrazine-3,6-dicarbonyl dichloride, prepared from dihydro-1,2,4,5-tetrazine-3,6-dicarboxylic acid and thionyl chloride, failed to react with alcohols and underwent ring-opening of tetrazine. Hence, Boger chose to mix the alcohol and thionyl chloride at a low temperature before adding dihydro-1,2,4,5-tetrazine-3,6-dicarboxylic acid. These dihydro-tetrazines **1a–d** were easily oxidised by nitrous gases, but they were neither stable to silica gel nor when warmed. When bright red dimethyl 1,2,4,5-tetrazine-3,6-dicarboxylate **6a** (*m/z* = 198) was recrystallised by heating in ethanol, the solution turned yellow, and an unidentified product precipitated without a trace of a peak of *m/z* = 198 in the mass spectrum.

It was not clear whether these dihydrotetrazines possessed a 1,2-dihydro- or 1,4-dihydro-tetrazine structure. Single crystals were grown of compound **1b** and the molecular X-ray structure is shown in Fig. 1.[9] In molecule **1b**, the N(2)–C(3) [1.272 (3) Å] and N(5)–C(6) [1.277 (3) Å] bond lengths correspond to typical double bonds, and the N(1)–N(2) [1.408 (3) Å], C(3)–N(4) [1.394 (3) Å], N(4)–N(5) [1.416 (3) Å] and C(6)–N(1) [1.392 (3) Å] are typical for single bonds. Therefore, the tetrazine ring has a 1,4-dihydro structure rather than a 1,2-dihydro structure. Moreover, it was found that atoms N(2), C(3), N(5), C(6) were coplanar, deviation within 0.0195 (12) Å, whilst atoms N(1) and N(4) deviated from the plane by 0.443 (4) Å and 0.462 (4) Å, respectively, indicating a boat conformation for the central six-membered tetrazine ring.

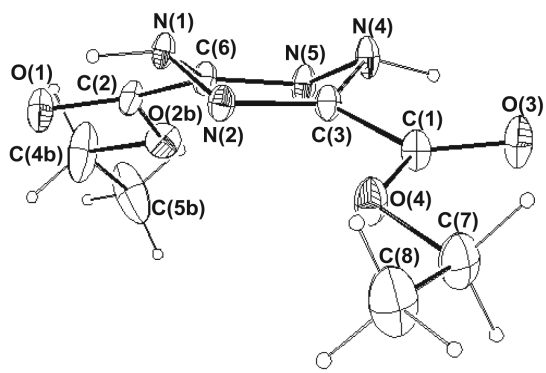
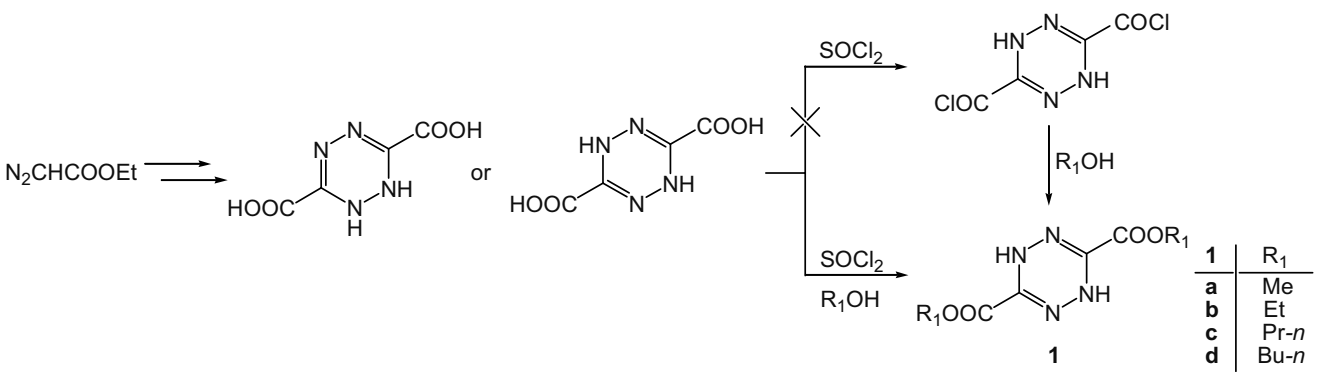
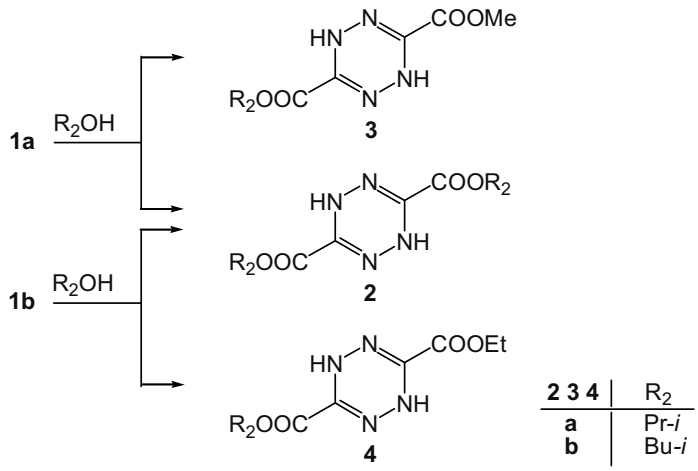


