Received: 19 April 2021 | Revised: 3 June 2021 | Accepted: 7 June 2021

DOI: 10.1002/ardp.202100151

FULL PAPER

ARCH PHARM DPhG
Archiv der Pharmazie

# Another structural correction for 1-oxo-1*H*-phenalene-2,3-dicarbonitriles: Synthesis of a potent BCL-2 inhibiting 7-phenoxy derivative

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**Funding information**
Slovenian Research Agency, Grant/Award Number: J1-2485 and core funding P1-0208

## Abstract

Aromatic scaffolds are an important part of biologically active compounds and molecular probes used to study biochemical pathways and the involved targeted proteins of interest. 1-Oxo-1*H*-phenalene-2,3-dicarbonitrile-based compounds have been described as inhibitors of the BCL-2 family of proteins, and this core structure represents numerous possibilities for modifications that could lead to improved inhibitory potencies. Many studies demonstrated intriguing characteristics of these compounds in terms of reactivity and, interestingly, some contradictory literature reports appeared about reaction outcomes to synthesize them. Here, we initially provide a condensed overview of transformations performed on the phenalene scaffold, followed by the resynthesis of a 6-phenoxy-substituted derivative. We show that the initial determination of this particular structure was wrong and provide two-dimensional nuclear magnetic resonance (NMR) evidence to assign the structure properly. When preparing new derivatives using the same synthetic route, we observed 6- and 7-substituted regioisomers. After confirming their structures by NMR experiments, the ability of these compounds to inhibit BCL-2 was evaluated. The most potent 1-oxo-1*H*-phenalene-2,3-dicarbonitrile derivatives inhibited BCL-2 in the nanomolar range and showed double-digit micromolar cytotoxicity against four different cancer cell lines.

**KEYWORDS**
2D NMR spectroscopy, BCL-2, phenalenones, ring expansion

## 1 | INTRODUCTION

Aromatic rings are frequently used structural components in drugs and represent a common part of different medicinal chemistry approaches. They offer an easy and well-characterized synthetic availability and manifold possibilities for systematic structural modifications, including those toward polycyclic aromatic compounds with electrophilic properties.[1–5] In 2005, a series of acenaphthylene-based molecules were synthesized, and the core structure of the most exciting compound was reported as 8-oxo-8*H*-acenaphtho[1,2-*b*]

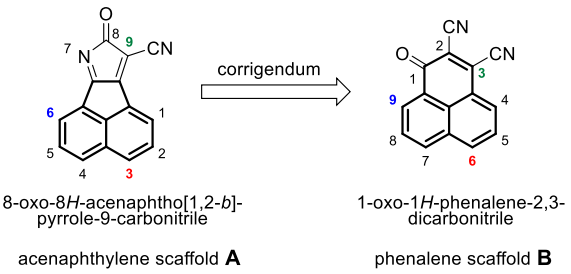



**FIGURE 1** The misassigned structure **A** and the corrected compound **B**. The numbering of both core scaffolds is shown. The colored numbers show the reactive carbon atoms, with red denoting the most reactive one

pyrrole-9-carbonitrile (compound **A**, Figure 1).[6] This highly electron-deficient scaffold was deemed as an attractive precursor for the preparation of useful fluorophores,[6,7] fluorescent markers for tumor cells,[8,9] and fluorescence probes for imaging cysteine and homocysteine.[10] Moreover, derivatives of **A** were shown to have different bioactive properties, such as cytotoxicity against tumor cells,[7,11] DNA intercalation and apoptosis induction,[12] fibroblast growth factor receptor 1 inhibition,[13] and inhibition of the B-cell lymphoma 2 (BCL-2) family of proteins.[14–20]

Despite numerous studies describing physicochemical and biological properties of 8-oxo-8*H*-acenaphtho[1,2-*b*]pyrrole-9-carbonitrile (**A**) derivatives, it was only in 2013/2014 when the original authors published two separate corrigendum papers,[21,22] in which the core structure **A** was revised. They used two-dimensional (2D) nuclear magnetic resonance (NMR) techniques to reassign the molecule as 1-oxo-1*H*-phenalene-2,3-dicarbonitrile (scaffold **B**, Figure 1). Moreover, the authors[23] and, independently, Lenk et al.[24] provided mechanistic explanations for the formation of 1-oxo-1*H*-phenalene-2,3-dicarbonitrile (**B**) from starting acenaphthenequinone, as well as an unambiguous structure confirmation by X-ray diffraction.[24]

The class of phenalenones is accessible through various preparative methods, some of which have been discovered recently.[25–27] The electronic characteristics of the aromatic system **B** allowed access to many derivatives thereof, which were primarily obtained via nucleophilic substitution of aromatic hydrogens ($S_NAr^H$) at positions C-6 and C-9 by *N*- and *S*-nucleophiles.[24] In Figure 2, a condensed overview of reactions performed on this planar and highly electron-deficient polycyclic system is represented, giving the reader an impression of the phenalene scaffold's reactivity.

The majority of reactions on the phenalenone scaffold were performed with primary or cyclic secondary amines at room temperature, almost exclusively with MeCN as solvent. Usually, an amine excess of 1.0–4.0 equivalents was used and the reaction times spanned from 1 to 24 h, yielding compounds in up to 50% yield (Figure 2a). In the initial paper,[7] primary amines (4.0 equiv, 1 h) gave 6-substituted products predominantly, whereas cyclic secondary amines (4.0 equiv, 24 h) resulted in 6-substituted, 9-substituted, and 6,9-disubstituted products. Later on, detailed structural analyses of such $S_NAr^H$ products were performed to confirm different regioselectivities.[23,24] It was revealed that the reaction of phenalene **B** with *n*-butylamine (4.0 equiv, 3 h) resulted in the formation of 6-, 9-, and 3-substituted derivatives (Figure 2a).[24] In contrast, a reaction of **B** with three different primary amines (1.1 equiv, 12 h) mainly gave 6-substituted products with trace amounts of 9-substituted derivatives.[23] Secondary amines (1.1 equiv, 2 h) produced 6-substituted major products (30–49% yield) accompanied by 9- and 6,9-disubstituted derivatives (Figure 2a).[23]

The $S_NAr^H$ reactions with thiol-based reagents resulted in a single 6-substituted regioisomer (Figure 2b) with yields in a similar range as for 6-amino-substituted compounds. Usually, for aromatic thiols, 4 equivalents were needed, and the $S_NAr^H$ proceeded in MeCN at room temperature in 2–48 h.[16,18,19,28] To react aliphatic thiols with phenalene **B**, MeOH was applied as a solvent. Chen et al.[13] synthesized different 6-thiol-substituted derivatives using 4.0 equivalents of the appropriate *S*-nucleophile at 0–5°C in an overnight reaction (Figure 2b). For the reaction with mercaptopropionic acid in MeOH, opposing outcomes were described. Zhang et al.[10] noted that for a successful reaction only 2.0 equivalents of the nucleophile were required (2 h, 46% yield), whereas Li et al.[23] reported only 9% conversion in refluxing MeOH for 48 h despite using 5.0 equivalents of mercaptopropionic acid.

Another contrasting observation in the chemistry of these compounds was the outcome of reacting primary amines for 1–3 h in MeCN with phenalenones that possessed thiol- and secondary amine-based substituents at position 6. Some studies reported substitutions (3.0 equiv amine) to occur at position 6 (Figure 2c),[7,23] whereas others[18,20,28] reported Michael additions (10 equiv amine) to give 3-substituted derivatives in up to 35% yield (Figure 2d) without the nucleophilic substitution at position 6.

A different route was applied for the 6-phenoxy-substituted phenalenones (Figure 2e). These were synthesized in high overall yields (approx. 70%) by initial nucleophilic aromatic substitution of 5-bromoacenaphthylene-1,2-dione, followed by sequential Knoevenagel condensation with malononitrile and base-mediated cyclization.[16–18]

We became aware of compounds with the 1-oxo-1*H*-phenalene-2,3-dicarbonitrile scaffold because of their ability to potently inhibit members of the BCL-2 family of proteins. Based on our interest in BCL-2 as an anticancer target and the in-depth overview of synthetic access toward such compounds, our initial motivation was to resynthesize one of the previously described pan-BCL-2 inhibitors.[18] Considering the generally low yields reported for 6-amino- and 6-thio-substituted derivatives by $S_NAr^H$ reactions and the contrasting data on the products obtained, we decided to initially prepare a BCL-2 inhibitor with the 6-phenoxy-phenalene core. As the next step, it was intended to employ the same synthetic route toward novel 6-substituted phenalene derivatives and evaluate their potential to inhibit BCL-2.

