


 Cite this: *RSC Adv.*, 2024, 14, 28659

Synthesis of quinoxalines and assessment of their inhibitory effects against human non-small-cell lung cancer cells†

 Jia-Hua Liang,^a Shu-Tse Cho,^b Tzeng-Lien Shih^{*b} and Jih-Jung Chen^{ID*acde}

Twenty-six quinoxaline derivatives were synthesized to assess their biological activities against human non-small-cell lung cancer cells (A549 cells). Compound **4b** (IC₅₀ = 11.98 ± 2.59 μM) and compound **4m** (IC₅₀ = 9.32 ± 1.56 μM) possess anticancer activity comparable to 5-fluorouracil (clinical anticancer drug) (IC₅₀ = 4.89 ± 0.20 μM). Western blot tests further confirmed that compound **4m** effectively induced apoptosis of A549 cells through mitochondrial- and caspase-3-dependent pathways. The introduction of bromo groups instead of nitro groups into the quinoxaline skeleton has been shown to provide better inhibition against lung cancer cells in this article. This modification in the molecular structure could enhance the biological activity and effectiveness of quinoxaline derivatives in the design and synthesis of anticancer drugs, making bromo-substituted quinoxalines a promising avenue for further research and development in anticancer therapeutics.

 Received 18th June 2024
 Accepted 3rd September 2024

DOI: 10.1039/d4ra04453c

rsc.li/rsc-advances

1 Introduction

Quinoxaline, known as benzopyrazine, is naphthalene with some carbon atoms replaced with nitrogen atoms¹ (Fig. 1a). The nitrogen atoms of quinoxalines contribute to the stabilization of radical ion species. Naturally occurring quinoxalines are scarce,^{2,3} and their derivatives are mostly derived from synthetic products. Quinoxaline's skeleton is in frame with some natural antibiotics, such as echinomycin,^{4,5} quinornycin A,⁶ triostins⁷ and actinoleutin which show activities against Gram-positive bacteria⁸ and tumors.⁹ Furthermore, the synthetic quinoxaline derivatives possess antimicrobial,^{1,10,11} anti-inflammatory,^{12,13} antiviral,^{1,14} antioxidant,^{13,15} antitumor,^{1,9} receptor antagonist,¹⁶ antitubercular,^{1,17,18} antifungal,^{1,11,15,19–22} antiparasitic²³ and antidiabetic^{1,24–28} activities. Besides the pharmacological interest in quinoxalines, they are also employed in material sciences, such as in OLEDs²⁹ and dye-sensitized solar cells.³⁰

Quinoxaline urea derivatives were evaluated in the treatment of pancreatic therapy.³¹ Therefore, we have reported the synthesis of cinnamils and 2,3-dialkenyl-substituted quinoxalines to evaluate their activities against pancreatic cancer cells.³² Among them, one of cinnamils exhibited a more potent anticancer effect than that of quinoxalines. However, that series of quinoxalines presented a moderate inhibitory effect. Compared with other quinoxalines, synthesizing 2,3-dialkenyl-substituted quinoxalines is relatively rare.^{33,34} They exhibited photophysical properties³⁴ or as a detector,³⁵ however, their biological activity is not well studied. A C6-fluoro substituted quinoxaline was reported as a potent inhibitor of JNK stimulatory phosphatase-1 (JSP-1) in an *in vitro* biological assay (Fig. 1c).³⁶ Due to quinoxalines for their pharmacological interests, it prompted us to introduce an electron-withdrawing group to the C6-position of 2,3-dialkenyl-substituted quinoxalines. Therefore, the bromo or nitro groups were selected to assess their activities against non-small-cell lung cancer cells (Fig. 1b, X = Br, or NO₂) and pursue hit compounds. According to reports, the NO₂ functional group may be genotoxic, so the genotoxicity of **5a–5m** needs further discussion. However, this article aims to investigate the apoptotic mechanisms of the most effective compound **4m**.

^aInstitute of Traditional Medicine, National Yang Ming Chiao Tung University, Taipei 112304, Taiwan. E-mail: jjungchen@nycu.edu.tw; Tel: +886-2-2826-7195

^bDepartment of Chemistry, Tamkang University, 251301 Tamsui Dist., New Taipei City, Taiwan. E-mail: tlshih@mail.tku.edu.tw; Tel: +886-2-8631-5024

^cDepartment of Pharmacy, School of Pharmaceutical Sciences, National Yang Ming Chiao Tung University, Taipei 112304, Taiwan

^dDepartment of Medical Research, China Medical University Hospital, China Medical University, Taichung 404333, Taiwan

^eTraditional Herbal Medicine Research Center, Taipei Medical University Hospital, Taipei 110301, Taiwan

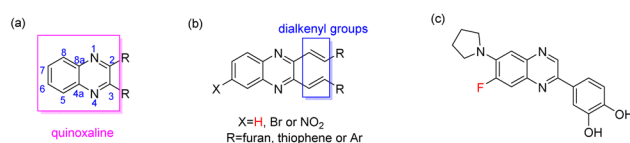
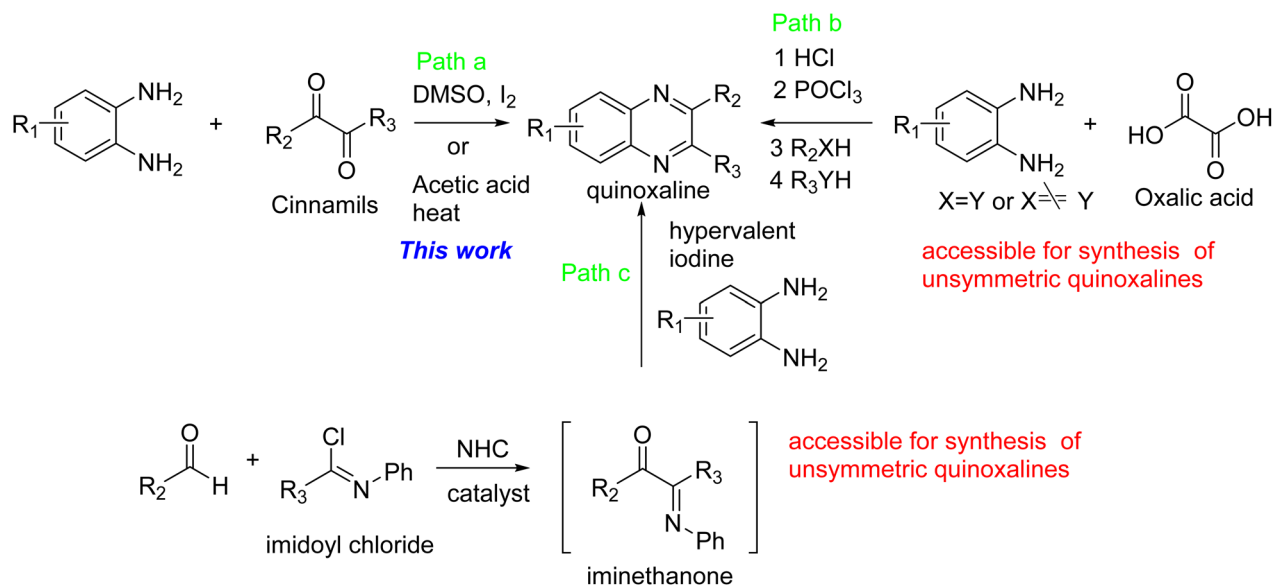
 † Electronic supplementary information (ESI) available: Spectroscopic data of ¹H NMR and ¹³C NMR for new compounds. See DOI: <https://doi.org/10.1039/d4ra04453c>


Fig. 1 The skeletons of quinoxaline (a) and C6-substituted alkenyl quinoxalines (b) are reported in this article, and a quinoxaline derivative (c) is reported in the literature with potent biological activity.





Scheme 1 The reported strategies for the synthesis of quinoxalines.

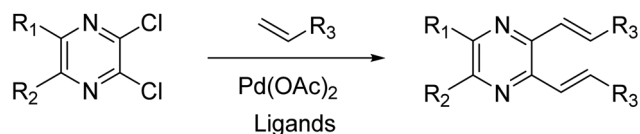


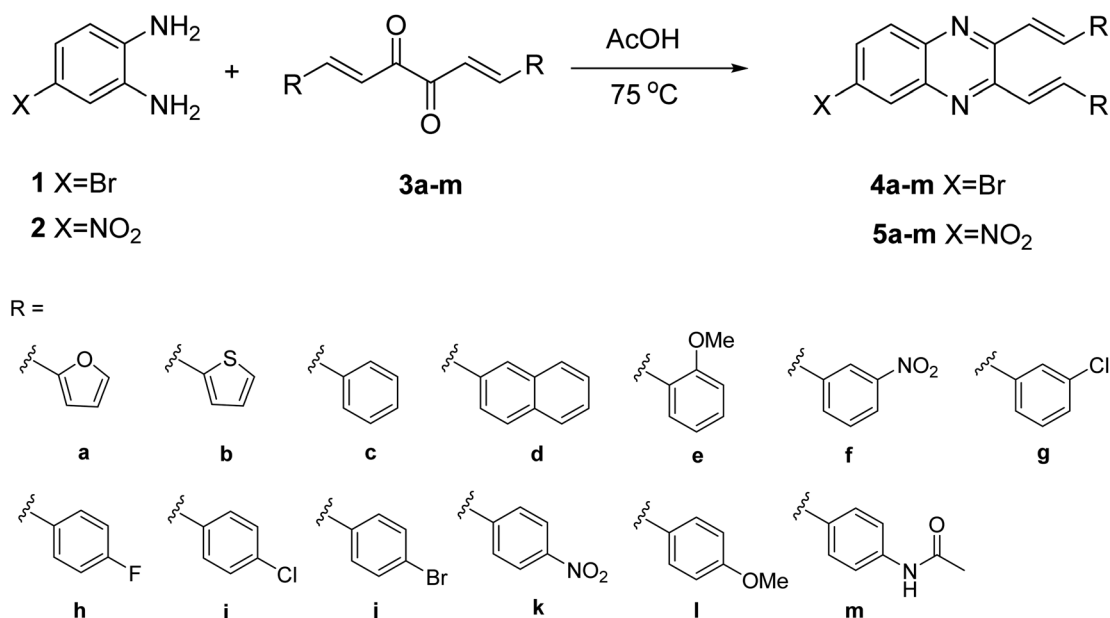
Fig. 2 The synthesis of 2,3-dialkenyl-substituted quinoxalines via the Heck method.

