

Scientific paper

Synthesis and Biological Activity of New Diazenedicarboxamides as Potential Anticancer Agents

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Abstract

To increase the effectiveness of cancer treatment, more effective anti-cancer drugs, as well as the new improved strategies of cancer treatment, are urgently needed. Our previous results have shown that various diazenes are cytotoxic to different tumor cells and can even revert the resistance to cisplatin and vincristine. We also demonstrated that unsymmetrical diazenedicarboxamides **1** and **2** exhibited promising cytotoxicity. The aim of the present study was to synthesize new diazenedicarboxamides with acceptable solubility and good cytotoxicity. Here we report the synthesis and biological evaluation of new *N,N'*-disubstituted diazenedicarboxamides. We found that a modification of either **1** or **2** led to the more active compounds. The most effective among them was diazenedicarboxamide **11**, which can be considered as a new potential anticancer agent for the tumors of different origin, as well as for the drug resistant tumors.

Keywords: Diazenedicarboxamides, tumor cells, anticancer drugs

1. Introduction

Cancer is currently the leading cause of death in many high-developed countries and will probably become a major cause of mortality worldwide. One of the most widely applied modality for cancer treatment is chemotherapy. However, the major obstacle for the successful treatment of cancer patients with standard chemotherapy is drug resistance. It is based on the alterations that are induced in the cells during normal malignant transition process,¹ or those alterations that are developed during the treatment of patients with anti-cancer drugs.² To increase the effectiveness of cancer treatment, new, target oriented drugs have been developed. These, so called “smart drugs”, are designed to act selectively against tumor cells by targeting only specific bio-molecules present. However, in spite of the initial enthusiasm, these drugs did not adequately increase the success of cancer treatment.^{3–5} Therefore, in order to increase the effectiveness of cancer treatment, more effective anti-cancer drugs, as well as the

new improved strategies of cancer treatment are urgently required.

During the last ten years, we have synthesized and screened the cytotoxic activity of a number of compounds from diazene family. We have shown that these compounds have antiproliferative activity against different tumor cell lines.⁶ If diazenedicarboxamides were administered in combination with cisplatin, one of the most commonly used anti-cancer drug for the treatment of various solid tumors,⁷ synergistic effect was detected on several tumor cells from different origins, as well as the reversion of cisplatin and vincristine resistance.^{6a,6c,6d,6h} Moreover, it was shown that antiproliferative activity of selected diazenedicarboxamides against tumor cells was significantly higher in comparison to the normal cells.⁶ⁱ

Regarding the molecular mechanisms of their biological activity we found that diazenedicarboxamides can significantly reduce the intracellular level of glutathione (GSH).^{6a,6c–6e} Under quasi-physiological conditions (0.15 M aqueous solution of KCl, pH 7.4, presence of air, con-

centration of a thiol 10 mmol L⁻¹, an equimolar amount of a diazene, 30 °C), diazenecarboxamides can transform different thioles to the corresponding disulphides. The same activity of diazenecarboxamides against the most important intracellular thiol, glutathione (GSH), was later identified to take place in the living cells.^{6d} Glutathione is the major antioxidant in the cells.⁸ In addition, it interacts with several different cytotoxic compounds, reducing their bioavailability and thus their cytotoxicity.^{8b} It has been recently recognized that GSH is responsible for cellular redox state maintaining,^{9a,9b} a process that is essential for a number of physiological processes in the leaving cell including cell proliferation, DNA synthesis, sensitivity to cytotoxic compounds, cell death etc.^{8b,9b,9c} Regarding the type of the cell death that can be triggered by diazenecarboxamides, both apoptosis like,^{6g} and necrosis type of the cell death were observed.^{6h}

Although diazenecarboxamides that have been prepared and tested so far possess several desired characteristics, their main disadvantage in view of potential clinical application is the low solubility in aqueous media. Therefore the aim of the present study was to synthesize new diazenedicarboxamides, structurally similar to the compounds **1** and **2**, with improved solubility and a good cytotoxicity.

2. Chemistry

Unsymmetrical diazenedicarboxamides, also shortly referred to as diazenes, employed in this study were obtained starting from the commercially available isocyanates **A**. The only exception was 4-butylphenyl isocyanate, which we prepared in-situ from 4-butylaniline and triphosgene following the procedure described for the formation of isocyanates from primary amines.¹⁰ The reaction of a selected isocyanate **A** with ethyl carbazate **B** gave the corresponding 1,4-disubstituted semicarbazide (compounds **C1–C6**). This was oxidized with *N*-bromosuccinimide (NBS) leading to the ethyl diazenecarboxylate (compounds **D1–D6**) and in the final stage, through a nucleophilic substitution of the ethoxy group in **D** with the

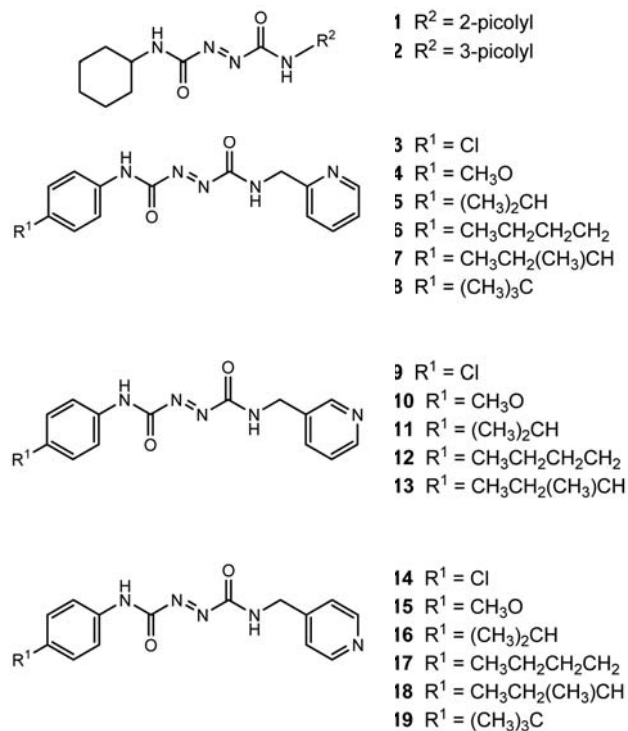


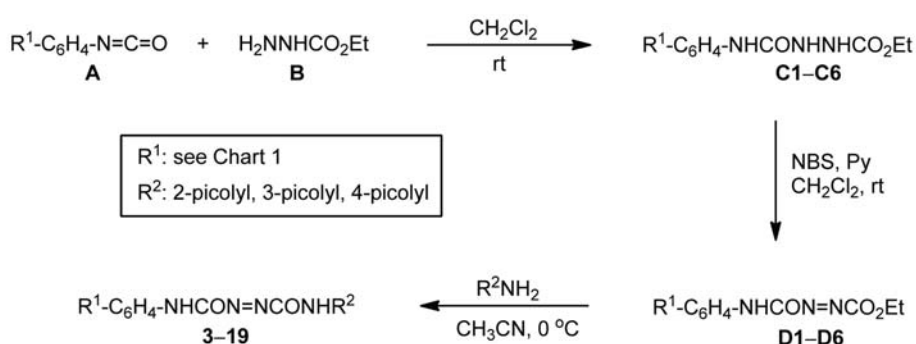
Chart 1. The structures of the diazenedicarboxamides **1–19**.

appropriate picolylamine, transformed into the diazenedicarboxamide shown in Scheme 1 (compounds **3–19**).

The entire sequence was performed under mild reaction conditions and led to high yields of the desired compounds **3–19** (Chart 1). The final products are crystalline compounds that are stable at room temperature in a solid state for months.

3. Results and Discussion

Our recent study revealed that diazenedicarboxamides **1** and **2** exhibited promising cytotoxicity.¹¹ Interestingly, however, compound **2** containing 3-picolyl moiety



Scheme 1. Synthetic approach to diazenedicarboxamides **3–19**.

at the amide nitrogen atom turned out to be more active than the analogue **1** having 2-picolyl group at the same position. This prompted us to check cytotoxicity of unsymmetrical diazenedicarboxamides possessing 2-picolyl, 3-picolyl or 4-picolyl group at one of the amide nitrogen atom and 4-substituted phenyl group at the other. The screening of their cytotoxic activity was carried out on human cervical carcinoma (HeLa) cells (Table 1).

Table 1. Cytotoxic activity of diazenedicarboxamides against human cervical carcinoma (HeLa) cells.

Compound	IC ₅₀ (μM) ^a
1	155 ¹¹
2	86 ¹¹
3	141.7 ± 0.1
4	88.9 ± 33.3
5	28.2 ± 4.1
6	23.9 ± 3.1 ^b
7	56.6 ± 14.5
8	61.3 ± 24.3
9	46.1 ± 1.0
10	34.5 ± 4.1
11	18.9 ± 4.0
12	12.4 ± 6.0 ^b
13	25.4 ± 5.6
14	92.5 ± 15.9
15	233.9 ± 69.1
16	25.8 ± 5.7
17	14.1 ± 4.3 ^b
18	57.4 ± 2.6
19	257.0 ± 2.3

^a IC₅₀ is the concentration of the diazene inducing 50% cell growth inhibition after 72 h incubation.