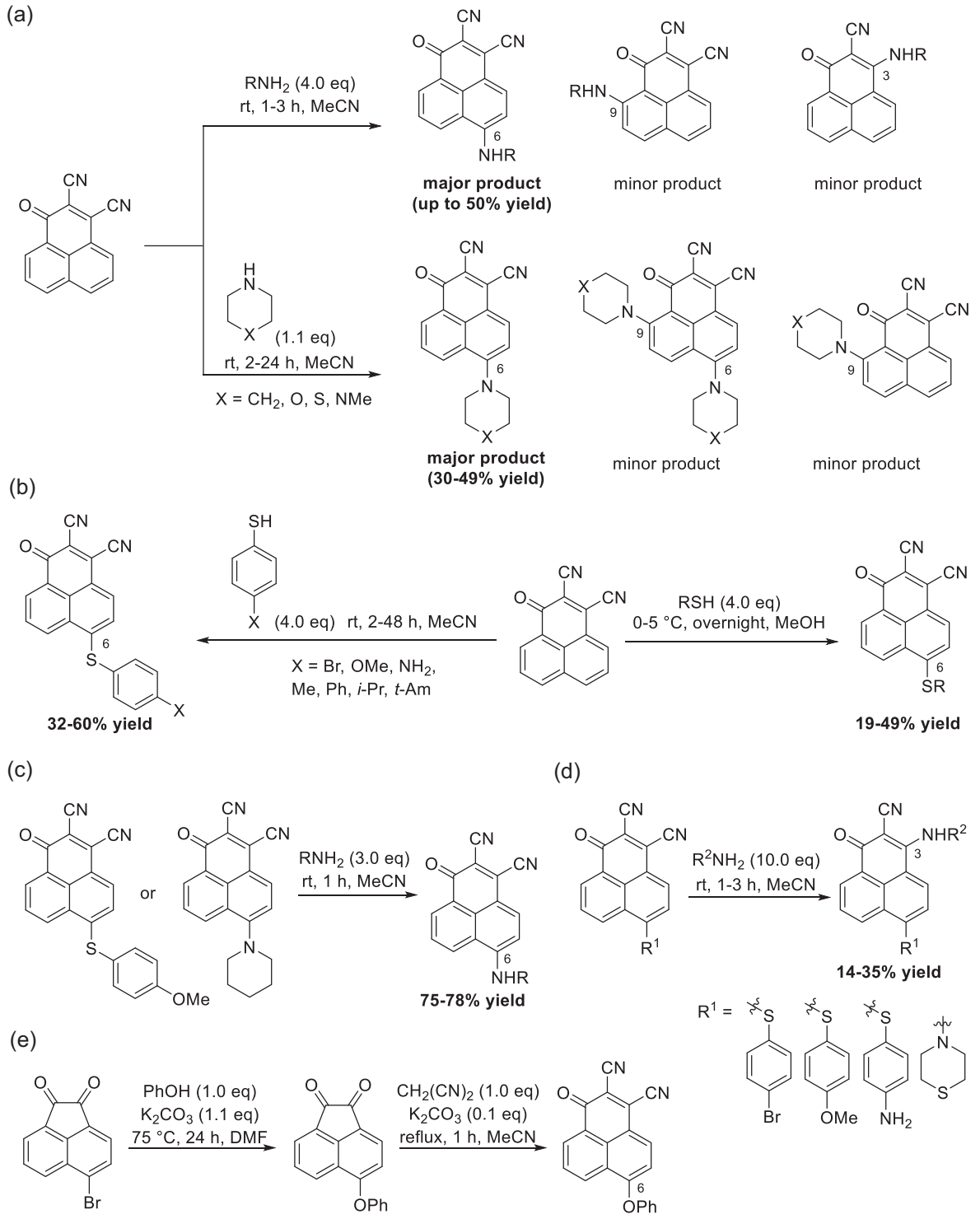
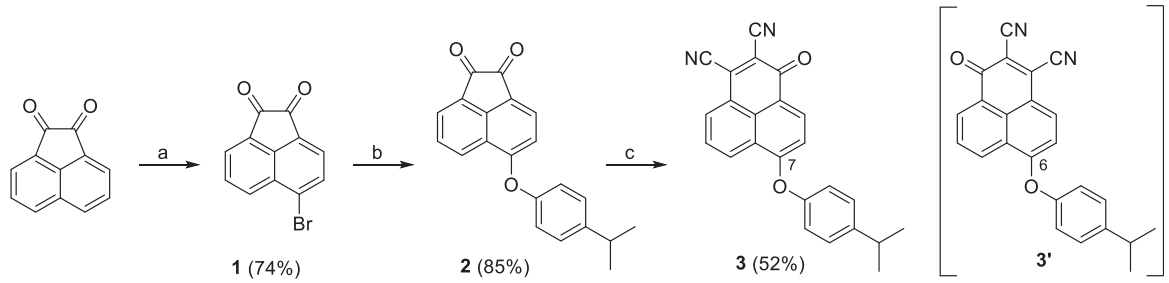



**FIGURE 2** Schematic overview of phenalene scaffold reactivity. Please note that not all of these structures were officially corrected. For the sake of clarity, however, all reactions and positions of substitution are shown on the revised core structure **B** despite initially described on the misassigned scaffold **A**. (a) Reaction with amines; (b) reaction with thiols; (c) nucleophilic substitution at position 6; (d) reactions at position 3; (e) reactions with phenols




**SCHEME 1** Resynthesis of the previously published 6-(4-isopropylphenoxy)-1-oxo-1*H*-phenalene-2,3-dicarbonitrile (**3′**). Reagents and conditions: (a) $Br_2$, reflux; (b) 4-isopropylphenol, $K_2CO_3$, dimethylformamide, 75°C, 24 h; (c) $CH_2(CN)_2$, 10 mol% $K_2CO_3$, wet $CH_3CN$, reflux, 1 h

## 2 | RESULTS AND DISCUSSION

### 2.1 | Chemistry

To prepare the 6-(4-isopropylphenoxy)-substituted phenalene **3′** (Scheme 1), literature procedures were used.[16–18] Bromination of the starting acenaphthenequinone yielded the 5-bromo derivative **1**, which was subjected to nucleophilic aromatic substitution with 4-isopropylphenol to obtain the diaryl ether **2**. The final product was obtained in a one-pot, two-step sequence by reacting **2** with malononitrile and catalytic amounts of $K_2CO_3$.[24] Its $^1$H NMR spectrum corresponded entirely to the previously described data, which presumed the formation of 6-substituted phenalene (structure **3′** in square brackets, Scheme 1).[16–18] However, when performing the complete assignment of $^1$H and $^{13}$C chemical shifts with $^1$H-$^1$H homonuclear correlation spectroscopy (COSY), $^1$H-$^{13}$C heteronuclear single-quantum correlation (HSQC) spectroscopy, nuclear Overhauser effect spectroscopy (NOESY), and $^1$H-$^{13}$C heteronuclear multiple-bond correlation (HMBC) spectroscopy, we could demonstrate that this product was the 7-(4-isopropylphenoxy)-substituted