Fig. 1 The crystal structure of **1b** with 10% ellipsoid probability and one component of disordered ethoxy group are shown for clarity.



Scheme 1 Synthesis of **1**

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



Scheme 2 Synthesis of esters of tetrazine by transesterification.

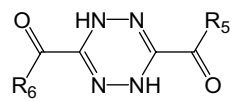
When using isopropanol and isobutanol as alcohols, the corresponding esters could not be prepared by Boger's method. Heating **1a** or **1b** under reflux with the corresponding alcohols, the transesterification proceeded to afford the expected products including partially asymmetrically substituted 3,6-dicarboxylates (Scheme 2). Asymmetric tetrazines **3b** and **4b** were difficult to isolate in very low yields. Recently, Almonasy's group has established the transesterification of dimethyl 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxylate in the presence of aluminium triethoxide, but their products limited to unbranched symmetric dialkyl esters of 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxylic acid.[10]

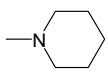
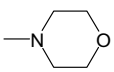
Amidation was also developed to obtain the 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxamide of alkyl amines with good to excellent yields by heating in ethanol solution. However, the reaction proceeded with difficulty with tert-butylamine or aniline, probably due to steric hindrance or the low nucleophilicity of the amines. It was impossible to obtain the amides through the reaction of 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarbonyl dichloride with the corresponding amines. This was explained by the ring cleavage of the tetrazine.

All esters and amides of 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxylic acid synthesised were listed in Table 1.

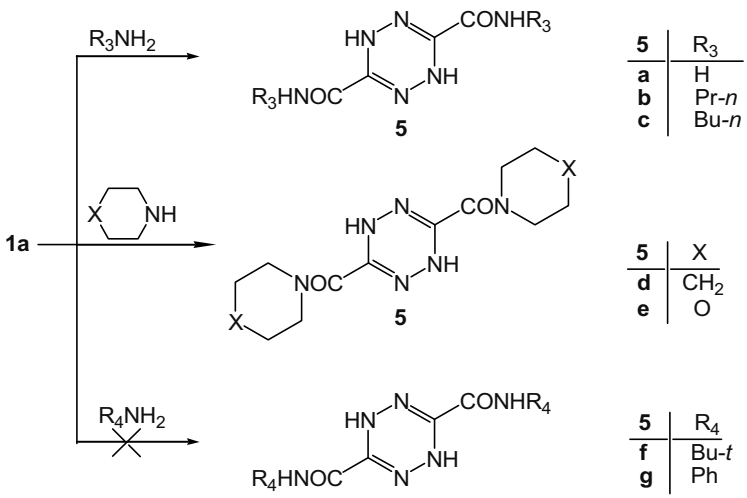
The *in vitro* anticancer activities for some selected compounds were evaluated by method MTT for P-388 cell lines and SRB for A-549 (Table 2). To our disappointment, these compounds do not possess as good anticancer activities

Table 1 The preparation of esters and amides



Entry	R_5	R_6	M.p./°C (lit.)	Yield/%
1a	OMe		171–172 (171–172)[8]	63
1b	OEt		101–102 (101–102)[9]	31
1c	OPr-*n*		78–79 (78–81)[10]	27
1d	OBu-*n*		53–54 (56–61)[10]	26
2a	OPr-*i*		56–57	31
2b	OBu-*i*		94–96	21
3a	OMe	OPr–*i*	83–84	44
3b[a]	OMe	OPr–*i*	–	–
4a	OEt	OPr–*i*	80–82	35
4b[a]	OEt	OPr–*i*	–	–
5a	NH_2		> 280	97
5b	NHPr-*n*		235–238	79
5c	NHBu-*n*		230–233	89
5d			262–265	98
5e			221–223	98
5f[b]	Bu-*t*		–	–
5g[b]	Ph		–	–

[a]Not isolated.
[b]No reaction.