2 Results and discussion

2.1 Chemistry

There are at least three methods available for the synthesis of quinoxalines. First, the condensation of cinnamils with 1,2-

phenylenediamine derivatives under either the catalytic amount of I₂ in DMSO³⁷ or reflux acetic acid,³⁸ which condition is used for the synthesis of target molecules in this article (Path a). Secondly, oxalic acid is condensed with 1,2-phenylenediamine in an acidic condition and then treated with POCl₃ followed by reacting with nucleophiles (Path b).¹⁰ Finally, the key intermediate, iminoethanone, was prepared from aldehyde and imidoyl chloride under the NHC catalyst (path c). The intermediate, iminoethanone, was condensed with 1,2-phenylenediamine catalyzed by hypervalent iodine³⁹ to obtain quinoxaline. It is noticed that Path b and Path c are more readily available for the synthesis of unsymmetric quinoxalines (Scheme 1).



Scheme 2 Synthesis of quinoxalines 4a–m and 5a–m.

Table 1 Inhibitory effects of compounds against human non-small-cell lung cancer cells (A549)^a

Compounds	IC ₅₀ ^b (μM)
4a	54.94 ± 2.13**
4b	11.98 ± 2.59***
4c	>100
4d	>100
4e	35.23 ± 9.29*
4f	>100
4g	42.39 ± 1.40**
4h	>100
4i	>100
4j	>100
4k	>100
4l	38.17 ± 0.48*
4m	9.32 ± 1.56**
5a	>100
5b	>100
5c	>100
5d	>100
5e	>100
5f	>100
5g	75.02 ± 4.50**
5h	>100
5i	>100
5j	>100
5k	>100
5l	>100
5m	>100
5-FU ^c	4.89 ± 0.20**

^a Results are presented as averages ±SD (*n* = 3). ^b Concentration necessary for 50% inhibition (IC₅₀). ^c 5-Fluorouracil (5-FU) was used as a positive control.

Table 2 *In vitro* cytotoxic effects of compound 4m and 5-fluorouracil against Hs68 (human foreskin fibroblast) cells^a

Compounds	IC ₅₀ ^b (μM)
4m	9.29 ± 1.25
5-FU ^c	3.08 ± 0.40

^a Results are presented as averages ±SD (*n* = 3). ^b Concentration necessary for 50% inhibition (IC₅₀). ^c 5-Fluorouracil (5-FU) was used as a positive control.

The previous synthesis of 2,3-dialkenyl-substituted quinoxalines employed a Heck method (Fig. 2).³³ We applied the condensation of **1** or **2** with the corresponding **3a–m** in acetic acid under reflux conditions.³² The resulting solid was filtered by suction and washed with distilled water to obtain **4a–m** and **5a–m** in moderate to high yields without column chromatography (Scheme 2, see experimental section).

2.2 Biological evaluation

The cytotoxic effects of all twenty-six synthesized compounds were evaluated by their activity to suppress human non-small cell lung cancer cells (A549 cells). As shown in Table 1,

compound **4m** (IC₅₀ = 9.32 ± 1.56 μM) exhibited the most potent inhibitory activity against A549 cells. Although compound **4m** showed slightly weaker anticancer activity than 5-fluorouracil (5-FU) against A549 cells. We further compared the cytotoxicity of **4m** and the clinical anticancer drug 5-FU on normal human cells. The cytotoxicity of **4m** (IC₅₀ = 9.29 ± 1.25 μM) to human foreskin fibroblast (Hs68) cells is approximately 3 times lower than that of 5-FU (IC₅₀ = 3.08 ± 1.40 μM) (Table 2). Therefore, it shows that **4m** possesses research and development value. In addition, compounds **4a**, **4b**, **4e**, **4g**, **4l**, and **5g** also exhibited cytotoxic activities against A549 cells.

The effect of treating A549 cells with compound **4m** on the expression of apoptosis-related proteins was investigated. Fig. 3a shows that the expression of the pro-apoptotic protein Bax treated with 20 μM of compound **4m** was higher than that treated with 5 or 10 μM of **4m**. In contrast, cells treated with 20 μM of compound **4m** showed lower Bcl-2 (anti-apoptotic protein) expression than those treated with 5 or 10 μM (Fig. 3b). The results show that compound **4m** suppressed the expression of Bcl-2 and increased the expression of Bax.

Besides, caspase 3 activation is a hallmark of apoptosis. We further investigated whether compound **4m** could influence the enzymatic activities of caspase-3. The results show that compound **4m** suppressed pro-caspase 3 and increased the cleaved caspase-3 (Fig. 3c and d). Furthermore, compound **4m** markedly induced apoptosis of A549 cells through caspase-3-dependent pathways.

3 Conclusions

The development of quinoxalines as anticancer agents has gained attention in recent years.^{40,41} Synthesis of quinoxalines **4a–m** and **5a–m** was facile. Their purifications were simple filtration, then washed with water to obtain the pure target molecules. This series of compounds showed less potency on A549 cells than 5-FU. However, it provides important information that the C6-bromo rather than C6-nitro group is a suitable quinoxaline substitute for designing potential drug candidates. Furthermore, we found compounds **4f**, **4k**, **5f**, and **5k**, which are with two nitro groups, reduced the solubility. Compound **4m** was found to have the highest cytotoxic activity against A549 cells out of all synthesized molecules. The activity of compound **4m** was comparable to that of 5-FU (clinical anticancer drug). Furthermore, compound **4m** markedly induced apoptosis of A549 cells through the mitochondrial- and caspase-3-dependent pathways (Fig. 4). This suggests that the halo substituted at C6-position of 2,3-dialkenyl quinoxalines may be necessary. Thus, compound **4m** is worth further investigation and might be developed as a candidate for treating or preventing human non-small-cell lung cancer.

4 Experimental

All chemicals were purchased from either Acros or Alfa from Uniworld in Taiwan and used from received without further purification except otherwise mentioned. The structure's determinations were based on ¹H and ¹³C-NMR data recorded

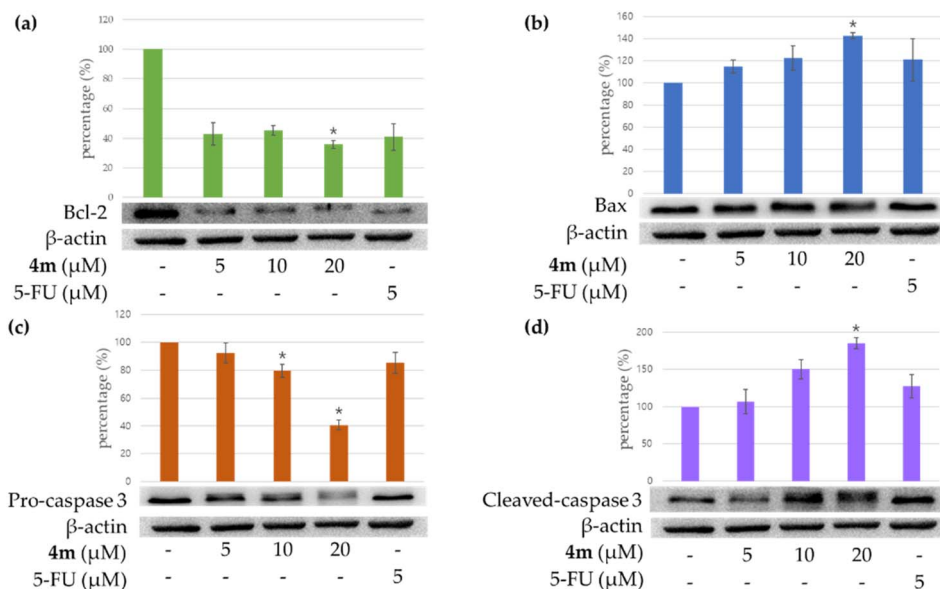


Fig. 3 Western blot analysis for Bcl-2 (a), Bax (b), pro-caspase 3 (c), and cleaved caspase 3 (d) in each group on A549 cells. **4m** means compound **4m**. 5-Fluorouracil (5-FU) was used as a positive control. Asterisks indicate significant differences ($*p < 0.05$) compared with the control group.