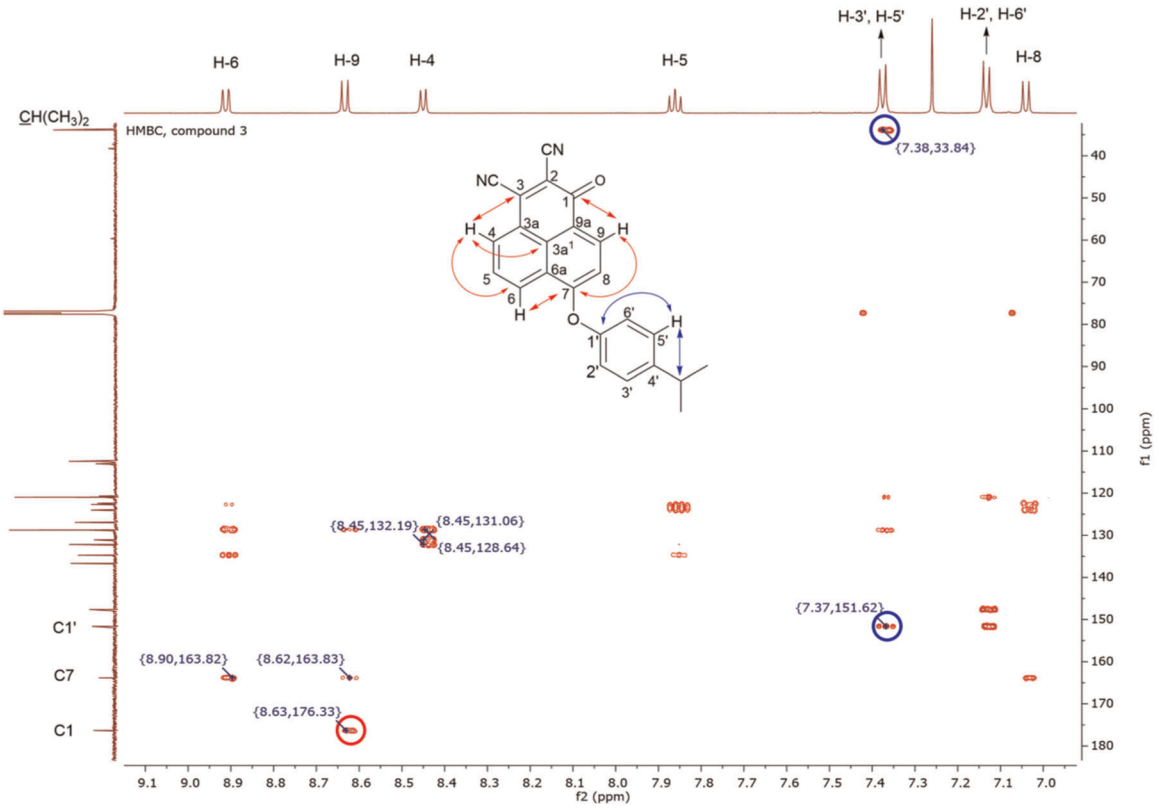



**FIGURE 3** $^1$H-$^{13}$C heteronuclear multiple-bond correlation spectrum ($CDCl_3$) of compound **3**. Only the most significant cross-peaks that correlate protons and carbons, which are connected through three covalent bonds, are noted. Circled cross-peaks in blue indicate correlations of H-3′, H-5′ (7.38 ppm) to $\underline{C}H(CH_3)_2$ (33.84 ppm) and to C1′ (151.62 ppm), whereas the red circle indicates a key correlation of H-9 (8.63 ppm) to C1 (176.33 ppm)

phenalene **3**, the regioisomer of the presumed compound **3′** (Scheme 1). Our attempts to additionally obtain compound **3′** failed.

We assigned the structure by first performing a $^{1}$H-$^{1}$H COSY experiment (Figure S1) to distinguish between *ortho*-coupled aromatic protons (H-8 and H-9, doublets, $J$ = 8.6 Hz) and protons at positions 4, 5, and 6. Next, we used the $^{1}$H-$^{13}$C HSQC experiment (Figure S2) to determine which hydrogen atoms are connected to which carbons. In the $^{1}$H-$^{1}$H NOESY data (Figure S3), the through-space correlation between the signal at 7.37 and 3.00 ppm indicated the spatial proximity of the aromatic H-3′ and H-5′ and the CH proton of the isopropyl group at 4′. Based on $^{1}$H-$^{13}$C HSQC and $^{1}$H-$^{1}$H NOESY, carbons of the phenoxy ring C2′, C6′ and C3′, C5′ could be appropriately assigned. Other cross-peaks in the $^{1}$H-$^{1}$H NOESY spectrum showing the proximity of aromatic protons corroborated data from the $^{1}$H-$^{1}$H COSY experiment.

The crucial correlations were, however, demonstrated in the $^{1}$H-$^{13}$C HMBC spectrum. Namely, a three-bond correlation of H-9 (8.63 ppm) to C1 (176.33 ppm) evidenced the 4-isopropylphenoxy moiety at position 7 of the phenalene ring (Figure 3, red circle). The long-range coupling correlations of H-9 (8.62 ppm) to C7 (163.83 ppm) as well as H-6 (8.90 ppm) to C7 (163.82 ppm) additionally confirmed the suggested substitution pattern (Figure 3). As a result of simultaneous correlations of H-4 (8.45 ppm) to C3 (131.06 ppm), C3a$^{1}$ (128.64 ppm), and C6 (132.19 ppm), other carbons assignations could be assigned with this experiment (Figure 3). Correlation peaks of H-3′ and H-5′ (7.38 ppm) to $\underline{C}H(CH_3)_2$ (33.84 ppm) and to C1′ (151.62 ppm) (Figure 3, blue circles) enabled further proof of the correct assignment of carbons and protons of the phenoxy ring.

We next subjected 5-bromo derivative **1** to the same reaction conditions that would enable access to a bromo-substituted phenalene (Scheme 2). After consumption of the starting material, two distinct products were visible by thin-layer chromatography (TLC) of the crude reaction mixture. Despite significant difficulties, we separated and purified both products by several consecutive runs of chromatographic purification. Interestingly, 2D NMR analyses showed that regioisomers, that is, 7-bromo- (**4**; Figures 4, S4, and S5) and 6-bromo-substituted (**5**; Figures 5, S6, and S7) phenalenones were formed (Scheme 2).

Evidently, the ring expansion of **1** can occur in two ways depending on which carbonyl group reacts with malononitrile in the initial Knoevenagel condensation. However, following a postulated mechanism, an intermediate tetracyclic 2,3-dihydroxycyclopropane-1,1-dicarbonitrile is formed whose deprotonation determines the position of the carbonyl group in the final phenalenone.[24] Hence, the regiopreference of the reaction is hardly predictable.

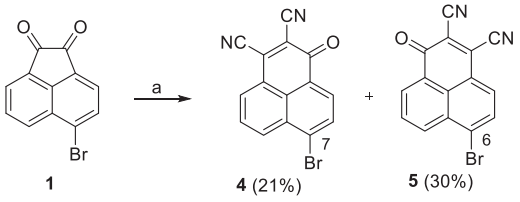
To determine the structures of both regioisomers, the same step-by-step combination of 2D NMR experiments as for compound **3** was employed, whereby key correlations to indirectly suggest the position of the Br atom were obtained in the $^{1}$H-$^{13}$C HMBC spectra. The most evident three-bond correlations for 7-bromo-substituted phenalene **4** are depicted in Figure 4. In particular, a correlation of H-9 (8.44 ppm) to C1 (177.20 ppm) indicated the 7-bromo substitution pattern, which was further supported by correlation peaks of both H-9 (8.44 ppm) and H-6 (8.69 ppm) to C7 (127.29 ppm) (Figure 4). For phenalenone **5**, a key three-bond correlation between H-9 (8.28 ppm) and C1 (185.30 ppm) suggested the 6-position of the Br atom (Figure 5).

When we attempted to prepare the 6-benzyl(methyl)amino-substituted derivative (Scheme 3), the same phenomenon occurred as we again observed two distinct products. Several rounds of chromatographic purifications eventually led to their successful isolation and characterization. 2D NMR data showed that these were indeed the 7- and 6-substituted regioisomers **7** and **8**, respectively (Scheme 3).

After assigning the most evident protons and carbons of compound **7** by analyzing $^{1}$H-$^{1}$H COSY (Figure S8), $^{1}$H-$^{13}$C HSQC (Figure S9), and $^{1}$H-$^{1}$H NOESY (Figures S10a and S10b) spectra, the remaining carbons and the substitution pattern were determined with the $^{1}$H-$^{13}$C HMBC experiment (Figure 6). Again, three-bond couplings between H-9 (8.41 ppm) and C1 (173.96 ppm), H-9 (8.41 ppm) and C7 (159.65 ppm), as well as H-6 (8.69 ppm) and C7 (159.65 ppm) indicated the 7-substituted phenalene ring (Figure 6). The phenyl carbons were unambiguously assigned via three-bond $^{1}$H, $^{13}$C coupling correlation (blue circles in Figure 6) of aliphatic $CH_2$ protons (4.98 ppm) to C2′, C6′ (127.13 ppm) and H-3′, H-5′ (7.40 ppm) to C1′ (136.13 ppm). The signals of other quaternary phenalene carbons were resolved by analyzing simultaneous couplings of H-4 (8.35 ppm) to C3 (128.11 ppm) and C6 (135.35 ppm), H-9 (8.41 ppm) to C3a$^{1}$ (129.09; ppm), and H-8 (7.39 ppm) to C9a (119.36 ppm) and C6a (122.05 ppm) (Figure 6; the latter two correlations are not shown with arrows on the structure).