^b Diazenedicarboxamide precipitated promptly after the addition to the growth medium and thus the cytotoxicity cannot be measured accurately.

As evident from Table 1, out of the diazenes having the same aryl group in their structures, the compounds bearing 3-picolyl moiety are more cytotoxic than those with either 2-picolyl or 4-picolyl substituent. The comparison should be made among 4-chlorophenyl (**3**, **9** and **14**), 4-methoxyphenyl (**4**, **10** and **15**), 4-isopropylphenyl (**5**, **11** and **16**), 4-butylphenyl (**6**, **12** and **17**), and 4-*sec*-

butylphenyl functionalized analogous (**7**, **13** and **18**). Unfortunately, the diazenes **6**, **12** and **17** precipitated promptly after their addition to the growth medium and thus the cytotoxicity could not be determined accurately. For other diazenes having 4-alkylphenyl group at the amide functionality, the bulkiness rather than the length of the alkyl fragment seems to have an effect on their activity. Namely, steric hindrance of the alkyl moiety increases as follows: isopropyl, *sec*-butyl, *tert*-butyl. In the same sequence cytotoxicity of the compounds decreases; compare the results for **5**, **7** and **8** in 2-picolyl series, **11** and **13** in 3-picolyl and the results for **16**, **18** and **19** in 4-picolyl series. Comparing a biological activity and the nature of the substituent on the benzene ring one can conclude that a nonpolar alkyl group at the *para* position is more appropriate than the chloro or methoxy substituent at the same position.

The above figures clearly demonstrate that 3-picolyl diazenedicarboxamides are more active against HeLa cell lines than analogous 2-picolyl and 4-picolyl derivatives. This stimulated us to screen the biological activity of three mostly cytotoxic 3-picolyl representatives, namely **10**, **11** and **13** against several other tumor cell lines, including drug-resistant subline. The results outlined in Table 2 indicate that all three diazenedicarboxamides inhibited the growth of all examined tumor cell lines. This antiproliferative activity was strongly expressed for the compounds **11** and **13**, and significantly less for the diazene **10**. Although the diazene **11** exhibited some tumor cell-type specific cytotoxicity, being mostly toxic against HeLa cells, similar cytotoxic to laryngeal carcinoma HEP-2 and colorectal carcinoma HCT-116 cells, and the least active against lung adenocarcinoma H460 cells, the difference in its biological vary less than 1.5 fold (as determined by IC₅₀). Contrary, the diazene **13** was similarly cytotoxic against all tumor cells lines. It is important to point out that both, **11** and **13**, were similarly active against parental HEP-2 cells and their drug-resistant subline, what is highly important keeping in mind that the drug-resistant cells are the major cause for an unsuccessful chemotherapy with the standard anti-cancer drugs. The cytotoxic activity of the least active of the three compounds, the diazene **10**, depends on the origin of the tumor cells: it was mostly cytotoxic against cervical carcinoma HeLa cells, followed by laryngeal carcinoma HEP-2 cells. Unfortunately, carboplatin resistant cells 7T were more than two times more

Table 2. Cytotoxic activity of the diazenedicarboxamides **10**, **11** and **13** against different tumor cell lines expressed as IC₅₀ value (μM).

Compound	HeLa	HEP2	7T	H460	HCT-116
10	34.5 ± 4.9	40.1 ± 2.3	88.8 ± 8.4	148.6 ± 4.6	164.0 ± 11.3
11	18.9 ± 4.0	22.5 ± 3.8	26.2 ± 2.4	27.6 ± 7.7	21.1 ± 2.1
13	25.4 ± 5.6	25.3 ± 4.3	23.3 ± 3.8	26.5 ± 3.8	21.6 ± 2.6

IC₅₀ is the concentration of test compound inducing 50% cell growth inhibition after 72 h incubation; HeLa = human cervical carcinoma cells; HEP-2 = laryngeal carcinoma cells; 7T = drug-resistant HEP-2 subline; H460 = lung adenocarcinoma H460 cells; HCT-116 = colorectal carcinoma cells.

resistant to **10** as their parental HEp-2 cells. Furthermore, the lung adenocarcinoma H460 cells were more than four times more resistant to **10** comparing to the sensitivity of HeLa cells. Finally, the colorectal carcinoma HCT-116 cells were found to be the most resistant among the examined cell lines.

While several diazenecarboxamides can reduce intracellular glutathione level,^{6a,6c–6e} thus reducing the survival of treated cells, we investigated whether glutathione is involved in the cell response to **10**. In order to get this answer we pretreated the cells with buthionine sulfoximine (BSO), the well-known inhibitor of glutathione synthesis.¹² The results presented in Table 3 clearly show that the depletion of glutathione did not alter the survival of the cells treated with **10**. Such a pretreatment slightly increases the cytotoxicity of **11** and **13** indicating that glutathione is also not involved in the cell response to these diazenes.

Table 3. Survival of HeLa cells following the treatment with **10**, **11** and **13**, with or without a pretreatment with BSO^a expressed as IC₅₀ value (μM).

Diazenes	Control	+ BSO
10	36.5 ± 2.3	35.6 ± 1.9
11	17.7 ± 2.9	14.3 ± 4.8
13	23.0 ± 2.7	18.6 ± 0.2

^a BSO: buthionine sulfoximine.

Finally, as diazenecarboxamides can induce different types of cell death,^{6g,6h} we examined which type is triggered by the compounds **10**, **11** and **13**. As evident from Figure 1 all three selected diazenecarboxamides induced very fast necrosis that could be observed within 1 hour after cell treatment.

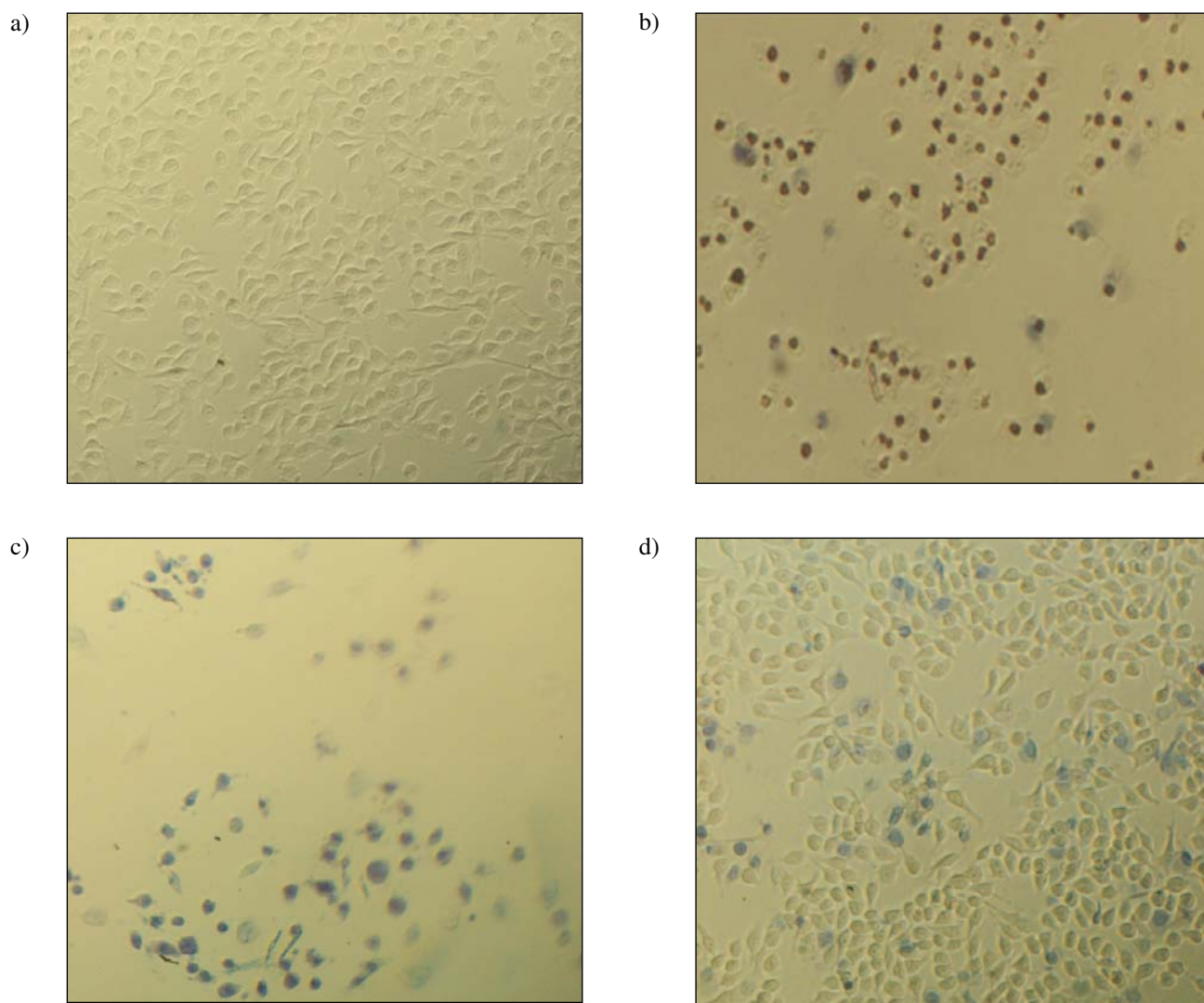


Figure 1. The diazenes **10**, **11** and **13** induce necrosis in HeLa cells. a) Control cells. Cells, stained with tripan blu dye 1 hour after cell treatment with: b) 34 μM of **10**; c) 100 μM of **11**; d) 80 μM of **13**. Phase contrast images of cells grown in culture.