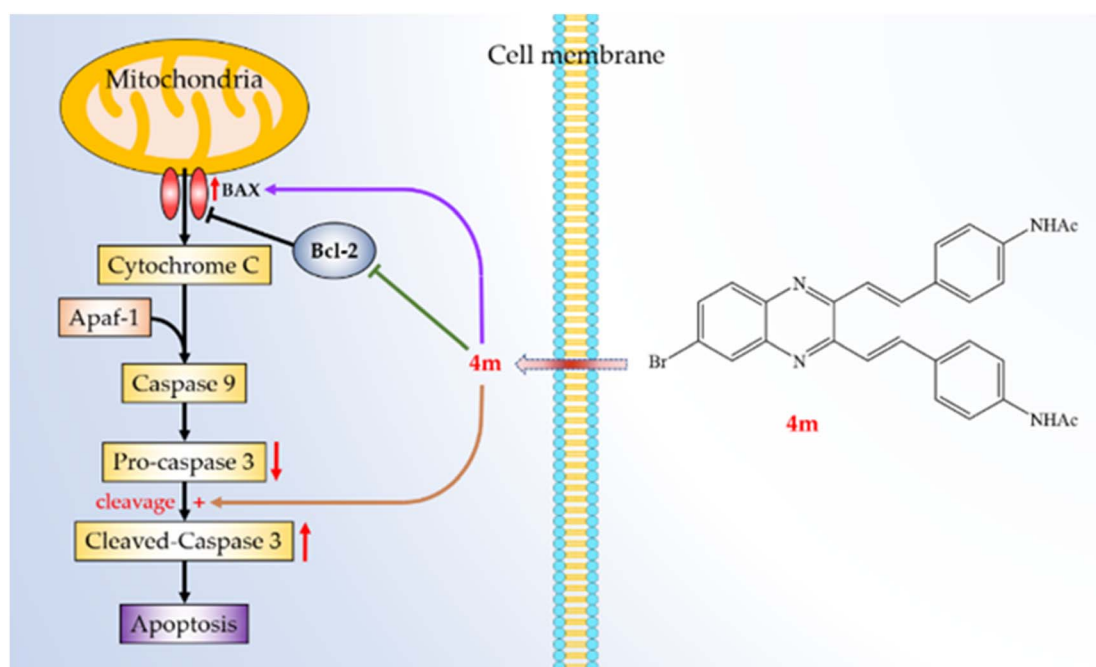


Fig. 4 Schematic diagram for cancer cell apoptosis mechanism of compound **4m** in A549 cells.

on a 600 MHz Bruker Ultrashield instrument. The chemical shifts were reported in part per million (ppm) for the residual solvent (CDCl_3 : 7.26 ppm for ^1H ; 77.0 ppm for ^{13}C). The splitting constant (J) was represented in hertz (Hz), and the splitting patterns were designated as s (singlet), br s (broad singlet), d (doublet), dd (doublet of doublets), t (triplet), and m (multiplet). The purity of compounds was performed by high-resolution mass spectrometry (HRMS) using a Bruker Ultra-Flex II instrument (from Bruker Taiwan Co. Ltd) for electrospray ionization (ESI). The reaction processes were monitored by thin

layer chromatography (Analtech Silica gel HLF UV254) and visualized by UV (256 or 365 nm) or stained with KMnO_4 or *p*-anisaldehyde solution. The melting points were determined using an MP-2D apparatus and were uncorrected.

4.1 General procedure for the synthesis of compounds **4a–m** and **5a–m**

Compound **1** (4-bromo-1,2-diaminobenzene, 1.0 equivalent) and the corresponding cinnamil **3a–m** (1.2 equivalent) were

dissolved in acetic acid (0.2 M). This resulting mixture was heated at 75 °C for 6 h and then cooled in an ice bath until a precipitate formed. The precipitate was filtrated by suction and washed with H₂O to obtain the target molecules **4a–m**, respectively. Synthesis of compounds **5a–m** followed the method mentioned above.

4.2 Spectra data

4.2.1 6-Bromo-2,3-bis[(E)-2-(furan-2-yl)vinyl]quinoxaline (4a). Use compound **1** (0.093 g, 0.495 mmol) and compound **3a** (0.10 g, 0.413 mmol) to afford compound **4a** (0.144 g, 0.366 mmol). Yield: 89%. Mp 213–214 °C. A brown solid. ¹H NMR (600 MHz, CDCl₃) δ 8.15 (d, *J* = 1.7 Hz, 1H), 7.83 (d, *J* = 13.4 Hz, 2H), 7.82 (d, *J* = 9.6 Hz, 1H), 7.70 (dd, *J* = 8.9, 1.6 Hz, 1H), 7.55 (d, *J* = 3.2 Hz, 1H), 7.54 (d, *J* = 15.2 Hz, 2H), 7.53 (s, 1H), 6.62 (t, *J* = 4.8 Hz, 2H), 6.51 (d, *J* = 1.4 Hz, 2H). ¹³C NMR (150 MHz, CDCl₃) δ 152.9, 152.8, 149.4, 148.9, 143.8, 143.7, 142.1, 140.3, 132.7, 131.0, 130.0, 125.1, 124.8, 123.1, 119.8, 119.7, 113.1, 112.9, 112.3. HRMS (ESI) calculated for C₂₀H₁₄BrN₂O₂ [M + H]⁺ 393.0239. Found: 393.0225.

4.2.2 6-Bromo-2,3-bis[(E)-2-(thiophen-2-yl)vinyl]quinoxaline (4b). Use compound **1** (0.082 g, 0.437 mmol) and compound **3b** (0.10 g, 0.364 mmol) to afford compound **4b** (0.118 g, 0.276 mmol). Yield: 76%. Mp 210–211 °C. A yellow solid. ¹H NMR (600 MHz, CDCl₃) δ 8.17 (d, *J* = 2.1 Hz, 1H), 8.15 (d, *J* = 3.5 Hz, 1H), 8.12 (d, *J* = 3.5 Hz, 1H), 7.84 (d, *J* = 8.8 Hz, 1H), 7.71 (d, *J* = 8.8, 2.1 Hz, 1H), 7.38 (d, *J* = 15.3 Hz, 2H), 7.37 (d, *J* = 15.3 Hz, 2H), 7.32 (t, *J* = 3.4 Hz, 2H), 7.11–7.08 (m, 2H). ¹³C NMR (150 MHz, CDCl₃) δ 149.2, 148.7, 142.1, 141.9, 141.8, 140.3, 132.8, 131.2, 131.0, 130.9, 129.4, 129.3, 128.1, 126.9, 126.8, 123.2, 121.1, 121.0. HRMS (ESI) calculated for C₂₀H₁₄BrN₂S₂ [M + H]⁺ 424.9782. Found: 424.9798.

4.2.3 6-Bromo-2,3-di[(E)-styryl]quinoxaline (4c). Use compound **1** (0.257 g, 1.373 mmol) and compound **3c** (0.30 g, 1.144 mmol) to afford compound **4c** (0.448 g, 1.084 mmol). Yield: 95%. Mp 190–191 °C. A yellow solid. ¹H NMR (600 MHz, CDCl₃) δ 8.20 (d, *J* = 2.0 Hz, 1H), 8.00 (d, *J* = 15.5 Hz, 1H), 7.99 (d, *J* = 15.5 Hz, 1H), 7.87 (d, *J* = 8.9 Hz, 1H), 7.72 (d, *J* = 8.9, 2.0 Hz, 1H), 7.67 (d, *J* = 7.7 Hz, 4H), 7.60 (d, *J* = 15.5 Hz, 1H), 7.59 (d, *J* = 15.5 Hz, 1H), 7.44 (d, *J* = 7.4 Hz, 1H), 7.43 (d, *J* = 7.4 Hz, 2H), 7.38 (t, *J* = 7.3 Hz, 2H). ¹³C NMR (150 MHz, CDCl₃) δ 149.7, 149.2, 142.0, 140.3, 138.6, 138.3, 136.3, 136.2, 132.9, 131.1, 130.1, 129.3, 129.2, 128.9, 127.7, 127.6, 123.3, 122.2, 122.1. HRMS (ESI) calculated for C₂₄H₁₈BrN₂ [M + H]⁺ 413.0653. Found: 413.0640.

4.2.4 6-Bromo-2,3-bis[(E)-2-(naphthalen-2-yl)vinyl]quinoxaline (4d). Use Compound **1** (0.031 g, 0.165 mmol) and compound **3d** (0.050 g, 0.138 mmol) to afford compound **4d** (0.065 g, 0.126 mmol). Yield: 91%. Mp 234–235 °C. A yellow solid. ¹H NMR (600 MHz, CDCl₃) δ 8.25 (d, *J* = 1.5 Hz, 1H), 8.20 (d, *J* = 15.5 Hz, 2H), 8.07 (s, 2H), 7.93–7.85 (m, 9H), 7.78–7.75 (m, 3H), 7.55–7.51 (br m, 4H). ¹³C NMR (150 MHz, CDCl₃) δ 149.8, 149.4, 142.2, 140.4, 138.8, 138.5, 133.9, 133.8, 133.6, 132.9, 131.2, 130.2, 129.1, 129.0, 128.6, 128.5, 128.4, 127.8, 126.8, 126.7, 126.6, 123.7, 123.3, 122.4, 122.3. HRMS (ESI) calculated for C₃₂H₂₂BrN₂ [M + H]⁺ 513.0966. Found: 513.0971.