Scheme 3 Synthesis of amides of tetrazine by amidation.

Table 2 The inhibition for P-388 and A-549 growth

Entry	Inhibition of P-388/% concentration (mol L^{-1})					Inhibition of A-549/% concentration (mol L^{-1})				
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
1b	0	0	0	0	0	31.1	2.4	0	9.9	23.9
1c	7.1	0	0	0	0	30.8	1.0	3.4	1.5	0
1d	23.4	0	16.1	0	5.9	36.2	0	0	1.9	0
2b	25.9	1.4	7.3	0	1.2	32.4	7.1	12.1	4.2	4.9
5a	0	1.4	6.3	0	13.8	26.1	2.2	1.2	0	0
5d	28.9	4.8	0	15.0	0	29.5	6.8	8.6	0	10.1
5e	1.2	2.9	7.7	10.7	0	25.7	2.7	0	8.8	19.5

as the 3,6-aryl substituted 1,2,4,5-tetrazine derivatives which we had previously tested before.[5-7]

Conclusion

Unbranched dialkyl dihydro-1,2,4,5-tetrazine-3,6-dicarboxylates were synthesised by Boger's method. A crystal structure of **1b** showed that the central six-membered tetrazine ring has a 1,4-dihydro structure with a boat conformation. Branched dialkyl esters of 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxylic acid were synthesised by transesterification with the corresponding alcohols and this was accompanied 3,6-asymmetric substituted esters. Amidation was developed to obtain dialkyl amides of 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxylic acid with good to excellent yields. Some selected compounds were evaluated *in vitro* against P-388 and A-549 cancer cell lines, but showed poor inhibitory activities.

Experimental

Melting points were taken on XRC-1 apparatus and are uncorrected. IR spectra were obtained on a PK-6000 spectrophotometer or a Thermo Nicolet Avatar 370 FT-IR spectrophotometer. ^{1}H NMR spectra were recorded on a Bruker AC 400 spectrometer operating at 400 MHz or a Bruker AVANCE III spectrometer at 500 MHz using TMS as the internal standard. MS spectra were run on an HP5989B instrument or a Waters GCT Premier with EI source. Elemental analyses of C, H, N were performed on a Thermo Finnigan Flash EA 1112 instrument. All the chemicals and solvents were analytical reagent grade and were used as received.

General procedure for the synthesis of **1a–d** *according to Boger's method*[8]

To the corresponding alcohol (25 mL) with thionyl chloride (3.8 mL) at –20 °C was added 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxylic acid (3.4 g, 20 mmol) suspended in the alcohol (25 mL) in portions. Other procedures were identical to Boger's method.

Dimethyl 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxylate (**1a**): 2.5 g, 63%; m.p. 171–172 °C, (lit.[8] 171–172 °C). IR ν_{max}(KBr)/cm^{-1}: 3361 (NH), 2961 (CH), 1723 (C=O). ^{1}H NMR ($CDCl_3$, TMS, 400 MHz)/δppm: 7.49 (s, 2H), 3.92 (s, 6H). EI-MS (*m/z*): 200 (M^+).

Diethyl 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxylate (**1b**): 1.4 g, 31%, m.p. 101–102 °C, (lit.[9] 101–102 °C). IR ν_{max}(KBr)/cm^{-1}: 3378 (NH), 2983 (CH), 1717 (C=O). ^{1}H NMR ($CDCl_3$, TMS, 400 MHz)/δppm: 7.55 (s, 2H), 4.41–4.35 (m, 4H), 1.38 (t, J = 6.8 Hz, 6H). EI-MS (*m/z*): 228 (M^+). Anal. Calcd for $C_8H_{12}N_4O_4$: C, 42.1; H, 5.3; N, 24.55; Found: C, 42.4; H, 5.4; N, 25.0%. Crystal data: Monoclinic, $P2_1/c$, a = 8.4740 (10) Å, b = 13.4510 (15) Å, c = 10.159 (3) Å, β = 109.090 (17)°, V = 1094.3 (4) Å^3, Z = 4, D_x = 1.385 Mg m^{-3}, Mo $K\alpha$ radiation, μ = 0.11 mm^{-1}, T = 298 (2) K, 2262 measured reflections, 1954 independent reflections, R_{int} = 0.014, Final R^1 = 0.050, $wR(F^2)$ = 0.169.