4. Conclusions

We have shown that all new diazenedicarboxamides are highly cytotoxic against human carcinoma cells. Among the analogues, having the same aryl group, the compounds bearing 3-picolyl functionality are more cytotoxic than those possessing either 2-picolyl or 4-picolyl substituent. For the diazenedicarboxamides with 4-alkylphenyl group at one of the amide nitrogen atom it turned out that an increased bulkiness of the alkyl chain resulted in the decreased cytotoxicity.

From the series of the examined compounds, the diazene **11** was found to be the most cytotoxic that induced necrosis in the treated cells. Our results concerning the diazenedicarboxamide **11** indicate as follows: (a) it is mostly cytotoxic against human cervical carcinoma HeLa cells; (b) it is biologically active against a number of tumor cell lines; (c) it exhibits similar cytotoxicity against parental HeEp-2 cells and their drug-resistant subline. Therefore, the diazene **11** could be considered as a new potential anticancer agent for the tumors of different origin as well as for the drug-resistant laryngeal carcinoma tumors.

5. Experimental

5. 1. Chemistry

Starting materials for the synthesis of the examined compounds were used as obtained from the commercial sources (Aldrich, Fluka, Alfa Aesar, Maybridge Chemical Company Ltd). Melting points were determined on a Kofler micro hot stage and are uncorrected. NMR spectra were recorded at 29 °C with a Bruker Avance DPX 300 spectrometer (¹H NMR spectra at 300 MHz; ¹³C NMR spectra at 75 MHz) and with a Bruker Avance III spectrometer (¹H NMR spectra at 500 MHz; ¹³C NMR spectra at 126 MHz). Proton spectra are referenced to TMS as an internal standard; the carbon shifts are given against the central line of the solvent signal (DMSO-*d*₆ at $\delta = 39.5$ ppm; CDCl₃ at $\delta = 77.1$ ppm). IR spectra were obtained with a Perkin-Elmer Spectrum 100, equipped with a Specac Golden Gate Diamond ATR as a solid sample support. High-resolution mass spectra with atmospheric pressure electrospray ionization (HRMS ESI) were recorded with an Agilent 6224 Accurate Mass TOF LC/MS spectrometer. FAB spectra were obtained with VG-Analytical Autospec EQ instrument. Elemental analyses (C, H, N) were performed with Perkin Elmer 2400 Series II CHNS/O Analyzer. TLC was carried out on Fluka silica-gel TLC-cards.

Semicarbazides **C1**^{13a} and **C2**^{13b} were previously described in the literature but their syntheses and characterization details were not provided. For this reason they were prepared following our general synthesis to obtain semicarbazides **C** and all the missing data are reported.

Diazenedicarboxamides **1**,¹¹ **2**¹¹ and **17**,¹⁴ already described in the literature, as well as the new ones were prepared following a general procedure given underneath.

All tested compounds possessed a purity of $\geq 95\%$ as verified by ¹H NMR and ¹³C NMR spectroscopy and with elemental microanalysis. All results of C, H, N analyses were within $\pm 0.35\%$ of the calculated values.

5. 1. 1. General Procedure for the Synthesis of Semicarbazides C

A modified procedure we described earlier¹⁴ was employed for the synthesis of semicarbazides. A solution of the appropriate 4-substituted phenyl isocyanate **A** (5 mmol) in acetonitrile (3 mL) was added dropwise to the solution of ethyl carbazate **B** (0.520 g, 5 mmol) in acetonitrile (3 mL). The reaction mixture was stirred for 20 min at 0 °C and then for 18 h at room temperature, evaporated to dryness and treated with diethyl ether (7–10 mL). The solid material was filtered off and washed with hexane to afford the desired **C**. Crude product was crystallized from acetonitrile.

Ethyl 2-((4-chlorophenyl)carbamoyl)hydrazinecarboxylate (**C1**; R¹ = 4-Cl)

Reaction time: 0.5 h, colorless solid, yield: 0.938 g (73%), mp 200–201 °C (MeCN); mp^{13a} 193–195 °C; IR: ν 3281, 2981, 1737, 1685, 1636, 1590, 1554, 1524, 1493, 1403, 1369, 1308, 1254, 1232, 1208, 1088 cm⁻¹; ¹H NMR (500 MHz, DMSO-*d*₆): δ (ppm) 1.20 (t, *J* = 6.8 Hz, 3H), 4.06 (q, *J* = 6.8 Hz, 2H), 7.28–7.31 (m, 2H), 7.45–7.57 (m, 2H), 8.10 (s, 1H), 8.85–8.99 (m, 2H); ¹³C NMR (126 MHz, DMSO-*d*₆): δ (ppm) 14.4, 60.4, 119.9, 125.2, 128.3, 138.6, 155.4, 156.8; HRMS (ESI⁺) Calcd for C₁₀H₁₃ClN₃O₃ [M + H]⁺: 258.0640; Found 258.0642. Anal. Calcd for C₁₀H₁₂ClN₃O₃: C, 46.61; H, 4.69; N, 16.31. Found: C, 46.60; H, 4.51; N, 16.52.

Ethyl 2-((4-methoxyphenyl)carbamoyl)hydrazinecarboxylate (**C2**; R¹ = 4-MeO)

Reaction time: 0.5 h, colorless solid, yield: 0.772 g (61%), mp 164.5–166.5 °C (MeCN); mp^{13b} 170.5–171.5 °C; IR: ν 3320, 3279, 1739, 1681, 1637, 1612, 1598, 1559, 1515, 1455, 1297, 1236, 1206, 1189, 1032 cm⁻¹; ¹H NMR (500 MHz, DMSO-*d*₆): δ (ppm) 1.19 (t, *J* = 6.8 Hz, 3H), 3.70 (s, 3H), 4.05 (q, *J* = 6.8 Hz, 2H), 6.80–6.87 (m, 2H), 7.30–7.40 (m, 2H), 7.90 (s, 1H), 8.54 (s, 1H), 8.88 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆): δ (ppm) 14.4, 55.0, 60.4, 113.6, 120.1, 132.6, 154.3, 155.7, 156.9; HRMS (ESI⁺) Calcd for C₁₁H₁₆N₃O₄ [M + H]⁺: 254.1135; Found 254.1133. Anal. Calcd for C₁₁H₁₅N₃O₄: C, 52.17; H, 5.97; N, 16.59. Found: C, 52.25; H, 5.96; N, 16.66.

Ethyl 2-((4-isopropylphenyl)carbamoyl)hydrazinecarboxylate (**C3**; R¹ = 4-*i*-Pr)

Reaction time: 18 h, colorless solid, yield: 0.835 g (63%),

mp 138.5–139.5 °C (MeCN); IR: ν 3344, 3204, 3114, 2952, 2866, 1740, 1691, 1594, 1534, 1514 cm^{-1} ; ^1H NMR (500 MHz, DMSO- d_6): δ (ppm) 1.09–1.24 (m, 9H), 2.80 (sep, $J = 7.0$ Hz, 1H), 4.05 (q, $J = 7.0$ Hz, 2H), 7.07–7.15 (m, 2H), 7.29–7.40 (m, 2H), 7.92 (s, 1H), 8.60 (s, 1H), 8.89 (s, 1H); ^{13}C NMR (126 MHz, DMSO- d_6): δ (ppm) 14.5, 24.0, 32.8, 60.5, 118.6, 126.3, 137.4, 141.8, 155.6, 157.0; HRMS (ESI $^+$) Calcd for $\text{C}_{13}\text{H}_{20}\text{N}_3\text{O}_3$ [$\text{M} + \text{H}$] $^+$: 266.1499; Found 266.1495. Anal. Calcd for $\text{C}_{13}\text{H}_{19}\text{N}_3\text{O}_3$: C, 58.85; H, 7.22; N, 15.84. Found: C, 58.66; H, 7.36; N, 15.84.