4.2.5 6-Bromo-2,3-bis[(E)-2-methoxystyryl]quinoxaline (4e). Use compound **1** (0.139 g, 0.744 mmol) and compound **3e** (0.20 g, 0.620 mmol) to afford compound **4e** (0.284 g, 0.601 mmol). Yield: 97%. Mp 182–183 °C. A yellow solid. ¹H NMR (600 MHz, CDCl₃) δ 8.28 (d, *J* = 15.7 Hz, 1H), 8.27 (d, *J* = 15.7 Hz, 1H), 8.24 (d, *J* = 2.1 Hz, 1H), 7.91 (d, *J* = 8.9 Hz, 1H), 7.75 (d, *J* = 15.7 Hz, 1H), 7.73 (d, *J* = 15.7 Hz, 1H), 7.72 (dd, *J* = 8.7, 2.2 Hz, 1H), 7.70 (d, *J* = 7.6 Hz, 2H), 7.34 (t, *J* = 7.6 Hz, 2H), 7.02 (t, *J* = 7.5 Hz, 2H), 6.97 (d, *J* = 8.3 Hz, 2H). ¹³C NMR (150 MHz, CDCl₃) δ 158.0, 150.5, 150.1, 142.2, 140.3, 133.9, 133.6, 132.5, 131.2, 130.3, 130.2, 130.1, 128.2, 125.6, 125.5, 123.5, 123.3, 122.9, 120.8, 111.1, 55.5. HRMS (ESI) calculated for C₂₆H₂₂BrN₂O₂ [M + H]⁺ 473.0865. Found: 473.0858.

4.2.6 6-Bromo-2,3-bis[(E)-3-nitrostyryl]quinoxaline (4f). Use compound **1** (0.032 g, 0.170 mmol) and compound **3f** (0.050 g, 0.14 mmol) to afford compound **4f** (0.061 g, 0.121 mmol). Yield: 85%. Mp 290–291 °C. A yellow solid. ¹H and ¹³C-NMR spectra were not obtained in perfect forms because of insolubility in CDCl₃, DMSO-*d*₆, CD₃OD, and acetone-*d*₆. HRMS (ESI) calculated for C₂₄H₁₆BrN₄O₄ [M + H]⁺ 503.0355. Found: 503.0335.

4.2.7 6-Bromo-2,3-bis[(E)-3-chlorostyryl]quinoxaline (4g). Use compound **1** (0.068 g, 0.362 mmol) and compound **3g** (0.10 g, 0.302 mmol) to afford compound **4g** (0.123 g, 0.254 mmol). Yield: 85%. Mp 94–95 °C. A yellow-brown solid. ¹H NMR (600 MHz, CDCl₃) δ 8.21 (d, *J* = 1.9 Hz, 1H), 7.94 (d, *J* = 15.5 Hz, 2H), 7.88 (d, *J* = 8.9 Hz, 1H), 7.76 (dd, *J* = 8.9, 2.0 Hz, 1H), 7.66 (s, 2H), 7.57 (d, *J* = 3.6 Hz, 1H), 7.55 (d, *J* = 9.2 Hz, 1H), 7.54 (s, 1H), 7.39–7.34 (m, 4H). ¹³C NMR (150 MHz, CDCl₃) δ 149.1, 148.7, 142.2, 140.4, 138.1, 138.0, 137.3, 137.0, 134.9, 133.3, 131.2, 130.2, 130.1, 129.2, 129.1, 127.4, 125.9, 125.8, 123.7, 123.2, 123.1. HRMS (ESI) calculated for C₂₄H₁₆BrCl₂N₂ [M + H]⁺ 480.9874. Found: 480.9870.

4.2.8 6-Bromo-2,3-bis[(E)-4-fluorostyryl]quinoxaline (4h). Use compound **1** (0.113 g, 0.603 mmol) and compound **3h** (0.150 g, 0.503 mmol) to afford compound **4h** (0.179 g, 0.399 mmol). Yield: 80%. Mp 212–213 °C. A yellow solid. ¹H NMR (600 MHz, CDCl₃) δ 8.21 (s, 1H), 7.97 (d, *J* = 15.5 Hz, 1H), 7.96 (d, *J* = 15.5 Hz, 1H), 7.88 (d, *J* = 8.9 Hz, 1H), 7.75 (dd, *J* = 8.9, 1.2 Hz, 1H), 7.66 (d, *J* = 7.9 Hz, 2H), 7.65 (d, *J* = 7.9 Hz, 2H), 7.52 (d, *J* = 15.5 Hz, 1H), 7.50 (d, *J* = 15.5 Hz, 1H), 7.13 (t, *J* = 8.3 Hz, 4H). ¹³C NMR (150 MHz, CDCl₃) δ 164.1, 163.3 (¹*J*_{C-F} = 249.0 Hz), 149.5, 149.0, 142.1, 140.3, 137.4, 137.1, 133.0, 132.6, 132.5, 131.1, 130.1, 129.4 (³*J*_{C-F} = 7.5 Hz), 129.3, 123.4, 121.8, 121.7, 116.0 (²*J*_{C-F} = 22.5 Hz). HRMS (ESI) calculated for C₂₄H₁₆BrF₂N₂ [M + H]⁺ 449.0465. Found: 449.0455.

4.2.9 6-Bromo-2,3-bis[(E)-4-chlorostyryl]quinoxaline (4i). Use compound **1** (0.068 g, 0.362 mmol) and compound **3i** (0.10 g, 0.302 mmol) to afford compound **4i** (0.081 g, 0.168 mmol). Yield: 56%. Mp 219–220 °C. A brown solid. ¹H NMR (600 MHz, CDCl₃) δ 8.22 (s, 1H), 7.96 (d, *J* = 15.5 Hz, 2H), 7.89 (d, *J* = 8.8 Hz, 1H), 7.76 (dd, *J* = 8.8, 1.7 Hz, 1H), 7.61 (d, *J* = 8.3 Hz, 4H), 7.57 (d, *J* = 15.5 Hz, 1H), 7.56 (d, *J* = 15.5 Hz, 1H), 7.41 (d, *J* = 8.1 Hz, 4H). ¹³C NMR (150 MHz, CDCl₃) δ 149.3, 148.9, 142.2, 140.4, 137.4, 137.1, 135.1, 134.8, 134.7, 133.2, 131.1, 130.1,

129.2, 128.8, 128.7, 123.6, 122.5, 122.4. HRMS (ESI) calculated for $C_{24}H_{16}BrCl_2N_2 [M + H]^+$ 480.9880. Found: 480.9874.

4.2.10 6-Bromo-2,3-bis[(E)-4-bromostyryl]quinoxaline (4j).

Use compound 1 (0.053 g, 0.286 mmol) and compound 3j (0.10 g, 0.238 mmol) to afford compound 4j (0.116 g, 0.203 mmol). Yield: 85%. Mp 241–242 °C. A yellow solid. 1H NMR (600 MHz, $CDCl_3$) δ 8.22 (d, $J = 2.1$ Hz, 1H), 7.95 (d, $J = 15.5$ Hz, 1H), 7.94 (d, $J = 15.5$ Hz, 1H), 7.89 (d, $J = 8.9$ Hz, 1H), 7.76 (dd, $J = 8.9, 2.1$ Hz, 1H), 7.59–7.52 (m, 10H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 149.3, 148.9, 142.2, 140.4, 137.5, 137.2, 135.2, 135.1, 133.2, 132.1, 131.9, 131.5, 131.2, 130.4, 130.2, 129.1, 129.0, 123.6, 123.4, 123.3, 122.6, 122.5. HRMS (ESI) calculated for $C_{24}H_{16}Br_3N_2 [M + H]^+$ 568.8864. Found: 568.8877.

4.2.11 6-Bromo-2,3-bis[(E)-4-nitrostyryl]quinoxaline (4k).

Use compound 1 (0.032 g, 0.170 mmol) and compound 3k (0.050 g, 0.142 mmol) to afford compound 4k (0.061 g, 0.121 mmol). Yield: 85%. Mp 307–308 °C. A yellow solid. 1H NMR (600 MHz, $CDCl_3$) δ 8.32–8.28 (m, 5H), 8.11 (d, $J = 15.2$ Hz, 2H), 7.95 (d, $J = 8.6$ Hz, 1H), 7.84 (d, $J = 8.0$ Hz, 5H), 7.75 (d, $J = 15.2$ Hz, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 148.0, 147.9, 147.8, 142.3, 142.2, 140.5, 136.2, 135.9, 133.9, 131.2, 130.2, 128.1, 128.0, 125.7, 125.6, 124.4, 124.2. HRMS (ESI) calculated for $C_{24}H_{16}BrN_4O_2 [M + H]^+$ 503.0355. Found: 503.0356.

4.2.12 6-Bromo-2,3-bis[(E)-4-methoxystyryl]quinoxaline (4l).