The position of the *N*-benzylmethylamine moiety in compound **8** was deciphered similarly. After determining which phenalene protons are *ortho*-coupled, we searched for the key $^{1}$H-$^{13}$C HMBC correlation of a single aromatic proton with C1. From Figure 7, it can be observed that the proton at position 9 (8.66 ppm) is the only one that exhibited a notable three-bond correlation with a C1 peak at 176.90 ppm. Other 2D spectra of compound **8**, that is, $^{1}$H-$^{1}$H COSY and $^{1}$H-$^{13}$C HSQC, are shown in Figures S11 and S12, respectively.

It has to be emphasized that we encountered numerous difficulties with the purification of all final compounds, a phenomenon previously also observed by others.[24] This was probably due to the intrinsic reactivity of the phenalene scaffold and due to the structural similarity of regioisomeric products, which made purifications




**SCHEME 2** Synthesis of 7- and 6-bromo-substituted phenalenes **4** and **5**. Reagents and conditions: (a) $CH_2(CN)_2$, 10 mol% $K_2CO_3$, MeCN, reflux, 1 h

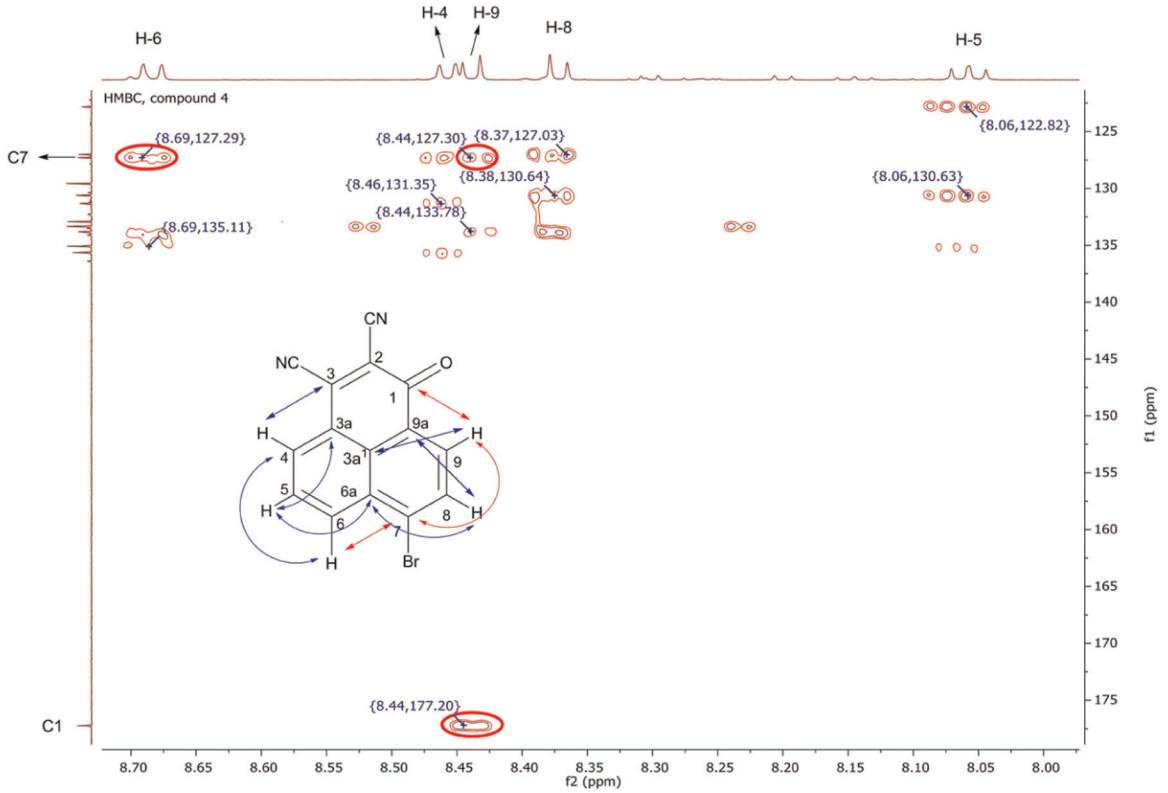



**FIGURE 4** $^{1}H$-$^{13}C$ HMBC spectrum (DMSO-$d_6$) of compound **4**. Only the most significant cross-peaks that correlate protons and carbons, which are connected through three covalent bonds, are noted. Circled cross-peaks in red indicate correlations of H-9 (8.44 ppm) to C1 (177.20 ppm) and C7 (127.29 ppm), as well as of H-6 (8.69 ppm) to C7 (127.29 ppm). Additional three-bond correlation peaks noted (blue arrows next to the structure) are H-4 (8.46 ppm) to C3 (131.35 ppm), H-5 (8.06 ppm) to C3a (122.82 ppm), and C6a (130.63 ppm), H-6 (8.69 ppm) to C4 (135.11 ppm), H-8 (8.37 ppm) to C9a (127.03 ppm) and C6a (130.64 ppm), as well as H-9 (8.44 ppm) to C3a$^{1}$ (133.78 ppm). DMSO, dimethyl sulfoxide; HMBC, heteronuclear multiple-bond correlation

of both compounds challenging. For instance, to obtain compounds pure enough for structural characterization and biochemical assays (see below), at least two rounds of silica gel chromatographic purifications were necessary. Of note, we tried to synthesize 6-substituted derivatives **3′** and **8** also by a direct $S_NAr^H$ reaction of 1-oxo-1*H*-phenalene-2,3-dicarbonitrile (**B**, Figure 1) with either 4-isopropylphenol or *N*-benzylmethylamine. However, a set of two different reaction conditions, both based on previous literature procedures, did not yield the desired products (Scheme S1).

## 2.2 | Biochemistry

The commitment of cells to apoptosis, an ancient cell suicide program, is controlled by the BCL-2 family of proteins, which contains both pro-apoptotic and pro-survival members. Cytotoxic stimuli can activate pro-apoptotic BH3-only proteins and some of them initiate apoptosis signaling by binding to pro-survival BCL-2 members. BCL-2 itself, for example, decreases the propensity of cells for apoptosis, confers cancer cells' resistance to therapeutic agents, and represents an important target for anticancer therapy.[29,30] BCL-2 forms a well-defined hydrophobic surface binding groove, into which pro-apoptotic proteins, such as BIM, can bind. Our interest in BCL-2 inhibition is also based on inhibitor of apoptosis (IAP)-addressing PROTACs with potential antitumor effects.[31]

The synthesized compounds **3**, **4**, **5**, **7**, and **8** were evaluated for their inhibition of BCL-2 using an enzyme-linked immunosorbent assay (ELISA) (Table 1), in which the ability of compounds to competitively displace a BIM-derived peptide from BCL-2 was measured. Briefly (see Section 4 for details), the biotinylated BIM peptide was attached to a streptavidin-coated well plate and mixtures of different inhibitor concentrations and His-tagged BCL-2 protein were added, followed by applying the anti-His secondary antibody conjugated to horseradish peroxidase and a colorimetric readout upon addition of *o*-phenylenediamine. The data were calculated as the residual activities (RAs) of BCL-2 in the presence of 50 μM of each compound. Compounds that showed more than 50% inhibition of the BCL-2 were subjected to dose-dependent inhibitory activity measurements. The binding curves for phenalenones **3**, **4**, **7**, and **8** are shown in Figure S13.