Ethyl 2-((4-(*sec*-butyl)phenyl)carbamoyl)hydrazine-carboxylate (C5; R 1 = 4-*sec*-Bu)

Reaction time: 0.5 h, colorless solid, yield: 0.962 g (69%), mp 97.5–99.5 °C (MeCN); IR: ν 3262, 2959, 2926, 2872, 1730, 1678, 1605, 1539, 1514, 1462 cm^{-1} ; ^1H NMR (500 MHz, DMSO- d_6): δ (ppm) 0.75 (t, $J = 7.5$ Hz, 3H), 1.09–1.24 (m, 6H), 1.45–1.57 (m, 2H), 4.05 (q, $J = 7.5$ Hz, 2H), 7.06 (d, $J = 8.5$ Hz, 2H), 7.36 (d, $J = 8.5$ Hz, 2H), 7.93 (s, 1H), 8.61 (s, 1H), 8.90 (s, 1H), (1 signal hidden beneath DMSO – proved by HSQC spectrum); ^{13}C NMR (126 MHz, DMSO- d_6): δ (ppm) 12.1, 14.6, 21.9, 30.7, 40.3, 60.5, 118.6, 126.9, 137.4, 140.5, 155.7, 157.0; HRMS (ESI $^+$) Calcd for $\text{C}_{14}\text{H}_{22}\text{N}_3\text{O}_3$ [$\text{M} + \text{H}$] $^+$: 280.1656; Found 280.1649. Anal. Calcd for $\text{C}_{14}\text{H}_{21}\text{N}_3\text{O}_3$: C, 60.20; H, 7.58; N, 15.04. Found: C, 59.92; H, 7.88; N, 15.06.

Ethyl 2-((4-(*tert*-butyl)phenyl)carbamoyl)hydrazine-carboxylate (C6; R 1 = 4-*t*-Bu)

Reaction time: 1 h, colorless solid, yield: 0.843 g (60%), mp 173.0–175.0 °C (MeCN); IR: ν 3346, 3206, 3116, 2948, 2903, 2868, 1742, 1689, 1593, 1530 cm^{-1} ; ^1H NMR (500 MHz, DMSO- d_6): δ (ppm) 1.19 (t, $J = 7.0$ Hz, 3H), 1.24 (s, 9H), 4.05 (q, $J = 7.0$ Hz, 2H), 7.22–7.28 (m, 2H), 7.30–7.40 (m, 2H), 7.92 (s, 1H), 8.62 (s, 1H), 8.90 (s, 1H); ^{13}C NMR (126 MHz, DMSO- d_6): δ (ppm) 14.6, 31.2, 33.8, 60.5, 118.3, 125.2, 137.0, 144.0, 155.6, 157.0; HRMS (ESI $^+$) Calcd for $\text{C}_{14}\text{H}_{22}\text{N}_3\text{O}_3$ [$\text{M} + \text{H}$] $^+$: 280.1656; Found 280.1648. Anal. Calcd for $\text{C}_{14}\text{H}_{21}\text{N}_3\text{O}_3$: C, 60.20; H, 7.58; N, 15.04. Found: C, 59.96; H, 7.73; N, 14.99.

Synthesis of Ethyl 2-((4-Butylphenyl)carbamoyl)hydrazinecarboxylate (C4; R 1 = 4-Bu)

A solution of 4-butylaniline (3.014 g, 20.2 mmol) in dry dichloromethane (60 mL, distilled over P_2O_5) was added dropwise to the solution of triphosgene (2.404 g, 8 mmol) in dry dichloromethane (100 mL) at room temperature. The reaction mixture was stirred at room temperature for 30 min. Then, a solution of triethylamine (5.86 mL, 42 mmol) in dry dichloromethane (60 mL) was added dropwise. After 30 min ethyl carbazate was added (2.120 g, 20.2 mmol). The reaction mixture was stirred at room temperature for additional 20 min and evaporated under reduced pressure. Then water (100 mL) was added and the aqueous phase was washed with ethyl acetate (3 \times 200 mL).

Combined organic phases were dried over anhydrous Na_2SO_4 , filtered and evaporated under reduced pressure to dryness, leading to **C4**. Crude product was crystallized from petroleum ether/ethyl acetate.

Colorless solid, yield: 3.45 g (61%), mp 155.0–157.5 °C (petroleum ether/ethyl acetate); IR: ν 3274, 2959, 2929, 2858, 1744, 1681, 1642, 1597, 1551, 1516 cm^{-1} ; ^1H NMR (500 MHz, DMSO- d_6): δ (ppm) 0.88 (t, $J = 7.5$ Hz, 3H), 1.19 (t, $J = 7.0$ Hz, 3H), 1.24–1.33 (m, 2H), 1.46–1.55 (m, 2H), 2.45–2.52 (m, 2H), 4.05 (q, $J = 7.0$ Hz, 2H), 7.02–7.09 (m, 2H), 7.30–7.40 (m, 2H), 7.93 (s, 1H), 8.61 (s, 1H), 8.90 (s, 1H); ^{13}C NMR (126 MHz, DMSO- d_6) δ (ppm) 13.8, 14.5, 21.7, 33.3, 34.1, 60.5, 118.6, 128.3, 135.6, 137.2, 155.6, 157.0; HRMS (ESI $^+$) Calcd for $\text{C}_{14}\text{H}_{22}\text{N}_3\text{O}_3$ [$\text{M} + \text{H}$] $^+$: 280.1656; Found 280.1656. Anal. Calcd for $\text{C}_{14}\text{H}_{21}\text{N}_3\text{O}_3$: C, 60.20; H, 7.58; N, 15.04. Found: C, 60.27; H, 7.71; N, 14.92.

5. 1. 2. General Procedure for the Synthesis of Diazenecarboxylates D

A modified procedure we described earlier¹⁵ was employed for the synthesis of diazenecarboxylates. Pyridine (0.808 mL, 10 mmol) was added to the solution of the appropriate semicarbazide **C** (5 mmol) in dichloromethane (18 mL). Then, NBS (0.907 g, 5.1 mmol) was added and the reaction mixture was stirred at room temperature for 1 h, washed with aqueous HCl (1:1, 18 mL), sodium thiosulfate (2.5%, 18 mL), saturated aqueous solution of NaHCO_3 (18 mL), brine (18 mL). Organic phase was dried over anhydrous Na_2SO_4 , filtered and evaporated under reduced pressure to give product **D**. Solid products were crystallized from dichloromethane.

Ethyl 2-((4-chlorophenyl)carbamoyl)diazenecarboxylate (D1; R 1 = 4-Cl)

Orange solid, yield: 1.150 g (90%), mp 88.4–89.8 °C (CH_2Cl_2); IR: ν 3313, 1763, 1745, 1714, 1602, 1565, 1525, 1490, 1461, 1399, 1366, 1297, 1225, 1120, 1087, 1033 cm^{-1} ; ^1H NMR (500 MHz, DMSO- d_6): δ (ppm) 1.38 (t, $J = 7.1$ Hz, 3H), 4.52 (q, $J = 7.1$ Hz, 2H), 7.47–7.53 (m, 2H), 7.73–7.80 (m, 2H), 11.72 (s, 1H); ^{13}C NMR (126 MHz, DMSO- d_6): δ (ppm) 13.8, 65.4, 115.0, 121.2, 129.0, 136.1, 156.8, 161.2; HRMS (ESI $^+$) Calcd for $\text{C}_{10}\text{H}_{11}\text{ClN}_3\text{O}_3$ [$\text{M} + \text{H}$] $^+$: 256.0483; Found 256.0482. Anal. Calcd for $\text{C}_{10}\text{H}_{10}\text{ClN}_3\text{O}_3$: C, 46.98; H, 3.94; N, 16.44. Found: C, 47.00; H, 3.74; N, 16.32.

Ethyl 2-((4-methoxyphenyl)carbamoyl)diazenecarboxylate (D2; R 1 = 4-MeO)

Red solid, yield: 1.217 g (97%), mp 72.5–73.3 °C (CH_2Cl_2); IR: ν 3249, 1759, 1739, 1715, 1601, 1557, 1509, 1468, 1418, 1371, 1302, 1257, 1231, 1200, 1173, 1116, 1027, 1016 cm^{-1} ; ^1H NMR (500 MHz, DMSO- d_6): δ (ppm) 1.37 (t, $J = 7.1$ Hz, 3H), 3.76 (s, 3H), 4.52 (q, $J = 7.1$ Hz, 2H), 6.95–7.03 (m, 2H), 7.64–7.71 (m, 2H), 11.49

(s, 1H); ^{13}C NMR (126 MHz, DMSO- d_6): δ (ppm) 13.9, 55.2, 65.3, 114.2, 121.1, 130.2, 156.3, 156.6, 161.5; HRMS (ESI $^+$) Calcd for $\text{C}_{11}\text{H}_{14}\text{N}_3\text{O}_4$ [M + H] $^+$: 252.0979; Found 252.0975. Anal. Calcd for $\text{C}_{11}\text{H}_{13}\text{N}_3\text{O}_4$: C, 52.39; H, 5.22; N, 16.73. Found: C, 52.69; H, 5.82; N, 16.65.