Use compound 1 (0.069 g, 0.372 mmol) and compound 3l (0.10 g, 0.310 mmol) to afford compound 4l (0.092 g, 0.195 mmol). Yield: 63%. Mp 190–191 °C. A yellow solid. 1H NMR (600 MHz, $CDCl_3$) δ 8.20 (d, $J = 2.0$ Hz, 1H), 7.97 (d, $J = 15.5$ Hz, 1H), 7.96 (d, $J = 15.5$ Hz, 1H), 7.87 (d, $J = 8.9$ Hz, 1H), 7.71 (dd, $J = 8.9, 2.0$ Hz, 1H), 7.64 (d, $J = 8.3$ Hz, 4H), 7.50 (d, $J = 15.5$ Hz, 1H), 7.49 (d, $J = 15.5$ Hz, 1H), 6.97 (d, $J = 8.3$ Hz, 1H), 3.87 (s, 6H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.6, 160.5, 150.0, 149.6, 142.0, 140.2, 138.1, 137.8, 132.5, 131.0, 130.0, 129.2, 129.1, 122.8, 120.0, 119.9, 114.3, 55.4. HRMS (ESI) calculated for $C_{26}H_{22}BrN_2O_2 [M + H]^+$ 473.0865. Found: 473.0864.

4.2.13 N,N' -[[(1E,1'E)-(6-bromoquinoxaline-2,3-diyl)

bis(ethene-2,1-diyl)]bis(4,1-phenylene)] diacetamide (4m). Use compound 1 (0.029 g, 0.159 mmol) and compound 3m (0.050 g, 0.133 mmol) to afford compound 4m (0.057 g, 0.108 mmol). Yield: 81%. Mp 239 °C (decomposed). A yellow solid. 1H NMR (600 MHz, $CDCl_3$) δ 10.12 (s, 1H), 10.1 (s, 1H), 8.18 (d, $J = 1.6$ Hz, 1H), 7.95–7.82 (m, 10H), 7.68 (d, $J = 8.0$ Hz, 2H), 7.67 (d, $J = 8.0$ Hz, 2H), 2.07 (s, 6H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 168.6, 149.7, 149.3, 141.5, 140.4, 140.3, 139.7, 137.6, 132.6, 130.8, 130.7, 130.4, 130.3, 128.9, 128.7, 122.3, 120.3, 120.3, 119.0. HRMS (ESI) calculated for $C_{28}H_{24}BrN_4O_2 [M + H]^+$ 527.1083. Found: 527.1087.

4.2.14 2,3-Bis[(E)-2-(furan-2-yl)vinyl]-6-nitroquinoxaline (5a).

Use compound 2 (0.076 g, 0.495 mmol) and compound 3a (0.10 g, 0.413 mmol) to afford compound 5a (0.124 g, 0.345 mmol). Yield: 84%. Mp 218–219 °C. 1H NMR (600 MHz, $CDCl_3$) δ 8.83 (d, $J = 2.2$ Hz, 1H), 8.37 (dd, $J = 9.1, 2.2$ Hz, 1H), 8.03 (d, $J = 9.1$ Hz, 1H), 7.92 (d, $J = 15.2$ Hz, 1H), 7.89 (d, $J = 15.2$ Hz, 1H), 7.56 (s, 1H), 7.55 (s, 1H), 7.52 (d, $J = 15.2$ Hz, 1H), 7.51 (d, $J = 15.2$ Hz, 1H), 6.68 (d, $J = 3.1$ Hz, 1H), 6.67 (d, $J = 3.1$ Hz, 1H), 6.53 (d, $J = 1.6$ Hz, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 152.6, 151.4, 150.8, 147.3, 144.3, 144.1, 144.0, 140.2, 130.0, 126.6,

126.0, 125.1, 122.5, 119.0, 118.8, 114.2, 113.9, 112.5, 112.4. HRMS (ESI) calculated for $C_{20}H_{14}N_3O_4 [M + H]^+$ 360.0984. Found: 360.0995.

4.2.15 6-Nitro-2,3-bis[(E)-2-(thiophen-2-yl)vinyl]

quinoxaline (5b). Use compound 2 (0.067 g, 0.437 mmol) and compound 3b (0.10 g, 0.364 mmol) to afford compound 5b (0.109 g, 0.279 mmol). Yield: 91%. Mp 223–224 °C. 1H NMR (600 MHz, $CDCl_3$) δ 8.86 (d, $J = 2.4$ Hz, 1H), 8.26 (d, $J = 15.2$ Hz, 1H), 8.23 (d, $J = 15.2$ Hz, 1H), 8.06 (d, $J = 9.1$ Hz, 1H), 7.41 (d, $J = 5.1$ Hz, 1H), 7.40 (d, $J = 5.1$ Hz, 1H), 7.38–7.34 (m, 4H), 7.13–7.10 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 151.1, 150.6, 147.3, 144.0, 141.6, 140.2, 133.0, 132.4, 130.3, 130.1, 130.0, 128.3, 128.2, 127.8, 127.5, 125.1, 122.6, 120.3, 120.1. HRMS (ESI) calculated for $C_{20}H_{14}N_3O_2S_2 [M + H]^+$ 392.0527. Found: 392.0540.

4.2.16 6-Nitro-2,3-di[(E)-styryl]quinoxaline (5c).

Use compound 2 (0.070 g, 0.457 mmol) and compound 3c (0.10 g, 0.381 mmol) to afford compound 5c (0.109 g, 0.286 mmol). Yield: 75%. Mp 195–196 °C. A brown solid. 1H NMR (600 MHz, $CDCl_3$) δ 8.91 (d, $J = 2.5$ Hz, 1H), 8.41 (dd, $J = 9.1, 2.5$ Hz, 1H), 8.13 (d, $J = 15.5$ Hz, 1H), 8.11 (d, $J = 9.1$ Hz, 1H), 8.09 (d, $J = 15.5$ Hz, 1H), 7.70 (d, $J = 7.4$ Hz, 1H), 7.62 (d, $J = 15.5$ Hz, 1H), 7.61 (d, $J = 15.5$ Hz, 1H), 7.46 (t, $J = 7.4$ Hz, 4H), 7.42–7.39 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 151.6, 151.1, 147.4, 144.0, 140.5, 140.3, 140.0, 136.0, 135.9, 130.2, 129.9, 129.7, 129.0, 127.9, 127.8, 125.2, 122.7, 121.4, 121.1. HRMS (ESI) calculated for $C_{24}H_{18}N_3O_2 [M + H]^+$ 380.1399. Found: 380.1405.

4.2.17 2,3-Bis[(E)-2-(naphthalen-2-yl)vinyl]-6-

nitroquinoxaline (5d). Use compound 2 (0.051 g, 0.331 mmol) and compound 3d (0.10 g, 0.276 mmol) to afford compound 5d (0.094 g, 0.196 mmol). Yield: 70%. Mp 228–229 °C. An orange solid. 1H NMR (600 MHz, $CDCl_3$) δ 8.93 (d, $J = 2.3$ Hz, 1H), 8.42 (dd, $J = 9.1, 2.8$ Hz, 1H), 8.31 (d, $J = 15.4$ Hz, 1H), 8.28 (d, $J = 15.4$ Hz, 1H), 8.14 (d, $J = 9.1$ Hz, 1H), 8.08 (s, 2H), 7.92–7.86 (m, 9H), 7.78 (d, $J = 15.4$ Hz, 1H), 7.77 (d, $J = 15.4$ Hz, 1H), 7.55–7.53 (m, 5H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 151.7, 151.2, 147.4, 144.1, 140.7, 140.3, 140.0, 134.1, 134.0, 133.5, 133.4, 130.2, 130.0, 129.5, 128.8, 128.7, 128.5, 127.8, 127.1, 127.0, 126.8, 126.7, 125.2, 123.6, 122.7, 121.6, 121.4. HRMS (ESI) calculated for $C_{32}H_{22}N_3O_2 [M + H]^+$ 480.1712. Found: 480.1719.

4.2.18 2,3-Bis[(E)-2-methoxystyryl]-6-nitroquinoxaline (5e).

Use compound 2 (0.057 g, 0.372 mmol) and compound 3e (0.10 g, 0.310 mmol) to afford compound 5e (0.122 g, 0.278 mmol). Yield: 90%. Mp 219–220 °C. An orange solid. 1H NMR (600 MHz, $CDCl_3$) δ 8.95 (s, 1H), 8.40 (dd, $J = 9.4, 2.5$ Hz, 1H), 8.39 (d, $J = 15.7$ Hz, 1H), 8.38 (d, $J = 15.7$ Hz, 1H), 8.14 (d, $J = 9.1$ Hz, 1H), 7.78 (d, $J = 15.7$ Hz, 1H), 7.77 (d, $J = 15.7$ Hz, 1H), 7.71 (d, $J = 7.5$ Hz, 2H), 7.40–7.35 (m, 2H), 7.04 (t, $J = 7.4$ Hz, 2H), 6.99 (d, $J = 8.3$ Hz, 2H), 3.97 (s, 3H), 3.96 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 158.3, 158.2, 152.5, 152.0, 147.2, 135.8, 135.1, 130.9, 130.7, 130.2, 128.6, 128.4, 125.3, 125.2, 125.1, 122.7, 122.5, 122.4, 120.9, 120.8, 119.2, 55.6. HRMS (ESI) calculated for $C_{26}H_{22}N_3O_4 [M + H]^+$ 440.1610. Found: 440.1594.

4.2.19 6-Nitro-2,3-bis[(E)-3-nitrostyryl]quinoxaline (5f).

