Synthesis and cytotoxicity against human cancer cells of novel diazenecarboxamides

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Dedicated to Professor Miha Tišler on the occasion of his 75th birthday

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Abstract

The synthesis as well as the cytotoxic activity of a series of diazenecarboxamides **1a - 1j**, which are stable representatives of the diazene family, in a human cancer cell line panel are described. Compounds **1j** and especially **1g** showed a high cytotoxic activity against some leukemia cell lines, and can be considered as new leads for further development.

Keywords: Diazenecarboxamides, synthesis, cytotoxic activity

Introduction

Diazenecarboxylic acid esters have already been evaluated decades ago in several biological systems by Kosower.[1] Unfortunately, most of them were found to be unstable. Their degradation involved free radicals, causing fatal damage throughout the cell. Having those results in mind, as well as the fact that amides are more stable towards hydrolysis than the corresponding esters, unsymmetrical diazenecarboxamides were supposed to be more appropriate for such studies than diazenecarboxylic acid esters. We described recently an application of diazenecarboxamides as convenient and selective oxidants of glutathione (GSH), cysteamine, dithiothreitol, and other

thiols to disulfides.[2] Some of diazenecarboxamides were then demonstrated to act as potential modulators of drug resistance to cisplatin for certain types of tumours. Their effectiveness appears to be related to the ability to lower the intracellular level of GSH.[3] A particular diazene, i.e. a diazenecarboxamide similar to **1f**−**1i** with the 2-pyridyl group attached directly to the diazene functionality, was found to exhibit a cytotoxic activity on 10 human cancer cell lines, on parental cells as well as on their drug-resistant sublines.[4] As a continuation of this work we describe herein the synthesis and the evaluation of the cytotoxic activity in an *in vitro* human disease-oriented tumour cell line screening panel of a series of 10 selected diazenes (**1a**−**1j**; Figure 1).

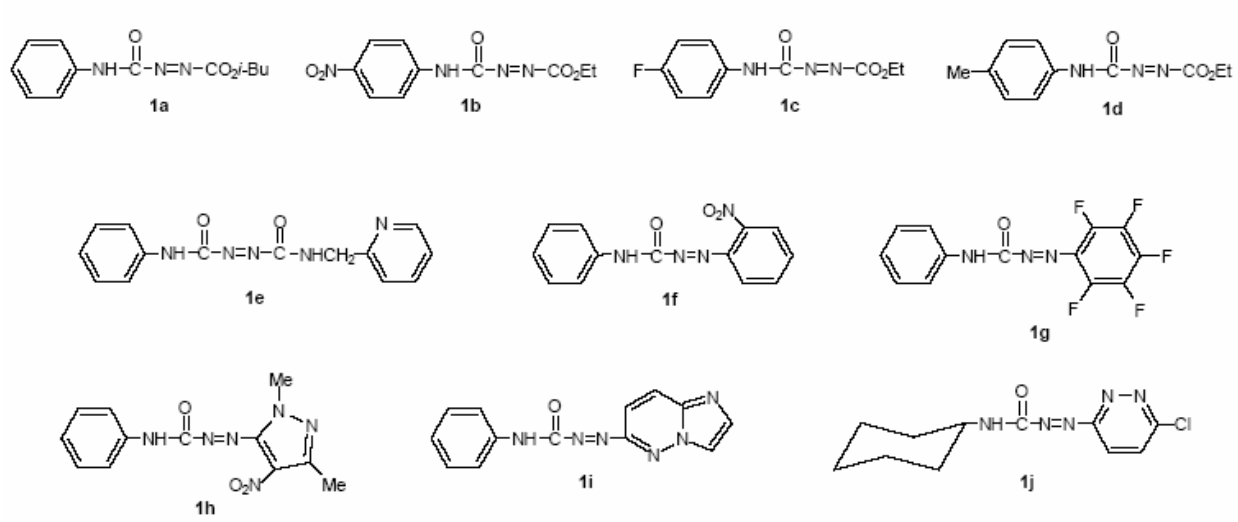
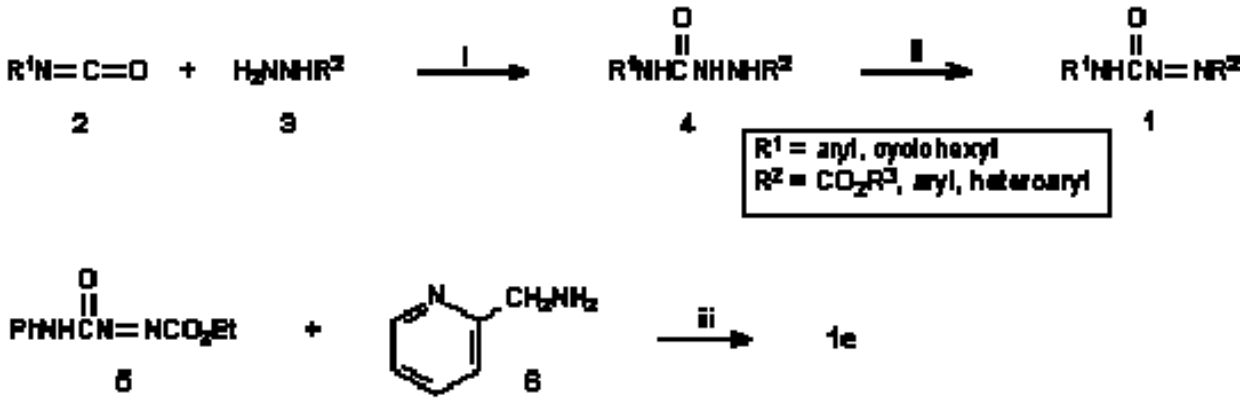