The 7-phenoxy-substituted compound **3** exhibited slightly different BCL-2 inhibition ($IC_{50}$ = 110 ± 30 nM) in comparison to previously published data,[18] where the $IC_{50}$ was determined to be 739 nM. All four new phenalenones proved to be less potent BCL-2

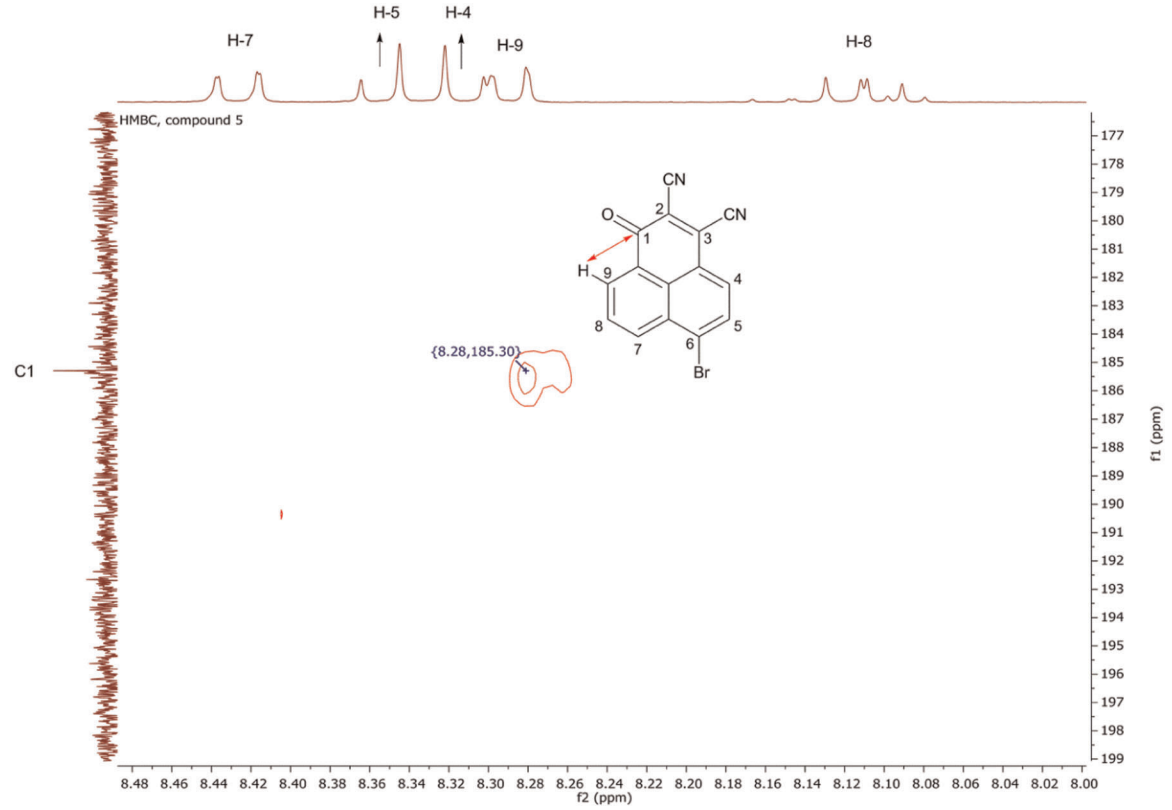
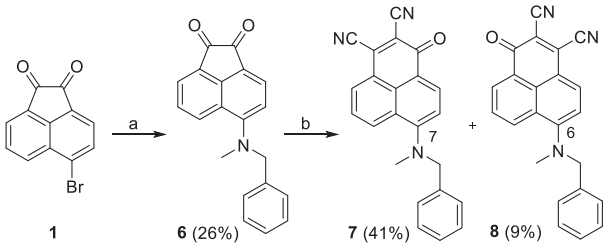



**FIGURE 5** A crucial segment of the $^{1}H$-$^{13}C$ HMBC spectrum (DMSO-$d_6$) of **5**. A three-bond correlation between H-9 (8.28 ppm) and C1 (185.30 ppm) is shown. DMSO, dimethyl sulfoxide; HMBC, heteronuclear multiple-bond correlation




**SCHEME 3** Synthesis of 7- and 6-benzyl(methyl)amino-substituted derivatives **7** and **8**. Reagents and conditions: (a) *N*-benzylmethylamine, $K_2CO_3$, dimethylformamide, 75°C, 24 h; (b) $CH_2(CN)_2$, 10 mol% $K_2CO_3$, MeCN, reflux, 1 h

inhibitors in comparison to **3**. Clearly, the replacement of the 7-aryloxy group by the 7-benzylamino was disadvantageous (**3** vs. **7**). Owing to the low number of new derivatives it was impossible to draw reliable conclusions about structure–activity relationships (SAR). Given that 7-substituted compounds **3**, **4**, and **7** inhibited BCL-2 to a different extent, a significantly greater number of compounds would be needed to gain a better understanding of SAR for 7-substituted compounds. Nevertheless, we assessed the cytotoxicity of the two most potent compounds **3** and **4** against four cancer cells (U87, human primary glioblastoma cell line; MV3, melanoma cell line; A2780, human ovarian cancer cell line; MDA-MB-231, human breast cancer cell line) by using 3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Both BCL-2 inhibitors exhibited double-digit micromolar cytotoxicity against all cancer cell lines (Table 1). The discrepancy between in vitro BCL-2 inhibition and cytotoxicity can be attributed to many factors, such as poor cellular permeability of compounds or the fact that the selected cell lines are less dependent on BCL-2, as indicated by CRISPR-based knockout screens from the DepMap database (see Figure S14).[32]

## 3 | CONCLUSION

In this study, we demonstrated the importance of using a combination of 2D NMR techniques to determine substituent patterns on the phenalene ring properly. The usefulness of this approach was clearly presented by correcting the structure of a previously misassigned 4-isopropylphenol-substituted phenalene **3′**. Despite showing this on a limited number of examples, our findings suggest that the one-pot Knoevenagel condensation and base-mediated ring expansion performed on 5-substituted-acenaphthylene-1,2-diones can generate two possible regioisomers (Schemes 2 and 3). If the acenaphthenequinone is first subjected to cyclization to yield the phenalene ring **B**, followed by the $S_NAr^H$ reactions, the final products obtained are mostly 6-substituted phenalenones (Figures 2a and 2b).

We are aware that the previously studied 6-arylthio derivatives performed even better as BCL-2 inhibitors than related phenoxy

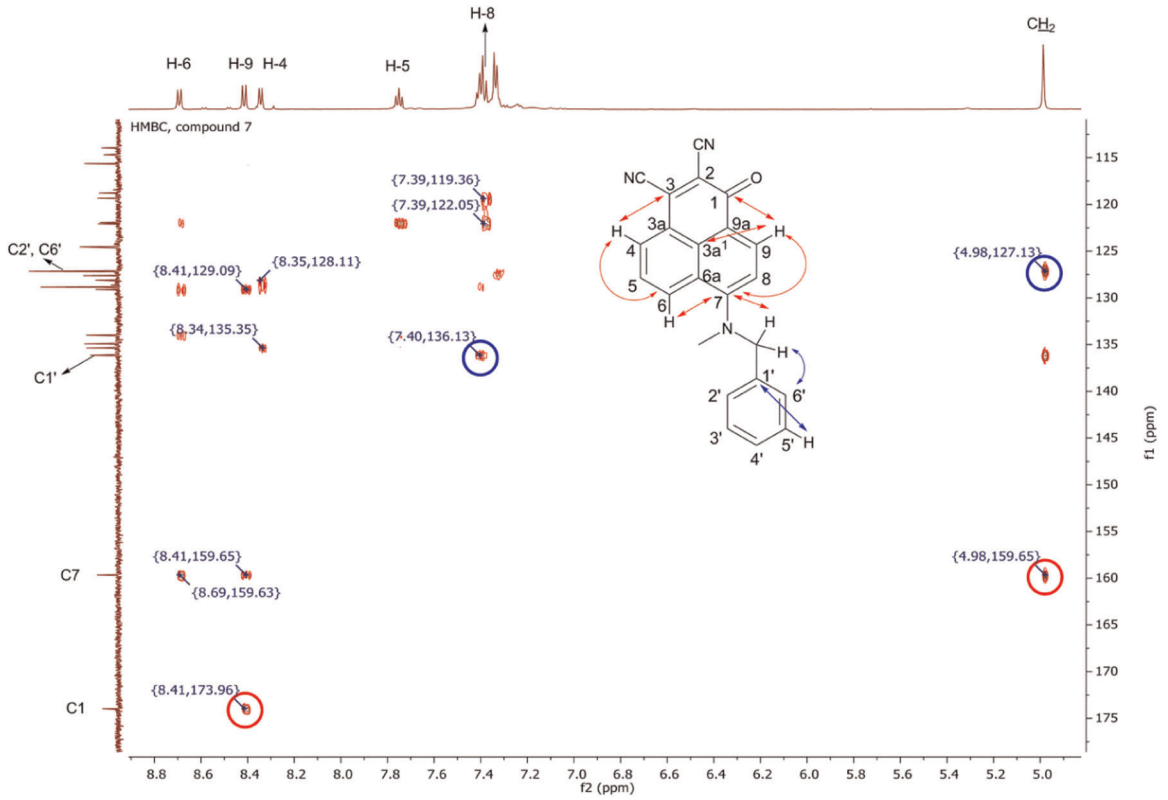



**FIGURE 6** $^{1}H$-$^{13}C$ HMBC spectrum (DMSO-$d_6$) of compound **7**. Only the most significant cross-peaks that correlate protons and carbons, which are connected through three covalent bonds, are noted. Circled cross-peaks in blue indicate correlations of aliphatic $CH_2$ protons (4.98 ppm) to C2′, C6′ (127.13 ppm) and H-3′, H-5′ (7.40 ppm) to C1′ (136.13 ppm). The red-circled cross-peaks indicate a key correlation of H-9 (8.41 ppm) to C1 (173.96 ppm) and a three-bond coupling between aliphatic $CH_2$ protons (4.98 ppm) and C7 (159.65 ppm). DMSO, dimethyl sulfoxide; HMBC, heteronuclear multiple-bond correlation

compounds.[16,18] However, the low-yielding reactions toward phenalene derivatives, their tedious purifications, and poor aqueous solubility[33] precluded our further work with these compounds despite encouraging results in terms of BCL-2 inhibition and despite knowing that several possibilities to explore the SAR still exist. Nevertheless, we believe that the findings presented in this report further clarify the structural issues of this compound class and thus provide additional guidelines for proper structure determination of substituted phenalene derivatives.