Use compound 2 (0.052 g, 0.341 mmol) and compound 3f (0.10 g, 0.284 mmol) to afford compound 5f (0.069 g, 0.146 mmol). Yield: 86%. Mp 197–198 °C. A yellow solid. 1H NMR (600 MHz, $CDCl_3$) δ 8.99 (d, $J = 2.4$ Hz, 1H), 8.57 (d, $J = 2.1$ Hz, 2H), 8.51

(dd, $J = 9.3, 2.7$ Hz, 1H), 8.28–8.24 (m, 3H), 8.21 (dd, $J = 9.3, 1.8$ Hz, 2H), 8.06–8.01 (m, 2H), 7.76 (d, $J = 15.6, 3.0$ Hz, 2H), 7.67 (t, $J = 7.8$ Hz, 2H). ^{13}C NMR (150 MHz, CDCl_3) δ 150.6, 150.1, 148.9, 148.0, 144.1, 140.6, 138.2, 137.6, 137.5, 133.7, 133.5, 130.6, 130.1, 125.4, 124.2, 124.1, 124.0, 123.8, 123.5, 122.4, 122.2. HRMS (ESI) calculated for $\text{C}_{24}\text{H}_{16}\text{N}_5\text{O}_6$ $[\text{M} + \text{H}]^+$ 470.1101. Found: 470.1094.

4.2.20 2,3-Bis[(*E*)-3-chlorostyryl]-6-nitroquinoxaline (5g). Use compound 2 (0.044 g, 0.289 mmol) and compound 3g (0.080 g, 0.242 mmol) to afford compound 5g (0.092 g, 0.205 mmol). Yield: 85%. Mp 200–201 °C. A brown solid. ^1H NMR (600 MHz, CDCl_3) δ 8.93 (s, 1H), 8.45 (d, $J = 8.9$ Hz, 1H), 8.15 (d, $J = 8.9$ Hz, 1H), 8.08 (d, $J = 15.6$ Hz, 1H), 8.05 (d, $J = 15.6$ Hz, 1H), 7.69 (s, 2H), 7.62–7.57 (br m, 4H), 7.45–7.38 (br m, 4H). ^{13}C NMR (150 MHz, CDCl_3) δ 151.1, 150.6, 147.7, 144.1, 140.4, 139.2, 138.6, 137.7, 135.0, 130.4, 129.7, 129.6, 127.7, 127.5, 126.2, 125.3, 123.0, 122.6, 122.4. HRMS (ESI) calculated for $\text{C}_{24}\text{H}_{16}\text{Cl}_2\text{N}_3\text{O}_2$ $[\text{M} + \text{H}]^+$ 448.0620. Found: 448.0521.

4.2.21 2,3-Bis[(*E*)-4-fluorostyryl]-6-nitroquinoxaline (5h). Use compound 2 (0.062 g, 0.402 mmol) and compound 3h (0.10 g, 0.335 mmol) to afford compound 5h (0.080 g, 0.195 mmol). Yield: 83%. Mp 258–259 °C. An orange-yellow solid. ^1H NMR (600 MHz, CDCl_3) δ 8.95 (s, 1H), 8.45 (d, $J = 9.5$ Hz, 1H), 8.15 (d, $J = 9.5$ Hz, 1H), 8.13 (d, $J = 15.5$ Hz, 1H), 8.09 (d, $J = 15.5$ Hz, 1H), 7.70 (br s, 4H), 7.56 (d, $J = 15.5$ Hz, 1H), 7.55 (d, $J = 15.5$ Hz, 1H), 7.16 (d, $J = 8.3$ Hz, 2H), 7.15 (d, $J = 8.3$ Hz, 2H). ^{13}C NMR (150 MHz, CDCl_3) δ 167.0, 163.7 ($^1J_{\text{C-F}} = 249.0$ Hz), 163.6 ($^1J_{\text{C-F}} = 249.0$ Hz), 151.5, 151.0, 147.5, 144.0, 140.3, 139.4, 138.7, 132.2, 130.2, 130.0, 129.7 ($^2J_{\text{C-F}} = 24.0$ Hz), 129.6 ($^3J_{\text{C-F}} = 7.5$ Hz), 125.2, 122.8, 121.1 ($^2J_{\text{C-F}} = 28.5$ Hz), 116.2 ($^4J_{\text{C-F}} = 4.5$ Hz), 116.0 ($^4J_{\text{C-F}} = 4.5$ Hz). HRMS (ESI) calculated for $\text{C}_{24}\text{H}_{16}\text{F}_2\text{N}_3\text{O}_2$ $[\text{M} + \text{H}]^+$ 416.1222. Found: 416.1215.

4.2.22 2,3-Bis[(*E*)-4-chlorostyryl]-6-nitroquinoxaline (5i). Use compound 2 (0.031 g, 0.199 mmol) and compound 3h (0.055 g, 0.166 mmol) to afford compound 5i (0.062 g, 0.138 mmol). Yield: 89%. Mp 221–222 °C. A yellow-brown solid. ^1H NMR (600 MHz, CDCl_3) δ 8.93 (s, 1H), 8.44 (d, $J = 7.4$ Hz, 1H), 8.14 (d, $J = 8.8$ Hz, 1H), 8.11 (d, $J = 15.8$ Hz, 1H), 8.06 (d, $J = 15.8$ Hz, 1H), 7.64 (d, $J = 6.8$ Hz, 1H), 7.59 (d, $J = 15.8$ Hz, 1H), 7.58 (d, $J = 15.8$ Hz, 1H), 7.43 (d, $J = 7.4$ Hz, 4H). ^{13}C NMR (150 MHz, CDCl_3) δ 151.3, 150.8, 147.6, 144.1, 140.4, 139.3, 138.6, 135.7, 135.6, 134.4, 130.3, 129.3, 129.1, 129.0, 125.3, 122.9, 121.9, 121.7. HRMS (ESI) calculated for $\text{C}_{24}\text{H}_{16}\text{Cl}_2\text{N}_3\text{O}_2$ $[\text{M} + \text{H}]^+$ 448.0620. Found: 448.0617.

4.2.23 2,3-Bis[(*E*)-4-bromostyryl]-6-nitroquinoxaline (5j). Use compound 2 (0.022 g, 0.143 mmol) and compound 3j (0.050 g, 0.119 mmol) to afford compound 5j (0.059 g, 0.111 mmol). Yield: 93%. Mp 247–248 °C. A yellow-brown solid. ^1H NMR (600 MHz, CDCl_3) δ 8.93 (d, $J = 2.3$ Hz, 1H), 8.44 (dt, $J = 9.2, 1.2$ Hz), 8.14 (d, $J = 9.2$ Hz, 1H), 8.08 (d, $J = 15.4$ Hz, 1H), 8.04 (d, $J = 15.4$ Hz, 1H), 7.62–7.55 (m, 11H). ^{13}C NMR (150 MHz, CDCl_3) δ 150.9, 150.4, 147.2, 143.7, 140.0, 138.9, 138.3, 134.5, 134.4, 131.9, 131.8, 129.9, 128.9, 128.8, 124.9, 123.7, 123.5, 122.6, 121.5, 121.3. HRMS (ESI) calculated for $\text{C}_{24}\text{H}_{16}\text{Br}_2\text{N}_3\text{O}_2$ $[\text{M} + \text{H}]^+$ 535.9609. Found: 535.9607.

4.2.24 6-Nitro-2,3-bis[(*E*)-4-nitrostyryl]quinoxaline (5k). Use compound 2 (0.052 g, 0.341 mmol) and compound 3k

(0.10 g, 0.284 mmol) to afford compound 5k (0.108 g, 0.231 mmol). Yield: 81%. Mp 297–298 °C. A yellow solid. ^1H NMR (600 MHz, CDCl_3) δ 9.00 (s, 1H), 8.53 (d, $J = 9.1$ Hz, 1H), 8.34 (d, $J = 8.5$ Hz, 4H), 8.25–8.20 (m, 3H), 7.87 (d, $J = 8.2$ Hz, 4H), 7.78 (d, $J = 16.2$ Hz, 1H), 7.76 (d, $J = 16.2$ Hz, 1H). No satisfaction of the ^{13}C NMR spectrum was obtained due to solubility. HRMS (FAB) calculated for $\text{C}_{24}\text{H}_{16}\text{N}_5\text{O}_6$ $[\text{M} + \text{H}]^+$ 470.1101. Found: 470.1101.

4.2.25 2,3-Bis[(*E*)-4-methoxystyryl]-6-nitroquinoxaline (5l). Use compound 2 (0.057 g, 0.372 mmol) and compound 3l (0.10 g, 0.335 mmol) to afford compound 5l (0.069 g, 0.184 mmol). Yield: 89%. Mp 144–145 °C. A dark-orange solid. ^1H NMR (600 MHz, CDCl_3) δ 8.85 (d, $J = 2.4$ Hz, 1H), 8.36 (dd, $J = 9.1, 2.4$ Hz, 1H), 8.05 (d, $J = 15.2$ Hz, 1H), 8.04 (d, $J = 9.1$ Hz, 1H), 8.02 (d, $J = 15.2$ Hz, 1H), 7.63 (d, $J = 8.5$ Hz, 4H), 7.45 (d, $J = 15.4$ Hz, 1H), 7.44 (d, $J = 15.4$ Hz, 1H), 6.97 (d, $J = 8.5$ Hz, 4H). ^{13}C NMR (150 MHz, CDCl_3) δ 161.0, 160.9, 151.9, 151.4, 147.1, 144.0, 140.1, 140.0, 139.3, 129.9, 129.5, 129.4, 128.9, 128.8, 125.0, 122.3, 119.1, 118.9, 114.4, 114.3, 55.4. HRMS (ESI) calculated for $\text{C}_{26}\text{H}_{22}\text{N}_3\text{O}_4$ $[\text{M} + \text{H}]^+$ 440.1610. Found: 440.1613.