Figure 1

Results and Discussion

Our recent reports on mono- and disubstituted hydrazines demonstrated their synthesis and a variety of applications in organic chemistry.[5] Diazenes are easily available from monosubstituted ones in a two-step procedure. Thus, the addition of the monosubstituted hydrazine derivative **3** to the isocyanate **2** resulted in the formation of the corresponding 1,4-disubstituted semicarbazide **4**. The oxidation of the latter type of compounds was performed with *N*-bromosuccinimide/pyridine (NBS/Py; the preparation of **1a**−**1d**), ceric(IV) ammonium nitrate (CAN; **1f**, **1g**, **1i** and **1j**) or

with Br_2/pyridine (Br_2/Py; **1h**; Scheme 1). The most convenient route to unsymmetrical diazenedicarboxamide **1e** was the substitution of the ethoxy group in the diazene **5**,[6] employing 2-picolylamine (**6**) as a nucleophile. The synthesis and spectroscopic data of **1a**, **1c**, **1d**, and **1i** have already been published.[2,7] Other diazenes were obtained following the reaction conditions, depicted in Scheme 1, and were characterised as described in experimental section.



Scheme 1. Reagents and conditions: (i) CH_2Cl_2 or CH_3CN or DMF, 0 °C or rt, 0.5 h−18 h, 85−97%. (ii) NBS/Py, CH_2Cl_2; or Br_2/Py, MeOH; or CAN, MeOH; rt, 0.2 h−2 h, 81−97%. (iii) CH_2Cl_2, 0 °C, 30 min then r.t., 10 min, 77%.

The cytotoxic activity against human cancer cell lines was evaluated first in a 3-cell line, one dose primary anticancer assay. Results for each test compound are reported as the percentage of growth of the treated cells when compared to the untreated control cells, and are listed for the diazenes **1a**–**1j** in Table 1.