CCDC 255702 contains the supplementary crystallographic data for **1b**. They can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request.cif.

Dipropyl 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxylate (**1c**): 1.4 g, 27%, m.p. 78–79 °C, (lit.[10] 78–81 °C). IR ν_{max}(KBr)/cm^{-1}: 3313 (NH), 2962 (CH), 1717 (C=O). ^{1}H NMR ($CDCl_3$, TMS, 400 MHz)/δppm: 7.56 (s, 2H), 4.27 (t, J = 7.2 Hz, 4H), 1.79–1.74 (m, 4H), 0.98 (t, J = 6.8 Hz, 6H). EI-MS (*m/z*): 256 (M^+). Anal. Calcd for $C_{10}H_{16}N_4O_4$: C, 46.9; H, 6.3; N, 21.9; Found: C, 46.9; H, 6.4; N, 22.2%.

Dibutyl 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxylate (**1d**): 1.5 g, 26%, m.p. 53–54 °C, (lit.[10] 56–61 °C). IR ν_{max}(KBr)/cm^{-1}: 3336 (NH), 2979 (CH), 1719 (C=O). ^{1}H NMR ($CDCl_3$, TMS, 400 MHz)/δppm: 7.49 (s, 2H), 4.31 (t, J = 7.2 Hz, 4H), 1.75–1.68 (m, 4H), 1.44–1.38 (m, 4H), 0.95 (t, J = 6.8 Hz, 6H). EI-MS (*m/z*): 284 (M^+). Anal. Calcd for $C_{12}H_{20}N_4O_4$: C, 50.7; H, 7.1; N, 19.7; Found: C, 50.9; H, 7.3; N, 19.7%.

General procedure for the synthesis of **2a–b**, **3a**, **4a**

To a solution of dialkyl 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxylate (20 mmol) in the corresponding alcohol (30 mL), was added *p*-toluenesulfonic acid (0.3 g, 2 mmol). The mixture was refluxed for 2–4 h and the crude product was purified by column chromatography to obtain the corresponding ester of 1,4-dihydro-1,2,4,5-tetrazine.

Diisopropyl 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxylate (**2a**): 1.6 g, 31%, m.p. 56–57 °C. IR ν_{max}(KBr)/cm^{-1}: 3330 (NH), 2981 (CH), 1718 (C=O). ^{1}H NMR ($CDCl_3$, TMS, 400 MHz)/δppm: 7.53 (s, 2H), 5.23–5.17 (m, 2H), 1.36 (d, J = 6.8 Hz, 12H). EI-MS (*m/z*): 256 (M^+). Anal. Calcd for $C_{10}H_{16}N_4O_4$: C, 46.9; H, 6.3; N, 21.9; Found: C, 46.95; H, 6.3; N, 21.8%.

Diisobutyl 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxylate (**2b**): 1.2 g, 21%, m.p. 94–96 °C. IR ν_{max}(KBr)/cm^{-1}: 3348 (NH), 2974 (CH), 1717 (C=O). ^{1}H NMR ($CDCl_3$, TMS, 400 MHz)/δppm: 7.52 (s, 2H), 4.09 (d, J = 7.2 Hz, 4H), 2.07–2.04 (m, 2H), 0.97 (d, J = 6.8 Hz, 12H). EI-MS (*m/z*): 284 (M^+). Anal. Calcd for $C_{12}H_{20}N_4O_4$: C, 50.7; H, 7.1; N, 19.7; Found: C, 51.0; H, 7.1; N, 19.7%.