4.2.26 *N,N'*-[(1*E*,1'*E*)-(6-nitroquinoxaline-2,3-diyl)bis(ethene-2,1-diyl)bis(4,1-phenylene)]diacetamide (5m). Use compound 2 (0.024 g, 0.159 mmol) and compound 3m (0.050 g, 0.133 mmol) to afford compound 5m (0.060 g, 0.121 mmol). Yield: 91%. Mp 325–326 °C. An orange solid. ^1H NMR (600 MHz, CDCl_3) δ 10.15 (s, 1H), 10.14 (s, 1H), 8.66 (d, $J = 2.1$ Hz, 1H), 8.34 (d, $J = 9.1$ Hz, 1H), 8.09 (d, $J = 9.1$ Hz, 1H), 7.99 (d, $J = 15.6$ Hz, 1H), 7.95 (d, $J = 15.6$ Hz, 1H), 7.85–7.81 (m, 6H), 7.67 (d, $J = 8.0$ Hz, 4H). ^{13}C NMR (150 MHz, CDCl_3) δ 168.7, 151.7, 151.1, 146.8, 143.6, 140.9, 140.7, 139.6, 139.5, 138.9, 130.6, 130.5, 130.1, 129.3, 129.2, 124.4, 122.6, 119.9, 119.7, 119.0, 24.2. HRMS (FAB) calculated for $\text{C}_{28}\text{H}_{24}\text{N}_5\text{O}_4$ $[\text{M} + \text{H}]^+$ 494.1828. Found: 494.1824.

4.3 Cell culture

Prof. Y. Su of National Yang-Ming Chiao Tung University, Taipei, Taiwan kindly provided human non-small lung cancer cells (A549 cells). The cells were stored in liquid nitrogen at -196 °C. After the cells were thawed, the cells were incubated at 37 °C in 5% CO_2 and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U per mL penicillin, 100 μg per mL streptomycin, 2 μM L-glutamine, and 1 mM sodium pyruvate.^{42,43}

4.4 *In vitro* cytotoxicity assay

The cell viability was evaluated with MTT assay, as previously reported to further assess the cytotoxicity.^{32,43} In brief, the cells were plated in 96-well culture plates at a concentration of 3×10^3 cells in 200 μl per well. After 24 hours, cells were treated with different concentrations (6.25, 12.5, 25, 50, and 100 μM) of all twenty-six synthesized compounds, and 5-fluorouracil (5-FU) was used as a positive control. After 72 hours, the attached cells were added with MTT reagent at 0.5 mg mL^{-1} in 100 μL per well and incubated at 37 °C for 3 h. Then, the MTT reagent was removed, 100 μL of DMSO was added per well to dissolve the formazan metabolite, and the amount of formazan was measured by the absorbance at 570 nm, using an ELISA plate

reader, TECAN Spark (Tecan Group Ltd) (μ Quant). The optical density recorded previously in the control group (untreated cells) was considered to be 100% cell viability.

4.5 Western blot

Western blot analysis was performed according to the method previously described.^{32,43} Briefly, the cells were seeded to 6-well culture plates. After reaching 85–90% confluence, cells were treated with compound **4m** (5, 10, and 20 μ M) and 5-FU (5 μ M). The cells were incubated for 48 h after treatment. Afterward, the cells were collected and lysed using radioimmunoprecipitation assay (RIPA) buffer. Lysates of total protein were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. Then, the membranes were blocked with 2% bovine serum albumin (BSA) solution and incubated with anti-Bax, anti-Bcl-2 (Cell Signaling Inc., Danvers, MA, USA), anti-caspase-3, and anti- β -actin (GeneTex Inc., Irvine, CA, USA) primary antibodies at 4 °C overnight. Each membrane was washed with tris-buffered saline containing 0.1% Tween 20 (TBST) three times and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 2 h. Finally, the membranes were developed with an enhanced chemiluminescence (ECL) detection kit and visualized by ImageQuant LAS 4000 Mini bio-molecular imager (GE Healthcare, MA, USA). ImageJ software quantified the band densities (BioTechniques, NY, USA).

Data availability

The data supporting this article have been included as part of the ESI.† No code was produced as part of this review.

Author contributions

Jia-Hua Liang performed the bioassay and analyzed the data and manuscript writing. Shu-Tse Cho conducted the isolation and structure elucidation of the constituents. Jih-Jung Chen and Tzeng-Lien Shih planned, designed, and organized this study's research and the manuscript's preparation. All authors read and approved the final version of the manuscript.

Conflicts of interest

The authors declare no conflicts of financial interest.

Acknowledgements

This research was supported by grants from the National Science and Technology Council (NSTC) and the Ministry of Science and Technology (MOST) of Taiwan (No. NSTC 112-2320-B-A49-028-MY3 and MOST 111-2113-M-032-004), awarded to J.-J. C. and T.-L. S., respectively.

Notes and references

1 J. A. Pereira, A. M. Pessoa, M. N. D. S. Cordeiro, R. Fernandes, C. Prudêncio, J. P. Noronha and M. Vieira, 'Quinoxaline, its

derivatives and applications: A state of the art review', *Eur. J. Med. Chem.*, 2015, **97**, 664–672.

- 2 K. Watanabe, H. Oguri and H. Oikawa, 'Diversification of echinomycin molecular structure by way of chemoenzymatic synthesis and heterologous expression of the engineered echinomycin biosynthetic pathway', *Curr. Opin. Chem. Biol.*, 2009, **13**, 189–196.
- 3 B. Dietrich and U. Diederichsen, 'Synthesis of cyclopeptidic analogues of triostin a with quinoxalines or nucleobases as chromophores', *Eur. J. Org. Chem.*, 2005, **13**, 147–153.
- 4 D. Kong, E. J. Park, A. G. Stephen, M. Calvani, J. H. Cardellina, A. Monks, R. J. Fisher and R. H. Shoemaker, 'Echinomycin, a small-molecule inhibitor of hypoxia-inducible factor-1 DNA-binding activity', *Cancer Res.*, 2005, **65**, 9047–9055.
- 5 Y. B. Kim, Y. H. Kim, J. Y. Park and S. K. Kim, 'Synthesis and biological activity of new quinoxaline antibiotics of echinomycin analogues', *Bioorg. Med. Chem. Lett.*, 2004, **14**, 541–544.
- 6 N. Steinerová, H. Lipavská, K. Stajner, J. Čáslavská, M. Blumauerová, J. Cudlín and Z. Vank, 'Production of quinomycin a in *Streptomyces lasaliensis*', *Folia Microbiol.*, 1987, **32**, 1–5.
- 7 A. Cornish, M. J. Waring and R. D. Nolan, 'Conversion of triostins to quinomycins by protoplasts of *Streptomyces echinatus*', *J. Antibiot.*, 1983, **36**, 1664–1670.
- 8 H. Ishikawa, T. Sugiyama, K. Kurita and A. Yokoyama, 'Synthesis and antimicrobial activity of 2,3-bis(bromomethyl)quinoxaline derivatives', *Bioorg. Chem.*, 2012, **41–42**, 1–5.
- 9 M. Montana, F. Mathias, T. Terme and P. Vanelle, 'Antitumoral activity of quinoxaline derivatives: a systematic review', *Eur. J. Med. Chem.*, 2019, **163**, 136–147.
- 10 M. A. El-Atawy, E. A. Hamed, M. Alhadi and A. Z. Omar, 'Synthesis and antimicrobial activity of some new substituted quinoxalines', *Molecules*, 2019, **24**, 4198.
- 11 H. Ishikawa, T. Sugiyama and A. Yokoyama, 'Synthesis and antimicrobial activity of some new substituted quinoxalines', *Chem. Pharm. Bull.*, 2013, **61**, 438–444.
- 12 E. A. Ahmed, M. F. A. Mohamed and O. A. Omrana, 'Novel quinoxaline derivatives as dual EGFR and COX-2 inhibitors: synthesis, molecular docking and biological evaluation as potential anticancer and anti-inflammatory agents', *RSC Adv.*, 2022, **12**, 25204–25216.
- 13 A. Burguete, E. Pontiki, D. Hadjipavlou-Litina, S. Ancizu, R. Villar, B. Solano, E. Moreno, E. Torres, S. Pérez, I. Aldana and A. Monge, 'Synthesis and biological evaluation of new quinoxaline derivatives as antioxidant and anti-inflammatory agents', *Chem. Biol. Drug Des.*, 2011, **77**, 255–267.
- 14 M. Montana, V. Montero, O. Khoumeri and P. Vanelle, 'Quinoxaline derivatives as antiviral agents: a systematic review', *Molecules*, 2020, **25**, 2784.
- 15 M. Zhang, Z.-C. Dai, S.-S. Qian, J.-Y. Liu, Y. Xiao, A.-M. Lu, H.-L. Zhu, J.-X. Wang and Y.-H. Ye, 'Design, synthesis, antifungal, and antioxidant activities of (E)-6-((2-