## 4 | EXPERIMENTAL

### 4.1 | Chemistry

#### 4.1.1 | General

Reagents and solvents were obtained from commercial sources (Acros Organics, Sigma-Aldrich, TCI Europe, Alfa Aesar, Fluorochem) and were used as received. For reactions involving air- or moisture-sensitive reagents, solvents were distilled before use, and these reactions were carried out under an argon atmosphere. Reactions were monitored using analytical TLC plates (Merck 60 F254, 0.20 mm), and the components were visualized under UV light and/or through staining with the relevant reagent. Preparative normal-phase flash column chromatography was performed on Merck Silica Gel 60 (particle size, 0.040–0.063 mm; Merck). For reversed-phase column chromatography Isolera Biotage One Flash Chromatography system (Biotage) and SNAP Biotage KP-C18-HS column (30 g) was used with a gradient of 0.1% trifluoroacetic acid (TFA) in deionized water and MeCN as eluent (gradient 0% MeCN for 2 column volumes; 10–90% MeCN in 15 column volumes; 90% MeCN for 5 column volumes). Melting points were determined on a Reichelt hot-stage apparatus and are uncorrected. $^{1}H$ and $^{13}C$ NMR spectra were recorded at 295 K on a Bruker Avance 500 MHz spectrometer (Bruker) or Bruker Avance III 400 MHz spectrometer operating at frequencies for $^{1}H$ NMR at 500 or 400 MHz, and for $^{13}C$ NMR at 125 or 101 MHz. The chemical shifts ($\delta$) are reported in parts per million (ppm) and are referenced to the deuterated solvent used. The coupling constants ($J$) are given in Hz, and the splitting patterns are designated as follows: s, singlet; d, doublet; app d, apparent doublet; dd, double doublet; t, triplet; m, multiplet. All $^{13}C$ NMR spectra were proton decoupled. Resonance assignments were made on the basis of one- and two-dimensional NMR techniques, which include $^{1}H$, $^{13}C$, $^{1}H$-$^{1}H$ COSY, $^{1}H$-$^{13}C$ HSQC, $^{1}H$-$^{13}C$ HMBC, and $^{1}H$-$^{1}H$ NOESY experiments. Standard parameter sets (COSYGPSW, NOESYPHSW, HSQCETGP,

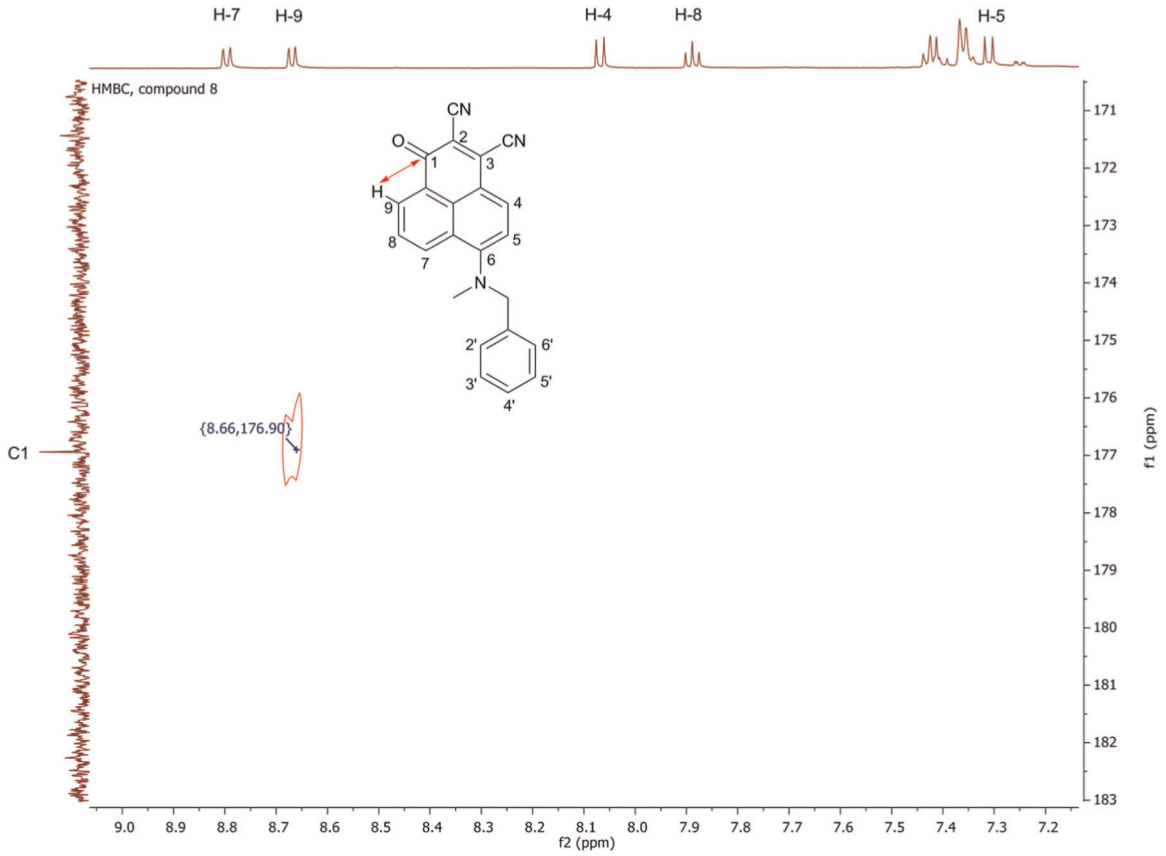



**FIGURE 7** A crucial segment of $^{1}H$-$^{13}C$ HMBC spectrum (DMSO-$d_6$) of compound **8.** A three-bond correlation between H-9 (8.28 ppm) and C1 (185.30 ppm) is shown. DMSO, dimethyl sulfoxide; HMBC, heteronuclear multiple-bond correlation

**TABLE 1** Inhibitory potencies of compounds **3**–**5**, **7**, and **8** toward BCL-2 determined using an ELISA assay[a] and cytotoxicity of compounds **3** and **4** against four cancer cell lines[b]

| | | Cytotoxicity | | | |
|---|---|---|---|---|---|
| Compound | $IC_{50}$ (μM) BCL-2 or RA (%) at 50 μM | U87 $IC_{50}$ (μM) | MV3 $IC_{50}$ (μM) | A2780 $IC_{50}$ (μM) | MDA-MB-231 $IC_{50}$ (μM) |
| **3** | 0.11 ± 0.03 | 39 ± 5 | 36 ± 4 | 39 ± 10 | 22 ± 7 |
| **4** | 0.61 ± 0.14 | 23 ± 4 | 31 ± 3 | 19 ± 4 | 22 ± 4 |
| **5** | 69 ± 2% | n.d. | n.d. | n.d. | n.d. |
| **7** | 2.14 ± 0.33 | n.d. | n.d. | n.d. | n.d. |
| **8** | 2.71 ± 0.20 | n.d. | n.d. | n.d. | n.d. |

Abbreviations: ELISA, enzyme-linked immunosorbent assay; n.d., not determined; RA, residual activity.

[a]Obtained from three independent experiments. For compound **5**, only RA of BCL-2 at 50 μM compound is given. Venetoclax was used as a positive control in the BCL-2 ELISA assay, and the $IC_{50}$ was determined to be 0.017 ± 0.005 μM.