Table 1. Growth percentages of diazenes **1a**–**1j** in the 3-cell line pre-screen (test concentration 100 μM)

Test compound	Cell line			Activity
	NCI-H460 (Lung)	MCF7 (Breast)	SF-268 (CNS)	
1a	116	84	92	inactive
1b	18	5	43	**active**
1c	58	32	42	inactive
1d	121	81	106	inactive
1e	−41	−76	−96	**active**
1f	26	14	−2	**active**
1g	−3	−66	−93	**active**
1h	116	−28	−37	**active**
1i	114	−89	−86	**active**
1j	165	67	10	**active**

Compounds which reduced the growth of any of the cell lines to less than 32% (negative numbers indicate cell kill), were passed on for evaluation in the full panel of 60 cell lines over a 5-log dose range. Since this condition was fulfilled for diazenes **1b**, **1e**, **1f**, **1g**, **1h**, **1i**, and **1j**, they were tested in the NCI's screening panel consisting of 60 human tumour cell lines, which is largely derived from solid tumours, plus some leukemia cell lines. Nine subpanels represent diverse histologies, i.e. non-small cell lung, colon, central nervous system, renal, ovarian, prostate, and breast cancers, melanoma, and leukemia. The average GI_{50} values calculated from all cell lines tested, as well as the GI_{50} values for two leukemia cell lines, i.e. CCRF-CEM and HL-60 (TB), are displayed in Table 2.

Table 2. GI_{50} values (μM)[a] of diazenes **1b** and **1e**–**1j** in the 60-cell line panel, and against leukemia cell lines CCRF-CEM and HL-60 (TB)

Test compound	Cell line		
	panel	CCRF-CEM	HL-60 (TB)
1b	61.7	64.6	85.1
1e	22.9	23.4	19.1
1f	51.3	14.8	25.7
1g	9.1	0.068	0.023
1h	55.0	10.5	7.4
1i	38.9	20.9	21.4
1j	60.3	1.7	1.0

[a] GI_{50} is the concentration of the diazene required for 50% growth inhibition.

The complete set of data obtained in the screening panel demonstrated that especially these leukemia cell lines were relatively more sensitive to diazenes **1f**–**1j** than were other cell lines. Diazene **1g** turned out to be the most active compound. Against the leukemia cell lines CCRF-CEM and HL-60 (TB) its GI_{50} values were in the nanomolar range (68 nM and 23 nM, respectively). The TGI values of **1g** against both cell lines were 1.5 μM and 55 nM, and the LC_{50} values 9.1 μM and 13.8 μM, respectively. Also diazene **1j** was an order of magnitude more active against these two cell lines (GI_{50} values 1.7 μM and 1.0 μM, respectively), than any other diazene tested. The TGI values of **1j** were 26.3 μM and 5.2 μM, respectively, whereas the LC_{50} values were >100 μM. In conclusion, the diazenecarboxamides **1j** and especially **1g** can be considered as new leads for the development of antitumoural agents, active against leukemia, which deserve further exploration.

Experimental Section

General Procedures. Melting points were determined on a hot stage and were uncorrected. IR spectra were measured as KBr pellets. NMR spectra were recorded at 300.13 and 75.5 MHz, respectively. Mass spectra were obtained on a VG-Analytical AutospecQ instrument. Elemental analyses (C, H, N) were performed in our analytical laboratory. Isobutyl

phenylaminocarbonyldiazenecarboxylate (**1a**),[7] ethyl (4-fluorophenyl)amino-carbonyldiazenecarboxylate (**1c**),[7] ethyl (4-tolyl)aminocarbonyldiazenecarboxylate (**1d**),[7] and *N*-phenyl-2-(imidazo[1,2-*b*]pyridazin-6-yl)diazenecarboxamide (**1i**)[2] were prepared as described earlier.