- phenylhydrazono) methyl) quinoxaline derivatives', *J. Agric. Food Chem.*, 2014, **62**, 9637–9643.
- 16 M. Kessler, M. Baudry and G. Lynch, 'Quinoxaline derivatives are high-affinity antagonists of the NMDA receptor-associated glycine sites', *Brain Res.*, 1989, **489**, 377–382.
- 17 S. Ancizu, E. Moreno, B. Solano, R. Villar, B. Asunción, E. Torres, S. Pérez-Silanes, I. Aldana and A. Monge, 'New 3-methylquinoxaline-2-carboxamide 1, 4-di-*N*-oxide derivatives as anti-*Mycobacterium tuberculosis* agents', *Bioorg. Med. Chem.*, 2010, **18**, 2713–2719.
- 18 M. Montana, V. Montero, O. Khoumeri and P. Vanelle, 'Quinoxaline moiety: a potential scaffold against *Mycobacterium tuberculosis*', *Molecules*, 2021, **26**, 4742.
- 19 X. Tang, Q. Zhou, W. Zhan, D. Hu, R. Zhou, N. Sun, S. Chen, W. Wu and W. Xue, 'Synthesis of novel antibacterial and antifungal quinoxaline derivatives', *RSC Adv.*, 2022, **12**, 2399–2407.
- 20 E. A. Fayed, M. A. Ebrahim, U. Fathy, H. S. El Saeed and W. S. Khalaf, 'Evaluation of quinoxaline derivatives as potential ergosterol biosynthesis inhibitors: design, synthesis, ADMET, molecular docking studies, and antifungal activities', *J. Mol. Struct.*, 2022, **1267**, 133578.
- 21 M. F. El Shehry, S. Y. Abbas, A. M. Farrag, S. I. Eissa, S. A. Fouad and Y. A. Ammar, 'Design, synthesis and biological evaluation of quinoxaline *N*-propionic and *O*-propionic hydrazide derivatives as antibacterial and antifungal agents', *Med. Chem. Res.*, 2018, **27**, 2287–2296.
- 22 M. J. Waring, B. H. Taibi, A. T. Kotchevar, A. Ramdani, R. Touzani, S. Elkadiri, A. Hakkou, M. Bouakka and T. Ellis, '2,3-Bifunctionalized quinoxalines: synthesis, DNA interactions and evaluation of anticancer, anti-tuberculosis and antifungal activity', *Molecules*, 2002, **7**, 641–656.
- 23 J. Soto-Sánchez and J. D. Ospina-Villa, 'Current status of quinoxaline and quinoxaline 1, 4-di-*N*-oxides derivatives as potential antiparasitic agents', *Chem. Biol. Drug Des.*, 2021, **98**, 683–699.
- 24 S. A. Shintre, D. Ramjugernath, M. S. Islam, R. Mopuri, C. Mocktar and N. A. Koorbanally, 'Synthesis, *in vitro* antimicrobial, antioxidant, and antidiabetic activities of thiazolidine–quinoxaline derivatives with amino acid side chains', *Med. Chem. Res.*, 2017, **26**, 2141–2151.
- 25 Y. Yang, S. Zhang, B. Wu, M. Ma, X. Chen, X. Qin, M. He, S. Hussain, C. Jing, B. Ma and C. Zhu, 'An efficient synthesis of quinoxalinone derivatives as potent inhibitors of aldose reductase', *ChemMedChem*, 2012, **7**, 823–835.
- 26 N. V. Kulkarni, V. K. Revankar, B. N. Kirasur and M. H. Hugar, 'Transition metal complexes of thiosemicarbazones with quinoxaline hub: an emphasis on antidiabetic property', *Med. Chem. Res.*, 2012, **21**, 663–671.
- 27 R. H. Bahekar, M. R. Jain, A. A. Gupta, A. Goel, P. A. Jadav, D. N. Patel, V. M. Prajapati and P. R. Patel, 'Synthesis and antidiabetic activity of 3,6,7-trisubstituted-2-(1*H*-imidazol-2-ylsulfanyl) quinoxalines and quinoxalin-2-yl isothioureas', *Arch. Pharm.*, 2007, **340**, 359–366.
- 28 D. Gupta, N. N. Ghosh and R. Chandra, 'Synthesis and pharmacological evaluation of substituted [5-[4-[2-(6, 7-dimethyl-1, 2, 3, 4-tetrahydro-2-oxo-4-quinoxalinyloxy) phenyl] methylene] thiazolidine-2,4-dione derivatives as potent euglycemic and hypolipidemic agents', *Bioorg. Med. Chem. Lett.*, 2005, **15**, 1019–1022.
- 29 P. Ledwon, R. Motyka, K. Ivaniuk, A. Pidluzhna, N. Martyniuk, P. Stakhira, G. Baryshnikov, B. F. Minaev and H. Ågren, 'The effect of molecular structure on the properties of quinoxaline-based molecules for OLED applications', *Dyes Pigm.*, 2020, **173**, 108008.
- 30 D. Kitazawa, N. Watanabe, S. Yamamoto and J. Tsukamoto, 'Quinoxaline-based donor polymers for organic solar cells', *J. Photopolym. Sci. Technol.*, 2010, **23**, 293–296.
- 31 S. Sagar, S. Singh, J. R. Mallareddy, Y. A. Sonawane, J. V. Napoleon, S. Rana, J. I. Contreras, C. R. E. L. Ezell, S. Kizhake, J. C. Garrison, P. Radhakrishnan and A. Natarajan, 'Structure activity relationship (SAR) study identifies a quinoxaline urea analog that modulates IKK β phosphorylation for pancreatic cancer therapy', *Eur. J. Med. Chem.*, 2021, **222**, 113579.
- 32 R.-Y. Wang, C.-W. Li, S.-T. Cho, C.-H. Chang, J.-J. Chen and T.-L. Shih, 'Synthesis of cinnamils and quinoxalines and their biological evaluation as anticancer agents', *Arch. Pharm.*, 2022, **355**, 2100448.
- 33 I. Malik, M. Hussain, A. Ali, S.-M. T. Togue, F. Z. Bash, C. Fischer and P. Langer, 'Synthesis of 2,3-disubstituted pyrazines and quinoxalines by Heck cross-coupling reactions of 2,3-dichloropyrazine and 2,3-dichloroquinoxaline. Influence of the temperature on the product distribution', *Tetrahedron*, 2010, **66**, 1637–1642.
- 34 P. Thirumurugan, D. Muralidharan and P. T. Perumal, 'The synthesis and photophysical studies of quinoxaline and pyridopyrazine derivatives', *Dyes Pigm.*, 2009, **81**, 245–253.
- 35 X. Luo and L.-T. Lim, 'Cinnamil- and Quinoxaline-Derivative Indicator Dyes for Detecting Volatile Amines in Fish Spoilage', *Molecules*, 2019, **24**, 3673.
- 36 L. Zhan, B. Qi, X. L. X. Wan, J. L. Y. Zhang, J. Li, J. Li and J. Shen, 'Preparation of 6-substituted quinoxaline JSP-1 inhibitors by microwave accelerated nucleophilic substitution', *Molecules*, 2006, **11**, 988–999.
- 37 R. S. Bhosale, S. R. Sarada, S. S. Ardhapure, W. N. Jadhav, S. R. Bhusare and R. P. Pawar, 'An efficient protocol for the synthesis of quinoxaline derivatives at room temperature using molecular iodine as the catalyst', *Tetrahedron Lett.*, 2005, **46**, 7183–7186.
- 38 Z. Zhao, D. D. Wisnoski, S. E. Wolkenberg, W. H. Leister, Y. Wang and C. W. Lindsley, 'General microwave-assisted protocols for the expedient synthesis of quinoxalines and heterocyclic pyrazines', *Tetrahedron Lett.*, 2004, **45**, 4873–4876.
- 39 Y. Suzuki, R. Takehara, K. Miura, R. Ito and N. Suzuki, 'Regioselective synthesis of trisubstituted quinoxalines mediated by hypervalent iodine reagents', *J. Org. Chem.*, 2021, **86**, 16892–16900.
- 40 S. K. Suthar, N. S. Chundawat, G. P. Singh, J. M. Padrón and Y. K. Jhala, 'Quinoxaline: A comprehension of current pharmacological advancement in medicinal chemistry', *Eur. J. Med. Chem. Rep.*, 2022, **5**, 100040.

- 41 M. S. Nafie, S. H. Kahwash, M. M. Youssef and K. M. Dawood, 'Recent advances on quinoxalines as target-orientate chemotherapeutic anticancer agents through apoptosis', *Arch. Pharm.*, 2024, **357**, e2400225.
- 42 D. K. Magozwi, M. Dinala, N. Mokwana, X. Siwe-Noundou, R. W. M. Krause, M. Sonopo, L. J. McGaw, W. A. Augustyn and V. J. Tembu, 'Flavonoids from the genus euphorbia: Isolation, structure, pharmacological activities and structure–activity relationships', *Pharmaceuticals*, 2021, **14**, 428.
- 43 T. Mosmann, 'Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays', *J. Immunol. Methods*, 1983, **65**, 55.