[b]The cytotoxicity data were obtained from three independent experiments.

and HMBCGP) from the Bruker pulse library were used. High-resolution mass measurements were performed on a Thermo Scientific Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) at the Faculty of Pharmacy, University of Ljubljana. The purity of the compounds was determined by HPLC-UV obtained on a liquid chromatography–mass spectrometry (LC-MS) instrument (Applied Biosystems API 2000 LC/MS/MS, HPLC Agilent 1100; Agilent) or on a Thermo Scientific Dionex UltiMate 3000 UHPLC modular system (Thermo Fisher Scientific), equipped with a photodiode array detector set to 254 nm, whereby an Acquity UPLC® BEH Phenyl Column (2.1 × 100 mm; 1.7 μm) was used, thermostated at 40°C, with a flow rate set to 0.3 ml/min. The purities of the test compounds used for the biological evaluations were confirmed to be ≥95% purity by LC, unless stated otherwise.

The InChI codes of the investigated compounds, together with some biological activity data, are provided as Supporting Information.

### 4.1.2 | Synthesis of compounds 1–3

*5-Bromoacenaphthylene-1,2-dione (1)*

Compound **1** was synthesized as described previously.[34,35] Acenaphthenequinone (6.02 g, 33.0 mmol) was weighed into a 250 ml round-bottom flask, followed by the addition of bromine (9.4 ml, 182 mmol, 5.5 equiv). The reaction mixture was stirred at 75°C for 2 h. During the reaction, the flask was fitted with a reflux condenser, a T-shaped adapter allowing a positive flow of argon, and an outlet that led to a trap of 1 M NaOH (200 ml). After the reaction was complete, it was cooled to room temperature and diluted with $H_2O$ (100 ml). Then, the mixture was cooled to 0°C and vigorously stirred while a 40% aqueous solution of $NaHSO_3$ (100 ml) was added. The orange solid was filtered and washed with $H_2O$ (200 ml). Pure compound was obtained by column chromatography using EtOAc/petroleum ether (1:4, v/v) as an eluent system. Yield (6.38 g, 74%); light orange solid; mp: 216–218°C (lit.[34] mp: 230–232°C); $R_f$ = 0.36 (EtOAc/petroleum ether, 1:3); $^1$H NMR (500 MHz, dimethyl sulfoxide [DMSO]-$d_6$) $\delta$ 7.96 (d, $J$ = 7.5 Hz, 1H, Ar–H), 8.04 (dd, $J$ = 8.3, 7.2 Hz, 1H, Ar–H), 8.15 (app d, $J$ = 7.2 Hz, 1H, Ar–H), 8.21 (d, $J$ = 7.5 Hz, 1H, Ar–H), 8.39 (app d, $J$ = 8.3 Hz, 1H, Ar–H); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 123.0, 123.0, 127.4, 129.8, 130.4, 130.6, 131.0, 131.5, 132.9, 145.2, 187.5, 187.7; LC-MS (ESI) (90% $H_2O$ to 100% MeCN in 10 min, then 100% MeCN to 20 min, diode array detector [DAD] 220–400 nm), $t_R$ = 10.40 min, 98% purity, $m/z$ $[M+H]^+$ calcd. for $C_{12}H_6BrO_2$: 260.95, found: 260.9; high-resolution mass spectrometry (HRMS) (electrospray ionization [ESI]) $m/z$ $[M+H]^+$ calcd. for $C_{12}H_6BrO_2$: 260.9546, found: 260.9549.

*5-(4-Isopropylphenoxy)acenaphthylene-1,2-dione (2)*

This compound was synthesized as described previously.[18] Briefly, to a suspension of 5-bromoacenaphthylene-1,2-dione (**1**, 261 mg, 1.0 mmol) and 4-isopropylphenol (136 mg, 1.0 mmol) in dimethylformamide (DMF) (15 ml), $K_2CO_3$ (152 mg, 1.1 mmol, 1.1 equiv) was added and the mixture stirred at 70°C for 5 h. The reaction mixture was then cooled to room temperature and poured into a saturated aqueous solution of NaCl (50 ml), followed by extraction with EtOAc (3 × 80 ml). The combined organic phases were extracted with saturated aqueous solution of NaCl (5 × 50 ml), dried with $Na_2SO_4$, filtered, and evaporated under reduced pressure. The product was purified by column chromatography using EtOAc/petroleum ether (1:3, v/v) as an eluent system. Yield (269 mg, 85%); beige solid; mp: 143–146°C; $R_f$ = 0.49 (EtOAc/petroleum ether, 1:3); $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 1.31 (d, $J$ = 7.0 Hz, 6H, $CH(CH_3)_2$), 2.99 (sep, $J$ = 7.0 Hz, 1H, $CH(CH_3)_2$), 6.98 (d, $J$ = 7.9 Hz, 1H, Ar–H), 7.12–7.16 (m, 2H, Ar–H), 7.33–7.37 (m, 2H, Ar–H), 7.84 (dd, $J$ = 8.4, 7.0 Hz, 1H, Ar–H), 8.00 (d, $J$ = 7.9 Hz, 1H, Ar–H), 8.11 (app d, $J$ = 7.0 Hz, 1H, Ar–H), 8.59 (app d, $J$ = 8.4 Hz, 1H, Ar–H); $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 1.25 (d, $J$ = 7.0 Hz, 6H, $CH(CH_3)_2$), 2.98 (hept, $J$ = 7.0 Hz, 1H, $CH(CH_3)_2$), 6.97 (d, $J$ = 7.9 Hz, 1H, Ar–H), 7.21–7.26 (m, 2H, Ar–H), 7.39–7.45 (m, 2H, Ar–H), 7.93 (dd, $J$ = 8.4, 7.1 Hz, 1H, Ar–H), 8.04 (d, $J$ = 7.9 Hz, 1H, Ar–H), 8.12 (dd d, $J$ = 7.0, 0.6 Hz, 1H, Ar–H), 8.54 (dd, $J$ = 8.4, 0.6 Hz, 1H, Ar–H); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 24.1, 33.7, 111.3, 120.7, 122.7, 123.1, 124.4, 127.8, 127.8, 128.1, 128.3, 128.4, 146.7, 148.1, 152.2, 160.1, 186.2, 189.0; LC-MS (ESI) (90% $H_2O$ to 100% MeCN in 10 min, then 100% MeCN to 20 min, DAD 220–400 nm), $t_R$ = 12.67 min, 94% purity, $m/z$ $[M+H]^+$ calcd. for $C_{21}H_{17}O_3$: 317.11, found: 316.9; HRMS (ESI) $m/z$ $[M+H]^+$ calcd. for $C_{21}H_{17}O_3$: 317.1172, found: 317.1162.

*7-(4-Isopropylphenoxy)-1-oxo-1H-phenalene-2,3-dicarbonitrile (3)*

This compound was synthesized based on a previously described one-pot sequence.[24] To a solution of 5-(4-isopropylphenoxy) acenaphthylene-1,2-dione (**2**, 104 mg, 0.33 mmol) in MeCN (15 ml) at room temperature, malononitrile (22 mg, 0.33 mmol) and $K_2CO_3$ (4.6 mg, 0.033 mmol, 0.1 equiv) were added consecutively. The reaction mixture was then heated at reflux for 1 h (note that the color of the mixture went from yellow to dark green during heating). After that time, the solvent was removed under reduced pressure and the pure product was obtained by two consecutive column chromatography steps using first EtOAc/petroleum ether (1:3, v/v) and second EtOAc/petroleum ether (1:4, v/v) as eluent systems. Yield (63 mg, 52%); orange wax-like solid; mp: 184–187°C; $R_f$ = 0.47 (EtOAc/petroleum ether, 1:3); $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 1.31 (d, $J$ = 6.9 Hz, 6H, $CH(CH_3)_2$), 3.00 (hept, $J$ = 6.9 Hz, 1H, $CH(CH_3)_2$), 7.04 (d, $J$ = 8.6 Hz, 1H, Ar–H, H-8), 7.12–7.16 (m, 2H, Ar–H, H-2′, H-6′), 7.35–7.39 (m, 2H, Ar–H, H-3′, H-5′), 7.86 (dd, $J$ = 8.4, 7.4 Hz, 1H, Ar–H, H-5), 8.45 (dd, $J$ = 7.4, 1.0 Hz, 1H, Ar–H, H-4), 8.63 (d, $J$ = 8.6 Hz, 1H, Ar–H, H-9), 8.91 (dd, $J$ = 8.4, 1.0 Hz, 1H, Ar–H, H-6); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 24.2 ($CH(CH_3)_2$), 33.9 ($CH(CH_3)_2$), 112.5 (C8), 113.0 (CN), 113.2 (CN), 120.7 (C2), 121.0 (C2′, C6′), 122.4 (C9a), 122.7 (C3a), 124.0 (C6a), 127.0 (C5), 128.7 (C3a$^1$), 128.8 (C3′, C5′), 131.1 (C3), 132.2 (C6), 134.7 (C4), 136.7 (C9), 147.6 (C4′), 151.6 (C1′), 163.8 (C7), 176.3 (C1); LC-MS (ESI) (90% $H_2O$ to 100% MeCN in 10 min, then 100% MeCN to 20 min, DAD 220–400 nm), $t_R$ = 12.70 min, 99% purity, $m/z$ $[M+H]^+$ calcd. for $C_{24}H_{17}N_2O_2$: 365.12, found: 364.7; HRMS (ESI) $m/z$ $[M+H]^+$ calcd. for $C_{24}H_{17}N_2O_2$: 365.1285, found: 365.1279.