Ethyl (4-nitrophenyl)aminocarbonyldiazenecarboxylate (1b). *N*-Bromosuccinimide (NBS, 0.748 g, 4.2 mmol) was slowly added to a stirred suspension of ethyl 2-(4-nitrophenyl)aminocarbonylhidrazinecarboxylate (1.073 g, 4 mmol) and pyridine (0.65 mL, 8 mmol) in CH_2Cl_2 (30 mL) at room temperature. The red solution was then stirred for 30 min, acidified with HCl (1:1, 20 mL) and the phases were separated. The organic phase, which was washed successively with a solution of sodium thiosulfate pentahydrate (0.400 g) in water (30 mL), saturated $NaHCO_3$ (30 mL), and finally water (30 mL), was then dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure to afford **1b** (991 mg, 93%): mp 138−141 °C (toluene); IR 3300, 3250, 3080, 1750, 1510, 1340, 1260 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 1.38 (t, 3H, *J* = 7.0 Hz), 4.54 (q, 2H, *J* = 7.0 Hz), 7.97 (m, 2H), 8.33 (m, 2H), 12.08 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ13.9, 65.6, 119.8, 125.1, 143.2, 143.7, 157.3, 161.0; MS (FAB) *m/z* 267 (M^+ + H, 21), 165 (19), 149 (27), 105 (40). Anal. Calcd for $C_{10}H_{10}N_4O_5$: C, 45.12; H, 3.79; N, 21.05. Found: C, 45.11; H, 3.78; N, 21.21.

1-(2-Picolyl)-2-phenyldiazenedicarboxamide (1e). Ethyl phenylaminocarbonyldiazenecarboxylate[6] (2.212 g, 10 mmol) was slowly added to the stirred solution of 2-picolylamine (1.082 g, 10 mmol) in CH_2Cl_2 (2 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 30 min and then at r.t. for 10 min. The solid material was filtered off and washed with cyclohexane to give the diazene **1e** (2.185 g, 77%): mp 112−115 °C (CH_2Cl_2); IR 3270, 3050, 1750, 1690, 1540, 1440, 1260, 1000, 760, 690 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 4.59 (d, 2H, *J* = 6.0 Hz), 7.20 (m, 1H), 7.32 (ddd, 1H, *J*1 = 7.5 Hz, *J*2 = 4.8 Hz, *J*3 = 1.1 Hz), 7.42 (m, 3H), 7.73 (m, 2H), 7.82 (ddd, 1H, *J*1 = 7.7 Hz, *J*2 = 7.5 Hz, *J*3 = 1.8 Hz), 8.55 (ddd, 1H, *J*1 = 4.8 Hz, *J*2 = 1.8 Hz, *J*3 = 1.0 Hz), 9.53 (d, 1H, *J* = 6.0 Hz), 11.30 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 160.8, 156.3, 155.0, 149.1, 137.2, 136.6, 129.3, 125.7, 123.0, 122.4, 119.8, 45.8; MS (FAB) *m/z* 286 (M^+ + 3, 20), 109 (24), 92 (35). Anal. Calcd for $C_{14}H_{13}N_5O_2$: C, 59.36; H, 4.63; N, 24.72. Found: C, 59.51; H, 4.63; N, 24.84.

***N*-Phenyl-2-(2-nitrophenyl)diazenecarboxamide (1f).** A solution of ceric(IV) ammonium nitrate (CAN, 1.206 g, 2.2 mmol) in methanol (5 mL) was added dropwise to the suspension of 4-phenyl-1-(2-nitrophenyl)semicarbazide (272 mg, 1 mmol) in methanol (5 mL). The reaction mixture was stirred for 1 h at r.t. and then treated with water (10 mL). The solid material was filtered off and washed with water to give the diazene **1f** (233 mg, 86%): mp 146−147 °C (MeOH); IR 3220, 1700, 1590, 1510, 1435, 1340, 750 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ

7.20 (m, 1H), 7.43 (m, 2H), 7.67 (m, 1H), 7.76 (m, 2H), 7.93 (m, 2H), 8.22 (m, 1H), 11.17 (broad, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 118.1, 119.5, 124.5, 124.7, 129.1, 133.5, 134.0, 137.6, 143.6, 147.4, 158.4. Anal. Calcd for $C_{13}H_{10}N_4O_3$: C, 57.78; H, 3.73; N, 20.73. Found: C, 57.58; H, 3.72; N, 20.72.