Ethyl 2-((4-isopropylphenyl)carbamoyl)diazene-carboxylate (D3; R 1 = 4-*i*-Pr)

Orange solid, yield: 1.24 g (94%), mp 65.5–67.0 °C (CH $_2$ Cl $_2$); IR: ν 3274, 2999, 2959, 2875, 1778, 1718, 1597, 1558, 1526, 1470 cm $^{-1}$; ^1H NMR (500 MHz, DMSO- d_6): δ (ppm) 1.19 (d, J = 6.5 Hz, 6H) 1.37 (t, J = 7.0 Hz, 3H), 2.87 (sep, J = 6.5 Hz, 1H) 4.51 (q, J = 7.0 Hz, 2H), 7.24–7.33 (m, 2H), 7.61–7.69 (m, 2H), 11.49 (s, 1H); ^{13}C NMR (126 MHz, DMSO- d_6): δ (ppm) 13.9, 23.8, 33.0, 65.4, 119.7, 126.9, 135.0, 145.4, 156.9, 161.5; HRMS (ESI $^+$) Calcd for $\text{C}_{13}\text{H}_{18}\text{N}_3\text{O}_3$ [M + H] $^+$: 264.1343; Found 264.1340. Anal. Calcd for $\text{C}_{13}\text{H}_{17}\text{N}_3\text{O}_3$: C, 59.30; H, 6.51; N, 15.96. Found: C, 59.09; H, 6.42; N, 15.67.

Ethyl 2-((4-butylphenyl)carbamoyl)diazene-carboxylate (D4; R 1 = 4-Bu)

Red oil, yield: 1.370 g (99%); IR: ν 3275, 2957, 2929, 2858, 1728, 1599, 1514, 1466, 1414, 1233 cm $^{-1}$; ^1H NMR (500 MHz, DMSO- d_6): δ (ppm) 0.90 (t, J = 7.5 Hz, 3H), 1.25–1.41 (m, 5H), 1.46–1.60 (m, 2H), 2.53–2.61 (m, 2H), 4.52 (q, J = 7.5 Hz, 2H), 7.21–7.27 (m, 2H), 7.60–7.63 (m, 2H), 11.50 (s, 1H); ^{13}C NMR (126 MHz, DMSO- d_6) δ (ppm) 13.7, 13.9, 21.7, 33.1, 34.2, 65.4, 119.6, 128.9, 134.9, 139.3, 156.8, 161.4; HRMS (ESI $^+$) Calcd for $\text{C}_{14}\text{H}_{20}\text{N}_3\text{O}_3$ [M + H] $^+$: 278.1499; Found 278.1498.

Ethyl 2-((4-*sec*-butyl)phenyl)carbamoyl)diazene-carboxylate (D5; R 1 = 4-*sec*-Bu)

Red oil, yield: 1.355 g (98%); IR: ν 3284, 2960, 2929, 2873, 1769, 1721, 1597, 1557, 1517, 1454 cm $^{-1}$; ^1H NMR (500 MHz, DMSO- d_6): δ (ppm) 0.76 (t, J = 7.5 Hz, 3H), 1.18 (d, J = 7.0 Hz, 3H), 1.37 (t, J = 7.0 Hz, 3H), 1.48–1.60 (m, 2H), 2.55–2.62 (m, 1H), 4.51 (q, J = 7.5 Hz, 2H), 7.20–7.28 (m, 2H), 7.61–7.69 (m, 2H), 11.49 (s, 1H); ^{13}C NMR (126 MHz, DMSO- d_6): δ (ppm) 12.0, 13.9, 21.7, 30.5, 40.4, 65.4, 119.7, 127.5, 135.0, 144.1, 156.9, 161.5; HRMS (ESI $^+$) Calcd for $\text{C}_{14}\text{H}_{20}\text{N}_3\text{O}_3$ [M + H] $^+$: 278.1499; Found 278.1491.

Ethyl 2-((4-*tert*-butyl)phenyl)carbamoyl)diazene-carboxylate (D6; R 1 = 4-*t*-Bu)

Orange oil, yield: 1.342 g (97%); IR: ν 3274, 2962, 2869, 1766, 1726, 1598, 1518, 1474, 1408, 1365 cm $^{-1}$; ^1H NMR (500 MHz, DMSO- d_6): δ (ppm) 1.28 (s, 9H), 1.37 (t, J = 7.0 Hz, 3H), 4.51 (q, J = 7.0 Hz, 2H), 7.40–7.47 (m, 2H), 7.61–7.69 (m, 2H), 11.49 (s, 1H); ^{13}C NMR (126 MHz, DMSO- d_6): δ (ppm) 13.9, 31.1, 34.2, 65.4, 119.5, 125.8, 134.7, 147.6, 156.9, 161.5; HRMS (ESI $^+$) Calcd for $\text{C}_{14}\text{H}_{23}\text{N}_4\text{O}_3$ [M + NH $_4$] $^+$: 295.1765; Found 295.1761.

5. 1. 3. General Procedure for the Synthesis Diazenedicarboxamides

A modified procedure we described earlier¹⁶ was employed for the synthesis of diazenedicarboxamides. A solution of the appropriate picolyl amine (0.108 g, 1 mmol) in acetonitrile (2.5 mL) was added to the solution of the selected diazenecarboxylate **D** (1 mmol) in acetonitrile (2.5 mL). The reaction mixture was stirred for 1 h at 0 °C, solid material was filtered off and washed with acetonitrile at 0 °C to give the final product. The synthesis of **5–8** was carried out in methanol. The reaction mixture was stirred at –15 °C for 1 h, then at rt for 20 h, evaporated to dryness and the residue was purified by column chromatography using ethyl acetate or ethyl acetate/petroleum ether (1:1) as a solvent to obtain the desired diazenedicarboxamide. Crude product was crystallized from the appropriate solvent.

***N* 1 -(4-Chlorophenyl)-*N* 2 -(pyridin-2-ylmethyl)diazene-1,2-dicarboxamide (3)**

Orange solid, yield: 0.216 g (68%), mp 149.5–151.5 °C (MeOH); IR: ν 3315, 3237, 2988, 1741, 1707, 1599, 1589, 1568, 1516, 1490, 1434, 1424, 1402, 1359, 1305, 1258, 1245, 1216, 1147, 1087 cm $^{-1}$; ^1H NMR (500 MHz, DMSO- d_6): δ (ppm) 4.59 (d, J = 6.1 Hz, 2H), 7.30–7.35 (m, 1H), 7.38–7.43 (m, 1H), 7.47–7.52 (m, 2H), 7.74–7.79 (m, 2H), 7.80–7.85 (m, 1H), 8.53–8.57 (m, 1H), 9.60 (t, J = 6.1 Hz, 1H), 11.53 (s, 1H); ^{13}C NMR (126 MHz, DMSO- d_6): δ (ppm) 45.3, 121.1, 121.3, 122.6, 128.7, 129.1, 136.4, 136.9, 149.1, 157.1, 158.1, 161.9; HRMS (ESI $^+$) Calcd for $\text{C}_{14}\text{H}_{13}\text{ClN}_5\text{O}_2$ [M + H] $^+$: 318.0752; Found 318.0752. Anal. Calcd for $\text{C}_{14}\text{H}_{12}\text{ClN}_5\text{O}_2$: C, 52.92; H, 3.81; N, 22.04. Found: C, 52.86; H, 3.62; N, 21.95.

***N* 1 -(4-Methoxyphenyl)-*N* 2 -(pyridin-2-ylmethyl)diazene-1,2-dicarboxamide (4)**

Red solid, yield: 0.152 g (49%), mp 139.5–141.0 °C (MeOH); IR: ν 3234, 2969, 1735, 1709, 1600, 1589, 1570, 1530, 1507, 1468, 1435, 1412, 1354, 1303, 1263, 1230, 1179, 1078, 1027 cm $^{-1}$; ^1H NMR (500 MHz, DMSO- d_6): δ (ppm) 3.76 (s, 3H), 4.58 (d, J = 6.1 Hz, 2H), 6.95–7.02 (m, 2H), 7.29–7.35 (m, 1H), 7.38–7.42 (m, 1H), 7.63–7.69 (m, 2H), 7.79–7.85 (m, 1H), 8.52–8.57 (m, 1H), 9.54 (t, J = 6.0 Hz, 1H), 11.28 (s, 1H); ^{13}C NMR (126 MHz, DMSO- d_6): δ (ppm) 45.3, 55.3, 114.3, 121.0, 121.3, 122.5, 130.5, 136.9, 149.1, 156.4, 157.1, 157.8, 162.2; HRMS (ESI $^+$) Calcd for $\text{C}_{15}\text{H}_{16}\text{N}_5\text{O}_3$ [M + H] $^+$: 314.1248; Found 314.1246. Anal. Calcd for $\text{C}_{15}\text{H}_{15}\text{N}_5\text{O}_3$: C, 57.50; H, 4.83; N, 22.35. Found: C, 57.28; H, 4.75; N, 22.11.