3-Isopropyl 6-methyl 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxylate (**3a**): 2.0 g, 44%, m.p. 83–84 °C. IR ν_{max}(KBr)/cm^{-1}: 3325 (NH), 2974 (CH), 1715 (C=O). ^{1}H NMR ($CDCl_3$, TMS, 400 MHz)/δppm: 7.52 (s, 1H), 7.47 (s, 1H), 5.22–5.19 (m, 1H), 3.92 (s, 3H), 1.36 (d, J = 6.8 Hz, 6H). EI-MS (*m/z*): 228 (M^+). Anal. Calcd for $C_8H_{12}N_4O_4$: C, 42.1; H, 5.3; N, 24.55; Found: C, 42.3; H, 5.4; N, 24.65%.

3-Ethyl 6-isopropyl 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxylate (**4a**): 1.7 g, 35%, m.p. 80–82 °C. IR ν_{max}(KBr)/cm^{-1}: 3381 (NH), 2996, 2978, 2941 (CH), 1714 (C=O). ^{1}H NMR ($CDCl_3$, TMS, 400 MHz)/δppm: 7.50 (s, 1H), 7.47 (s, 1H), 5.06–5.01 (m, 1H), 3.94–3.91 (m, 2H), 1.38–1.32 (m, 9H). EI-MS (*m/z*): 242 (M^+). Anal. Calcd for $C_9H_{14}N_4O_4$: C, 44.6; H, 5.8; N, 23.1; Found: C, 44.5; H, 5.9; N, 23.3%.

General procedure for the synthesis of **5a–e**

To a solution of dimethyl 1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxylate **1a** (4.0 g, 20 mmol) in ethanol (50 mL), was added amine (50 mmol). The mixture was stirred at 60–70 °C for 0.5–1 h and then cooled. The precipitate was collected and recrystallised to afford the corresponding amide of 1,4-dihydro-1,2,4,5-tetrazine. Amide **5a** was prepared by adding saturated ammonia in ethanol.

1,4-Dihydro-1,2,4,5-tetrazine-3,6-dicarboxamide (**5a**): 3.3 g, 97%, m.p. > 280 °C. IR ν_{max}(KBr)/cm^{-1}: 3391, 3274, 3219 (NH), 1686 (C=O). ^{1}H NMR ($CDCl_3$ + DMSO-d_6, TMS, 500 MHz)/δppm: 7.96 (br, 4H), 7.59 (s, 2H). EI-MS (*m/z*): 170 (M^+). Anal. Calcd for $C_4H_6N_6O_2$: C, 28.2; H, 3.55; N, 49.4; Found: C, 28.1; H, 3.5; N, 49.0%.

N^3,N^6-Dipropyl-1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxamide (**5b**): 4.0 g, 79%, m.p. 235–238 °C. IR ν_{max}(KBr)/cm^{-1}: 3338, 3272 (NH), 2965 (CH), 1663 (C=O). ^{1}H NMR ($CDCl_3$, TMS, 500 MHz)/δppm: 7.57 (s, 2H), 6.88 (br, 2H), 3.29 (q, J = 7.0 Hz, 4H), 1.58 (q, J = 7.0 Hz, 4H), 0.94 (t, J = 7.5 Hz, 6H). EI-MS (*m/z*): 254 (M^+). Anal. Calcd for $C_{10}H_{18}N_6O_2$: C, 47.2; H, 7.1; N, 33.05; Found: C, 47.4; H, 7.0; N, 33.2%.

N^3,N^6-Dibutyl-1,4-dihydro-1,2,4,5-tetrazine-3,6-dicarboxamide (**5c**): 5.0 g, 89%, m.p. 230–233 °C. IR ν_{max}(KBr)/cm^{-1}: 3341, 3273 (NH), 2957 (CH), 1661 (C=O). ^{1}H NMR ($CDCl_3$, TMS, 500 MHz)/δppm: 7.76 (s, 1H), 7.61 (s, 1H), 6.90 (br, 1H), 6.78 (br, 1H), 3.33 (q, J = 7.0 Hz, 4H), 1.52 (m, J = 7.0 Hz, 4H), 1.37 (m, J = 7.5 Hz, 4H), 0.93 (t, J = 7.5 Hz, 6H). EI-MS (*m/z*): 282 (M^+). Anal. Calcd for $C_{12}H_{22}N_6O_2$: C, 51.05; H, 7.85; N, 29.8; Found: C, 50.8; H, 8.0; N, 29.7%.