***N*-Phenyl-2-(pentafluorophenyl)diazenecarboxamide (1g).** A mixture of pentafluorohydrazine (396 mg, 2 mmol) and phenyl isocyanate (0.22 mL, 2 mmol) in acetonitrile (5 mL) was stirred at r.t. for 1 h. Then, CAN (2.203 g, 4 mmol) in methanol (10 mL) was added dropwise. The reaction mixture was stirred for additional 0.5 h and treated with water (15 mL). The solid material was filtered off and washed with water to afford **1g** (610 mg, 97%): mp 142.5−143 °C (MeOH); IR 3420, 1710, 1505, 1480, 1310, 1025, 755 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.20 (m, 1H), 7.43 (m, 2H), 7.78 (m, 2H), 11.26 (broad, 1H); ^{19}F NMR (282 MHz, DMSO-d_6) δ−161.4 (m, 2F), −148.0 (m, 2F), −147.7 (m, 1F); MS (EI) *m/z* 317 (M^+ + 2, 3), 120 (100), 92 (55), 77 (91). Anal. Calcd for $C_{13}H_6F_5N_3O$ x 0.25 H_2O: C, 48.84; H, 2.05; N, 13.14. Found: C, 48.92; H, 2.35; N, 13.46.

***N*-Phenyl-2-(1,3-dimethyl-4-nitropyrazol-5-yl)diazenecarboxamide (1h).** A solution of bromine (230 mg, 1.4 mmol) in methanol (4 mL) was added dropwise to the mixture of 4-phenyl-1-(1,3-dimethyl-4-nitropyrazol-5-yl)semicarbazide (290 mg, 1 mmol) and pyridine (0.16 mL, 2 mmol) in methanol (10 mL). The reaction mixture was stirred at r.t. for 20 min and then treated with water (50 mL). The solid material was filtered off and washed with water to give **1h** (258 mg, 90%): mp 157−158.5 °C (EtOH/H_2O); IR 3310, 1725, 1590, 1480, 1335, 960, 750 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.55 (s, 3H), 4.14 (s, 3H), 7.26 (tt, 1H, *J*1 = 7.4 Hz, *J*2 = 1.1 Hz), 7.47−7.72 (m, 2H), 7.74 (dd, 2H, *J*1 = 8.9 Hz, *J*2 = 1.1 Hz), 8.47 (s, 1H); MS (EI) *m/z* 290 (M^+ + 2, 15), 243 (55), 141 (100), 120 (34). Anal. Calcd for $C_{12}H_{12}N_6O_3$: C, 50.00; H, 4.20; N, 29.15. Found: C, 50.07; H, 4.04; N, 29.37.

***N*-Cyclohexyl-2-(3-chloropyridazin-6-yl)diazenecarboxamide (1j).** A solution of CAN (1.205 g, 2.3 mmol) in water (10 mL) was added dropwise to a stirred suspension of 4-phenyl-1-(3-chloropyridazin-6-yl)semicarbazide (269.7 mg, 1 mmol) in methanol (10 mL). After 10 min, the reaction mixture was extracted with chloroform (2 x 10 mL). The combined chloroform extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness to afford **1j** (244 mg, 91%): mp 194−196 °C (EtOAc); IR 3290, 3020, 2920, 2840, 1725, 1550, 1510, 1395 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.18 (m, 1H), 1.32 (m, 4H), 1.60 (m, 1H), 1.73 (m, 2H), 1.92 (m, 2H), 3.64 (m, 1H), 7.97 (d, 1H, *J* = 9.1 Hz), 8.22 (d, 1H, *J* = 9.1 Hz), 8.76 (broad, 1H, *J* = 7.8 Hz); ^{13}C NMR (75 MHz, DMSOd_6) δ 24.4, 24.9, 32.0, 49.4, 120.3, 131.9, 158.3, 161.1, 164.5. Anal. Calcd for $C_{11}H_{14}ClN_5O$: C, 49.35; H, 5.27; N, 26.16. Found: C, 49.37; H, 5.30; N, 26.20.