***N* 1 -(4-Isopropylphenyl)-*N* 2 -(pyridin-2-ylmethyl)diazene-1,2-dicarboxamide (5)**

Orange solid, yield: 0.238 g (73%), mp 119.0–120.5 °C (ethyl acetate); IR: ν 3227, 3048, 2953, 1741, 1708, 1591,

1571, 1516, 1473, 1435 cm^{-1} ; ^1H NMR (500 MHz, $\text{DMSO-}d_6$): δ (ppm) 1.20 (d, $J = 7.0$ Hz, 6H), 2.88 (sep, $J = 7.0$ Hz, 1H), 4.59 (d, $J = 6.0$ Hz, 2H), 7.25–7.35 (m, 3H), 7.38–7.43 (m, 1H), 7.62–7.69 (m, 2H), 7.78–7.86 (m, 1H), 8.53–8.58 (m, 1H), 9.56 (t, $J = 6.0$ Hz, 1H), 11.29 (s, 1H); ^{13}C NMR (126 MHz, $\text{DMSO-}d_6$) δ (ppm) 23.9, 32.9, 45.3, 119.6, 121.3, 122.5, 126.9, 135.2, 136.9, 145.0, 149.0, 157.1, 158.1, 162.1; HRMS (ESI⁺) Calcd for $\text{C}_{17}\text{H}_{20}\text{N}_5\text{O}_2$ [M + H]⁺: 326.1612; Found 326.1609. Anal. Calcd for $\text{C}_{17}\text{H}_{19}\text{N}_5\text{O}_2$: C, 62.75; H, 5.89; N, 21.52. Found: C, 62.76; H, 5.75; N, 21.45.

***N*¹-(4-Butylphenyl)-*N*²-(pyridin-2-ylmethyl)diazene-1,2-dicarboxamide (6)**

Yellow solid, yield: 0.239 g (70%), mp 122.5–124.0 °C (petroleum ether/ethyl acetate); IR: ν 3262, 2954, 2928, 2854, 1752, 1709, 1688, 1594, 1531, 1514 cm^{-1} ; ^1H NMR (500 MHz, $\text{DMSO-}d_6$): δ (ppm) 0.90 (t, $J = 7.5$ Hz, 3H), 1.25–1.35 (m, 2H), 1.50–1.60 (m, 2H), 2.57 (t, $J = 7.5$ Hz, 2H), 4.59 (d, $J = 6.0$ Hz, 2H), 7.20–7.26 (m, 2H), 7.30–7.35 (m, 1H), 7.38–7.43 (m, 2H), 7.61–7.69 (m, 1H), 7.79–7.85 (m, 1H), 8.53–8.58 (m, 1H), 9.56 (t, $J = 6.0$ Hz, 1H), 11.29 (s, 1H); ^{13}C NMR (126 MHz, $\text{DMSO-}d_6$) δ (ppm) 13.7, 21.7, 33.1, 34.2, 45.3, 119.5, 121.3, 122.5, 128.8, 135.1, 136.9, 139.0, 149.1, 157.1, 158.0, 162.1; HRMS (ESI⁺) Calcd for $\text{C}_{18}\text{H}_{22}\text{N}_5\text{O}_2$ [M + H]⁺: 340.1766; Found 340.1766. Anal. Calcd for $\text{C}_{18}\text{H}_{21}\text{N}_5\text{O}_2$: C, 63.70; H, 6.24; N, 20.64. Found: C, 63.51; H, 6.18; N, 20.39.

***N*¹-(4-(*sec*-Butyl)phenyl)-*N*²-(pyridin-2-ylmethyl)diazene-1,2-dicarboxamide (7)**

Orange solid, yield: 0.195 g (57%), mp 93.5–94.5 °C (petroleum ether/ethyl acetate); IR: ν 3230, 2959, 2926, 2872, 1709, 1593, 1571, 1516, 1473, 1435 cm^{-1} ; ^1H NMR (500 MHz, $\text{DMSO-}d_6$): δ (ppm) 0.77 (t, $J = 7.5$ Hz, 3H), 1.18 (d, $J = 7.0$ Hz, 3H), 1.48–1.60 (m, 2H), 2.55–2.62 (m, $J = 6.0$ Hz, 1H), 4.58 (d, $J = 6.0$ Hz, 2H), 7.21–7.27 (m, 2H), 7.30–7.34 (m, 1H), 7.38–7.42 (m, 1H), 7.62–7.67 (m, 2H), 7.79–7.85 (m, 1H), 8.53–8.57 (m, 1H), 9.56 (t, $J = 6.0$ Hz, 1H), 11.29 (s, 1H); ^{13}C NMR (126 MHz, $\text{DMSO-}d_6$) δ (ppm) 12.1, 21.7, 30.5, 40.0, 45.3, 119.6, 121.3, 122.6, 127.5, 135.2, 137.0, 143.9, 149.1, 157.1, 158.1, 162.1; HRMS (ESI⁺) Calcd for $\text{C}_{18}\text{H}_{22}\text{N}_5\text{O}_2$ [M + H]⁺: 340.1768; Found 340.1764. Anal. Calcd for $\text{C}_{18}\text{H}_{21}\text{N}_5\text{O}_2 \times 0.33 \text{H}_2\text{O}$: C, 62.59; H, 6.32; N, 20.28. Found: C, 62.59; H, 6.03; N, 20.00.

***N*¹-(4-(*tert*-Butyl)phenyl)-*N*²-(pyridin-2-ylmethyl)diazene-1,2-dicarboxamide (8)**

Orange solid, yield: 0.254 g (75%), mp 105.5–108.5 °C (petroleum ether/ethyl acetate); IR: ν 3228, 3049, 2952, 1739, 1709, 1592, 1571, 1516, 1571, 1516 cm^{-1} ; ^1H NMR (500 MHz, $\text{DMSO-}d_6$): δ (ppm) 1.28 (s, 9H), 4.59 (d, $J = 6.0$ Hz, 2H), 7.30–7.35 (m, 1H), 7.38–7.47 (m, 3H), 7.62–7.86 (m, 2H), 7.79–7.86 (m, 1H), 8.531–8.580

(m, 1H), 9.56 (t, $J = 6.0$ Hz, 1H) 11.29 (s, 1H); ^{13}C NMR (126 MHz, $\text{DMSO-}d_6$) δ (ppm) 31.1, 34.1, 45.3, 119.3, 121.3, 122.5, 125.8, 134.9, 136.9, 147.3, 149.1, 157.1, 158.1, 162.1; HRMS (ESI⁺) Calcd for $\text{C}_{18}\text{H}_{22}\text{N}_5\text{O}_2$ [M + H]⁺: 340.1768; Found 340.1763. Anal. Calcd for $\text{C}_{18}\text{H}_{21}\text{N}_5\text{O}_2$: C, 63.70; H, 6.24; N, 20.64. Found: C, 63.73; H, 6.12; N, 20.47.

***N*¹-(4-Chlorophenyl)-*N*²-(pyridin-3-ylmethyl)diazene-1,2-dicarboxamide (9)**

Orange solid, yield: 0.220 g (70 %), mp 168.0–169.5 °C (MeOH); IR: ν 3332, 2951, 1732, 1712, 1609, 1596, 1541, 1512, 1489, 1439, 1426, 1400, 1345, 1308, 1250, 1234, 1189, 1089 cm^{-1} ; ^1H NMR (500 MHz, $\text{DMSO-}d_6$): δ (ppm) 4.53 (d, $J = 6.0$ Hz, 2H), 7.39–7.44 (m, 1H), 7.46–7.51 (m, 2H), 7.72–7.79 (m, 3H), 8.50–8.53 (m, 1H), 8.57–8.61 (m, 1H), 9.63 (t, $J = 6.0$ Hz, 1H), 11.54 (s, 1H); ^{13}C NMR (126 MHz, $\text{DMSO-}d_6$): δ (ppm) 41.2, 121.1, 123.6, 128.7, 129.1, 133.6, 135.4, 136.4, 148.6, 149.0, 158.0, 161.8; HRMS (ESI⁺) Calcd for $\text{C}_{14}\text{H}_{13}\text{ClN}_5\text{O}_2$ [M + H]⁺: 318.0750; Found 318.0764. Anal. Calcd for $\text{C}_{14}\text{H}_{12}\text{ClN}_5\text{O}_2$: C, 52.92; H, 3.81; N, 22.04. Found: C, 52.86; H, 3.84; N, 22.05.

***N*¹-(4-Methoxyphenyl)-*N*²-(pyridin-3-ylmethyl)diazene-1,2-dicarboxamide (10)**

Orange solid, yield: 0.189 g (60%), mp 146.5–147.5 °C (MeOH); IR: ν 3295, 2928, 1741, 1698, 1608, 1580, 1545, 1509, 1484, 1463, 1444, 1432, 1422, 1336, 1301, 1273, 1241, 1184, 1171, 1112, 1034, 1013 cm^{-1} ; ^1H NMR (500 MHz, $\text{DMSO-}d_6$): δ (ppm) 3.75 (s, 3H), 4.51 (d, $J = 6.1$ Hz, 2H), 6.96–7.01 (m, 2H), 7.39–7.44 (m, 1H), 7.63–7.68 (m, 2H), 7.75–7.79 (m, 1H), 8.49–8.53 (m, 1H), 8.57–8.61 (m, 1H), 9.56 (t, $J = 6.1$ Hz, 1H), 11.28 (s, 1H); ^{13}C NMR (126 MHz, $\text{DMSO-}d_6$): δ (ppm) 41.1, 55.3, 114.3, 121.0, 123.6, 130.5, 133.7, 135.4, 148.6, 149.0, 156.4, 157.7, 162.2; HRMS (ESI⁺) Calcd for $\text{C}_{15}\text{H}_{16}\text{N}_5\text{O}_3$ [M + H]⁺: 314.1248; Found 314.1250. Anal. Calcd for $\text{C}_{15}\text{H}_{15}\text{N}_5\text{O}_3$: C, 57.50; H, 4.83; N, 22.35. Found: C, 57.44; H, 4.63; N, 22.28.