1,1'-(1,4-Dihydro-1,2,4,5-tetrazine-3,6-diyldicarbonyl)bispiperidine (**5d**): 6.0 g, 98%, m.p. 262–265 °C. IR ν_{max}(KBr)/cm^{-1}: 3289 (NH), 2950 (CH), 1623 (C=O). ^{1}H NMR ($CDCl_3$, TMS, 400 MHz)/ δppm: 7.64 (s, 2H), 3.94–3.63 (m, 8H), 1.67–1.61 (m, 12H). EI-MS (*m/z*): 306 (M^+). Anal. Calcd for $C_{14}H_{22}N_6O_2$: C, 54.9; H, 7.2; N, 27.4; Found: C, 54.7; H, 7.2; N, 27.5%.

4,4'-(1,4-Dihydro-1,2,4,5-tetrazine-3,6-diyldicarbonyl)bismorpholine (**5e**): 6.1 g, 98%, m.p. 221–223 °C. IR ν_{max}(KBr)/cm^{-1}: 3327 (NH), 2979 (CH), 1630 (C=O). ^{1}H NMR ($CDCl_3$, TMS, 400 MHz)/ δppm: 7.73 (s, 2H), 4.12–4.10 (m, 4H), 3.72–3.68 (m, 12H). EI-MS (*m/z*): 310 (M^+). Anal. Calcd for $C_{12}H_{18}N_6O_4$: C, 46.45; H, 5.85; N, 27.1; Found: C, 46.5; H, 5.8; N, 27.2%.

General procedure for the synthesis of **6a**[8]

CAUTION: Operations concerning toxic nitrous gases must be carried out carefully in a well-ventilated hood.

An ice-cold solution of **1a** (2.00 g, 10 mmol) in dichloromethane (80 mL) was treated with nitrous fumes prepared by stirring concentrated hydrochloric acid (12.5 mL) dropwise into a solution of sodium nitrite (120 mmol) in H_2O (20 mL), to afford the brown gases of nitrous fumes. Stirring was continued for 1.5 h at room temperature before the solvent and the excess nitrous gases were removed *in vacuo* to afford bright red dimethyl 1,2,4,5-tetrazine-3,6-dicarboxylate **6a**: 1.94 g, 98.0%, m.p. 170–173 °C, (lit.[8] 173–175 °C). IR ν_{max}(KBr)/cm^{-1}: 3479 (NH), 2970 (CH), 1752 (C=O). ^{1}H NMR ($CDCl_3$, TMS, 500 MHz)/ δppm: 7.23 (s, 6H). EI-MS (*m/z*): 198 (M^+).

The authors thank National Centre for Drug Screening, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China, for anticancer evaluation. Financial assistance from the Opening Foundation of Zhejiang Provincial Top Key Discipline of Biochemical Engineering and the National Natural and Scientific Foundation (grant No. 20802069), and the early work from the National Natural and Scientific Foundation (grant No. 20272053) are also gratefully acknowledged.

Received 17 August 2009; accepted 14 September 2009
Paper 09/0747 doi: 10.3184/030823409X12532880130972
Published online: 8 October 2009

References

1 N. Saracoglu, *Tetrahedron*, 2007, **63**, 4199.
2 A.V. Eremeev, D.A. Tikhomirov, V.A. Tyusheva and F. Liepins, *Khim. Geterotsikl. Soedin.*, 1978, 753.
3 H. Neunhoeffer, *Comprehensive heterocyclic chemistry I*, eds A.R. Katritzky, Pergamon, Frankfurt, 1984. Vol. 3, pp. 531-572.
4 J. Sauer, *Comprehensive heterocyclic chemistry II*, eds A.J. Boulton, Elsevier, Oxford, England, 1996. Vol. 6, pp. 901-955.
5 W.X. Hu, G.W. Rao and Y.Q. Sun, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 1177.
6 G.W. Rao and W.X. Hu, *Bioorg. Med. Chem. Lett.*, 2005, **15**, 3174.
7 G.W. Rao and W.X. Hu, *Bioorg. Med. Chem. Lett.*, 2006, **16**, 3702.
8 D.L. Boger, R.S. Coleman and J.S. Panek, *J. Org. Chem.*, 1985, **50**, 5377.
9 G.W. Rao and W.X. Hu, *Acta. Cryst.*, 2005, **E61**, o3664.
10 Š. Frebort, N. Almonasy, R. Hrdina, A. Lyčka, M. Lísa and M. Holčapek, *Collect. Czech. Chem. Commun.*, 2008, **73**, 107.