Cytotoxicity. The cytotoxic activity of the diazenes **1a**−**1j** was evaluated at the National Cancer Institute (NCI, Bethesda, Maryland, USA). The 3-cell line panel, consisting of MCF7 (breast

cancer), NCI-H460 (non-small cell lung cancer), and SF-268 (central nervous system cancer, CNS) was used as a pre-screen for the large 60 cell line panel. Briefly, each cell line was inoculated and preincubated on a microtiter plate. Test compounds were then added at a single dose (100 μM) and the culture was incubated for 48 h. End-point determinations were made with sulforhodamine B, a protein-binding dye. The diazenes **1b** and **1e**−**1j** were then tested at a minimum of five concentrations at 10fold dilutions, 100 μM being the highest test concentration, in the NCI's screening panel consisting of 60 human tumour cell lines. In this assay results are evaluated in terms of specificity and potency. The cytotoxic effects of each compound can be expressed as the molar drug concentrations required for 50% growth inhibition (GI_{50}), total growth inhibition (TGI), and 50% cell kill (LC_{50}).[8]

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References

1. Kosower, N. S.; Kosower, E. M. In *Glutathione: Chemical, Biochemical, and Medical Aspects*; Dolphin, D.; Poulson, R.; Avramović, O., Eds; Wiley-Interscience: New York, 1989; Part B, pp 319.
2. Košmrlj, J.; Kočevar, M.; Polanc, S. *J. Chem. Soc., Perkin Trans. 1* **1998**, 3917.
3. Osmak, M.; Bordukalo, T.; Košmrlj, J.; Kvajo, M.; Marijanović, Z.; Eljuga, D.; Polanc, S. *Neoplasma* **1999**, *46*, 201.
4. Osmak, M.; Bordukalo, T.; Jernej, B.; Košmrlj, J.; Polanc, S. *Anti-Cancer Drugs* **1999**, *10* , 853.
5. (a) Kočevar, M.; Mihorko, P.; Polanc, S. *Synlett* **1995**, 241. (b) Kočevar, M.; Mihorko, P.; Polanc, S. *J. Org. Chem.* **1995**, *60*, 1466. (c) Košmrlj, J.; Kočevar, M.; Polanc, S. *Synlett* **1996**, 652. (d) Štefane, B.; Kočevar, M.; Polanc, S. *J. Org. Chem.* **1997**, *62*, 7165. (e) Trebše, P.; Polanc, S.; Kočevar, M.; Šolmajer, T.; Golič-Grdadolnik, S. *Tetrahedron* **1997**, *53*, 1383. (f) Kepe, V.; Požgan, F.; Golobič, A.; Polanc, S.; Kočevar, M. *J. Chem. Soc.,*

Perkin Trans. 1 **1998**, 2813. (g) Vraničar, L.; Polanc, S.; Kočevar, M. *Tetrahedron* **1999**, *55*, 271. (h) Štefane, B.; Kočevar, M.; Polanc, S. *Tetrahedron Lett.* **1999**, *40*, 4429. (i) Kafka, S.; Trebše, P.; Polanc, S.; Kočevar, M. *Synlett* **2000**, 254.

6. Knight, G. T.; Loadman, M. J. R.; Saville, B.; Wildgoose, J. *J. Chem. Soc., Chem. Commun.* **1974**, 193.
7. Lenaršič, R.; Kočevar, M.; Polanc, S. *J. Org. Chem.* **1999**, *64*, 2558.
8. Boyd, M. R.; Paull, K. D. *Drug Dev. Res.* **1995**, *34*, 91.