***N*¹-(4-Isopropylphenyl)-*N*²-(pyridin-3-ylmethyl)diazene-1,2-dicarboxamide (11)**

Yellow crystals, yield: 0.276 g (85%), mp 123–126 °C (ethyl acetate); IR: ν 3227, 3050, 2691, 1713, 1551, 1514, 1431, 1422, 1262 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3): δ (ppm) 1.23 (d, $J = 6.9$ Hz, 6H), 2.90 (sep, $J = 6.9$ Hz, 1H), 4.63 (d, $J = 6.2$ Hz, 2H), 7.20–7.27 (m, 3H), 8.58 (d, $J = 8.5$ Hz, 2H), 7.67–7.70 (m, 1H), 8.19–8.24 (m, 1H), 8.48–8.51 (m, 1H), 8.59 (d, $J = 1.8$ Hz, 1H), 9.68 (s, 1H); ^{13}C NMR (126 MHz, $\text{DMSO-}d_6$) δ (ppm) 23.9, 33.0, 41.2, 119.6, 123.6, 126.9, 133.7, 135.2, 135.4, 145.2, 148.6, 149.0, 158.0, 162.0; HRMS (ESI⁺) Calcd for $\text{C}_{17}\text{H}_{20}\text{N}_5\text{O}_2$ [M + H]⁺: 326.1612; Found 326.1604. Anal. Calcd for $\text{C}_{17}\text{H}_{19}\text{N}_5\text{O}_2$: C, 62.75; H, 5.89; N, 21.52. Found: C, 62.23; H, 5.87; N, 21.29.

***N*¹-(4-Butylphenyl)-*N*²-(pyridin-3-ylmethyl)diazene-1,2-dicarboxamide (12)**

Yellow solid, yield: 0.266 g (78%), mp 135.5–137.0 °C (petroleum ether/ethyl acetate); IR: ν 3336, 2953, 2927, 2854, 1728, 1703, 1613, 1540, 1512, 1456 cm⁻¹; ¹H NMR (500 MHz, DMSO-*d*₆): δ (ppm) 0.89 (t, *J* = 7.5 Hz, 3H), 1.25–1.35 (m, 2H), 1.49–1.60 (m, 2H), 2.56 (t, *J* = 7.5 Hz, 2H), 4.52 (d, *J* = 6.0 Hz, 2H), 7.18–7.27 (m, 2H), 7.38–7.45 (m, 1H), 7.60–7.66 (m, 2H), 7.74–7.80 (m, 1H), 8.48–8.54 (m, 1H), 8.57–8.63 (m, 1H), 9.59 (t, *J* = 6.0 Hz, 1H), 11.30 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ (ppm) 13.7, 21.7, 33.1, 34.2, 41.1, 119.5, 123.6, 128.8, 133.6, 135.1, 135.4, 139.0, 148.6, 148.9, 157.9, 162.0; HRMS (ESI⁺) Calcd for C₁₈H₂₂N₅O₂ [M + H]⁺: 340.1766; Found 340.1767. Anal. Calcd for C₁₈H₂₁N₅O₂: C, 63.70; H, 6.24; N, 20.64. Found: C, 63.59; H, 6.34; N, 20.59.

***N*¹-(4-(*sec*-Butyl)phenyl)-*N*²-(pyridin-3-ylmethyl)diazene-1,2-dicarboxamide (13)**

Orange crystals, yield: 0.252 g (74%), mp 163–168 °C (ethyl acetate); IR: ν 3500, 3277, 2958, 1731, 1702, 1611, 1559, 1514, 1434, 1283 cm⁻¹; ¹H NMR (500 MHz, CDCl₃): δ (ppm) 0.81 (t, *J* = 7.4 Hz, 3H), 1.22 (d, *J* = 7.0 Hz, 3H), 1.53–1.62 (m, 2H), 2.57–2.63 (m, 1H), 4.65 (d, *J* = 6.2 Hz, 2H), 7.19 (d, *J* = 8.5 Hz, 2H), 7.23–7.28 (m, 1H), 7.59 (d, *J* = 8.5 Hz, 2H), 7.67–7.71 (m, 1H), 8.07–8.12 (m, 1H), 8.49–8.52 (m, 1H), 8.60 (d, *J* = 1.8 Hz, 1H), 9.55 (s, 1H); ¹³C NMR (126 MHz, CDCl₃) δ (ppm) 12.2, 21.8, 31.1, 41.1, 42.6, 119.8, 123.8, 127.9, 132.6, 134.2, 136.0, 145.8, 149.1, 149.2, 156.1, 160.2; HRMS (ESI⁺) Calcd for C₁₈H₂₂N₅O₂ [M + H]⁺: 340.1768; Found 340.1750. Anal. Calcd for C₁₈H₂₁N₅O₂: C, 63.70; H, 6.24; N, 20.64. Found: C, 63.94; H, 6.27; N, 20.21.

***N*¹-(4-Chlorophenyl)-*N*²-(pyridin-4-ylmethyl)diazene-1,2-dicarboxamide (14)**

Orange solid, yield: 0.211 g (67%), mp 168.8–170.5 °C (methanol); IR: ν 3675, 3328, 2987, 2901, 1739, 1722, 1609, 1565, 1552, 1510, 1490, 1421, 1402, 1376, 1313, 1279, 1258, 1217 cm⁻¹; ¹H NMR (500 MHz, DMSO-*d*₆): δ (ppm) 4.53 (d, *J* = 6.1 Hz, 2H), 7.33–7.38 (m, 2H), 7.47–7.53 (m, 2H), 7.74–7.79 (m, 2H), 8.54–8.59 (m, 2H), 9.69 (t, *J* = 6.10 Hz, 1H), 11.56 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ (ppm) 42.4, 121.1, 122.2, 128.7, 129.1, 136.4, 146.9, 149.7, 158.0, 162.0; HRMS (ESI⁻) Calcd for C₁₄H₁₁ClN₅O₂ [M - H]⁻: 316.0607; Found 316.0620. Anal. Calcd for C₁₄H₁₂ClN₅O₂: C, 52.92; H, 3.81; N, 22.04. Found: C, 52.91; H, 3.65; N, 22.04.

***N*¹-(4-Methoxyphenyl)-*N*²-(pyridin-4-ylmethyl)diazene-1,2-dicarboxamide (15)**

Yellow-orange solid, yield: 0.167 g (53%), mp 165.5–167.5 °C (MeOH); IR: ν 3267, 2781, 1701, 1609, 1568, 1550, 1509, 1463, 1439, 1419, 1371, 1323, 1303, 1262, 1246, 1224, 1170, 1022, 1006 cm⁻¹; ¹H NMR (500

MHz, DMSO-*d*₆): δ (ppm) 3.76 (s, 3H), 4.53 (d, *J* = 6.1 Hz, 2H), 6.97–7.02 (m, 2H), 7.33–7.38 (m, 2H), 7.64–7.70 (m, 2H), 8.54–8.59 (m, 2H), 9.62 (t, *J* = 6.1 Hz, 1H), 11.31 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆): δ (ppm) 42.4, 55.3, 114.3, 121.0, 122.2, 130.5, 147.0, 149.7, 156.5, 157.6, 162.3; HRMS (ESI⁺) Calcd for C₁₅H₁₆N₅O₃ [M + H]⁺: 314.1248; Found 314.1246. Anal. Calcd for C₁₅H₁₅N₅O₃: C, 57.50; H, 4.83; N, 22.35. Found: C, 57.54; H, 4.73; N, 22.28.

***N*¹-(4-Isopropylphenyl)-*N*²-(pyridin-4-ylmethyl)diazene-1,2-dicarboxamide (16)**

Yellow solid, yield: 0.264 g (81%), mp 162–165 °C (acetone/ethyl acetate); IR: ν 3201, 2960, 1708, 1611, 1535, 1261 cm⁻¹; ¹H NMR (300 MHz, DMSO-*d*₆): δ (ppm) 1.21 (d, *J* = 6.9 Hz, 6H), 2.88 (sep, *J* = 6.9 Hz, 1H), 4.53 (d, *J* = 6.0 Hz, 2H), 7.29 (m, 2H), 7.36 (m, 2H), 7.66 (m, 2H), 8.57 (m, 2H), 9.61 (t, *J* = 6.0 Hz, 1H), 11.27 (s, 1H); ¹³C NMR (75 MHz, DMSO-*d*₆): δ (ppm) 23.7, 32.8, 42.3, 119.6, 122.1, 126.8, 135.1, 145.0, 146.8, 149.6, 157.9, 162.1. MS (FAB): *m/z* 326 (MH⁺, 73), 107 (62), 71 (93) 55 (100). Anal. Calcd for C₁₇H₁₉N₅O₂: C, 62.75; H, 5.89; N, 21.52. Found: C, 62.72; H, 5.91; N, 21.51.

***N*¹-(4-Butylphenyl)-*N*²-(pyridin-4-ylmethyl)diazene-1,2-dicarboxamide (17)**

Yellow solid, yield: 0.273 g (80.5%), mp 156.0–157.0 °C (petroleum ether/ethyl acetate); IR: ν 3284, 2923, 2854, 1717, 1608, 1548, 1511, 1420, 1373, 1316 cm⁻¹; ¹H NMR (500 MHz, DMSO-*d*₆): δ (ppm) 0.90 (t, *J* = 7.5 Hz, 3H), 1.26–1.35 (m, 2H), 1.49–1.60 (m, 2H), 2.57 (t, *J* = 7.5 Hz, 2H), 4.53 (d, *J* = 6.0 Hz, 2H), 7.20–7.26 (m, 2H), 7.35–7.40 (m, 2H), 7.61–7.67 (m, 2H), 8.54–8.60 (m, 2H), 9.65 (t, *J* = 6.0 Hz, 1H), 11.33 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ (ppm) 13.7, 21.7, 33.1, 34.2, 42.4, 119.5, 122.2, 128.9, 135.1, 139.1, 147.0, 149.7, 157.9, 162.2; HRMS (ESI⁺) Calcd for C₁₈H₂₂N₅O₂ [M + H]⁺: 340.1768; Found 340.1769. Anal. Calcd for C₁₈H₂₁N₅O₂: C, 63.70; H, 6.24; N, 20.64. Found: C, 63.54; H, 6.24; N, 20.57.

***N*¹-(4-(*sec*-Butyl)phenyl)-*N*²-(pyridin-4-ylmethyl)diazene-1,2-dicarboxamide (18)**

Yellow solid, yield: 0.258 g (76%); mp 154.6–158.6 °C (ethyl acetate); IR: ν 3447, 3208, 2961, 2928, 1708, 1611, 1536, 1515, 1260 cm⁻¹; ¹H NMR (500 MHz, DMSO-*d*₆): δ (ppm) 0.77 (t, *J* = 7.5 Hz, 3H), 1.17 (d, *J* = 7.0 Hz, 3H), 1.49–1.59 (m, 2H), 2.55–2.62 (m, *J* = 6.0 Hz, 1H), 4.53 (d, *J* = 6.0 Hz, 2H), 7.21–7.27 (m, 2H), 7.33–7.39 (m, 2H), 7.60–7.68 (m, 2H), 8.52–8.60 (m, 2H), 9.64 (t, *J* = 6.0 Hz, 1H), 11.32 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ (ppm) 12.1, 21.8, 30.5, 40.4, 42.4, 119.7, 122.2, 127.5, 135.2, 144.0, 147.0, 149.8, 158.0, 162.2. MS (FAB): *m/z* 340 (MH⁺, 100), 107 (34), 93 (54), 69 (31). Anal. Calcd for C₁₈H₂₁N₅O₂: C, 63.70; H, 6.24; N, 20.64. Found: C, 63.65; H, 6.23; N, 20.67.

***N*¹-(4-(*tert*-Butyl)phenyl)-*N*²-(pyridin-4-ylmethyl) diazene-1,2-dicarboxamide (19)**

Yellow solid, yield: 0.256 g (75%); mp 180–185 °C (ethyl acetate/methanol); IR: ν 3198, 3030, 2961, 1707, 1610, 1537, 1516, 1262 cm^{-1} ; ¹H NMR (300 MHz, DMSO-*d*₆): δ (ppm) 1.28 (s, 9H), 4.53 (d, *J* = 5.8 Hz, 2H), 7.35 (m, 2H), 7.43 (m, 2H), 7.65 (m, 2H), 8.56 (m, 2H), 9.60 (t, *J* = 5.8 Hz, 1H), 11.3 (s, 1H); ¹³C NMR (75 MHz, DMSO-*d*₆) δ (ppm) 31.0, 34.1, 42.4, 119.3, 122.1, 125.7, 134.8, 146.9, 147.3, 149.6, 158.0, 162.1. MS (FAB): *m/z* 340 (MH⁺, 100), 107 (34), 71 (39), 55 (37). Anal. Calcd for C₁₈H₂₁N₅O₂: C, 63.70; H, 6.24; N, 20.64. Found: C, 63.56; H, 6.03; N, 20.79.

5. 2. Biology

5. 2. 1. Cell Culture

Human cervical carcinoma HeLa and laryngeal carcinoma HEp-2 cells were obtained from cell culture bank (GIBCO BRL, Invitrogen, Grand Island, USA). Development of HEp-2 subline resistant to carboplatin has been published previously.^{17a} These cells are cross-resistant to anti-cancer drug cisplatin^{17a} and natural compound curcumin as well.^{17b} Colorectal carcinoma HCT-116, and lung adenocarcinoma H460 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA). All mentioned cell lines were cultured as a monolayer culture in Dulbecco's medium supplemented with 10% fetal serum (Gibco BRL, Invitrogen, USA) and cultured in a humidified atmosphere of 5% CO₂ at 37 °C.

5. 2. 2. Compounds

Diazenedicarboxamides were dissolved in DMSO. They were stored at –20 °C, and diluted to the appropriate concentrations with growth medium just before use.

5. 2. 3. Cytotoxicity Assay

Cytotoxic activity of new compounds was determined by MTT assay,¹⁸ modified as described. Cells were seeded into 96-well tissue culture plates (3000 cells/0.18 mL medium/well). The next day different concentrations of new compounds were added (0.02 mL) to each well and each concentration was tested in quadruplicate. Following 72 h incubation at 37 °C, the medium was aspirated, and 20 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye (Sigma Aldrich, USA) /0.04 mL medium/well was added. Four hours later, formazan crystals were dissolved in DMSO (0.17 mL/well), the plates were mechanically agitated for 5 min and the optical density at 545 nm was determined on a microtiter plate reader (Awareness Technology Inc, Palm City, FL). Each experiment was repeated three times.

5. 2. 4. The Role of Glutathione (GSH) in Cell Response to 10, 11 and 13

To examine the possible role of GSH in cell response to the selected diazenedicarboxamides **10**, **11** and **13** we used HeLa cells, the model system that we applied for the screening of new compounds. The cells were seeded in the morning as described above for the examination of cytotoxic activity. After 6 h, when the cells were already attached to the surface of petri dishes, in each well the specific inhibitor of glutathione synthesis, buthionine sulfoximine (BSO)¹² was added at the final concentration of 0.01 mM. Following the next 18 h, the diazenedicarboxamides **10**, **11** or **13** were added at the final concentration from 2 to 200 mM and 72 h later the cell survival was determined by MTT assay.

5. 2. 5. Type of Cell Death Induced by 10, 11 and 13

While the cytotoxic assay has shown that diazenes **10**, **11** and **13** triggered cell death in all investigated cell lines, we used again the most sensitive, HeLa cells, to examine the type of cell death that is triggered by **10**, **11** and **13**. To determine if the necrosis has occurred, the loss of membrane integrity was examined, as measured by massive influx of trypan blue in cells. Cells (3.7×10^4) were plated in 24 well plates. The next day they were rinsed with a physiological saline, and treated with 34 μM of **10**, 100 μM of **11** and 80 μM of **13** for 1 hour. After rinsing with saline, the cells were treated with 0.2% (w / v) trypan blue for 15 minutes. After staining, the cells were again rinsed with saline, and incubated in saline for further investigation. The cells that have lost the membrane integrity, i.e. necrotic cells, are clearly stained blue. Images of stained and unstained cells were taken at a hundredfold magnification with a Pixera Pro150ES Camera.

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Povzetek

Za večjo uspešnost zdravljenja raka nujno potrebujemo učinkovitejša zdravila, kot tudi nove strategije zdravljenja. Naše dosedanje raziskave so pokazale, da so številni diazeni citotoksični za različne tumorske celice in da lahko izničijo odpornost le-teh na cisplatin in vinkristin. Izkazalo se je tudi, da imata nesimetrična diazendikarboksamida **1** in **2** obeta-jočo citotoksičnost. Cilj sedanje raziskave je bila sinteza novih diazendikarboksamidov, ki bi bili primerno topni in citotoksični. Poročamo o sintezi in biološki evalvaciji novih *N,N'*-diazendikarboksamidov. Pokazalo se je, da vodi modifikacija **1** in **2** do aktivnejših spojin. Najučinkovitejši med vsemi je diazendikarboksamid **11**. To spojino lahko obravnavamo kot potencialni protitumorski agens za različne tumorje in za nekatere sublinije, ki so odporne na doslej znana zdravila.