### 4.1.3 | Synthesis of compounds 4 and 5

This procedure was performed as described previously.[24] To a solution of 5-bromoacenaphthylene-1,2-dione (1, 522 mg, 2.0 mmol) in MeCN (20 ml) at room temperature, malononitrile (132 mg, 2.0 mmol) and $K_2CO_3$ (28 mg, 0.2 mmol, 0.1 equiv) were added consecutively. The reaction mixture was then heated at reflux for 1 h (note that the color of the mixture went from yellow to dark blue during heating). TLC showed the consumption of the starting material with two main products observed. The solvent was then removed under reduced pressure and the crude material loaded on silica for

purification by column chromatography using $CH_2Cl_2$ as an eluent. The first-eluting compound (**5**) and the second-eluting compound (**4**) were further purified by automated reversed-phase flash chromatography using 0.1% TFA in deionized water and MeCN as an eluent system. After the chromatographic purification, fractions containing each of the products were combined separately and organic volatiles were evaporated in vacuo. The remaining aqueous solution was made alkaline with a saturated aqueous solution of $NaHCO_3$ and extracted with $CH_2Cl_2$ (2 × 30 ml). The combined organic phases were dried with $Na_2SO_4$, filtered, and volatile components evaporated under reduced pressure to afford pure products.

*7-Bromo-1-oxo-1H-phenalene-2,3-dicarbonitrile (**4**)*

Yield (129 mg, 21%); orange solid; mp: 238–239°C; $R_f$ = 0.64 ($CH_2Cl_2$); $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 8.06 (dd, $J$ = 8.4, 7.5 Hz, 1H, Ar–H, H-5), 8.37 (d, $J$ = 8.0 Hz, 1H, Ar–H, H-8), 8.44 (d, $J$ = 8.0 Hz, 1H, Ar–H, H-9), 8.46 (app d, $J$ = 7.5 Hz, 1H, Ar–H, H-4), 8.69 (app d, $J$ = 8.4 Hz, 1H, Ar–H, H-6); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 113.4 (CN), 113.7 (CN), 120.1 (C2), 122.8 (C3a), 127.0 (C9a), 127.3 (C7), 129.6 (C5), 130.6 (C6a), 131.4 (C3), 132.9 (C9), 133.3 (C8), 133.8 (C3a1), 135.1 (C4), 135.7 (C6), 177.2 (C1); LC-MS (ESI) (90% $H_2O$ to 100% MeCN in 10 min, then 100% MeCN to 20 min, DAD 220–400 nm), $t_R$ = 10.97 min, 96% purity, $m/z$ $[M+H]^+$ calcd. for $C_{15}H_6BrN_2O$: 308.96, found: 308.9; HRMS (ESI) $m/z$ $[M+H]^+$ calcd. for $C_{21}H_{17}O_3$: 308.9658, found: 308.9646.

*6-Bromo-1-oxo-1H-phenalene-2,3-dicarbonitrile (**5**)*

Yield (186 mg, 30%); orange solid; mp: 241–244°C; $R_f$ = 0.67 ($CH_2Cl_2$); $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.11 (dd, $J$ = 8.3, 7.1 Hz, 1H, Ar–H, H-8), 8.29 (dd, $J$ = 7.1, 0.6 Hz, 1H, Ar–H, H-9), 8.32 (d, $J$ = 7.8 Hz, 1H, Ar–H, H-4), 8.35 (d, $J$ = 7.8 Hz, 1H, Ar–H, H-5), 8.43 (dd, $J$ = 8.3 Hz, 0.6 Hz, 1H, Ar–H, H-7); $^{13}$C NMR (101 MHz, DMSO-$d_6$) $\delta$ 111.7 (CN), 113.2 (CN), 124.3 (C9), 124.4 (C4), 126.8 (3a$^1$), 128.7 (C3a), 129.4 (C6a), 130.2 (C9a), 130.9 (C2), 130.9 (C7), 131.1 (C8), 132.8 (C5), 142.2 (C6), 155.5 (C3), 185.30 (C1); HPLC (95% $H_2O$ [with 0.1% TFA] to 95% MeCN in 10 min, then 95% MeCN for 4 min), $t_R$ = 7.21 min, 96% purity, detection at 254 nm; HRMS (ESI) $m/z$ $[M+H]^+$ calcd. for $C_{15}H_6BrN_2O$: 308.9658, found: 308.9650.

### 4.1.4 | Synthesis of compound **6**

*5-[Benzyl(methyl)amino]acenaphthylene-1,2-dione (**6**)*

5-Bromoacenaphthylene-1,2-dione (**1**, 350 mg, 1.34 mmol) was suspended in DMF (15 ml), followed by the addition of *N*-benzylmethylamine (163 mg, 1.34 mmol) and $K_2CO_3$ (204 mg, 1.47 mmol, 1.1 equiv). The reaction mixture was stirred at 80°C for 12 h. Because TLC showed the presence of the starting compound **1**, additional amounts of *N*-benzylmethylamine (81 mg, 0.67 mmol, 0.5 equiv) and $K_2CO_3$ (93 mg, 0.67 mmol, 0.5 equiv) were added and the mixture stirred at 80°C for additional 4 h. Then, it was cooled to room temperature and poured into a saturated aqueous solution of NaCl (100 ml), followed by extraction with EtOAc (4 × 80 ml). The combined organic phases were extracted with saturated aqueous solution of NaCl (5 × 50 ml), dried with $Na_2SO_4$, filtered, and evaporated under reduced pressure. The product was purified by column chromatography using EtOAc/petroleum ether (1:3, v/v) as an eluent system. Yield (104 mg, 26%); dark red solid; mp: 162–163°C; $R_f$ = 0.30 (EtOAc/petroleum ether, 1:3); $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 3.13 (s, 3H, NC$H_3$), 4.76 (s, 2H, PhC$H_2$), 7.15 (d, $J$ = 8.0 Hz, 1H, Ar–H), 7.34–7.38 (m, 1H, Ar–H), 7.39–7.45 (m, 4H, Ar–H), 7.60 (dd, $J$ = 8.4, 7.0 Hz, 1H, Ar–H), 7.96 (d, $J$ = 7.0 Hz, 1H, Ar–H), 8.05 (d, $J$ = 8.0 Hz, 1H, Ar–H), 8.28 (app d, $J$ = 8.4 Hz, 1H, Ar–H); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 40.4, 60.1, 113.9, 121.7, 121.8, 123.6, 124.3, 126.4, 127.2, 127.8, 128.7, 129.0, 130.0, 136.7, 148.8, 154.9, 185.6, 190.4; LC-MS (ESI) (90% $H_2O$ to 100% MeCN in 10 min, then 100% MeCN to 20 min, DAD 220–400 nm), $t_R$ = 11.58 min, 97% purity, $m/z$ $[M+H]^+$ calcd. for $C_{20}H_{16}NO_2$: 302.11, found: 301.8; HRMS (ESI) $m/z$ $[M+H]^+$ calcd. for $C_{20}H_{16}NO_2$: 302.1176, found: 302.1173.

### 4.1.5 | Synthesis of compounds **7** and **8**

This procedure was performed as described previously.[24] To a solution of 5-[benzyl(methyl)amino]acenaphthylene-1,2-dione (**6**, 75 mg, 0.25 mmol) in MeCN (15 ml) at room temperature, malononitrile (17 mg, 0.25 mmol) and $K_2CO_3$ (3.5 mg, 0.025 mmol, 0.1 equiv) were added consecutively. The reaction mixture was then heated at reflux for 1 h (note that the color of the mixture went from yellow to dark blue during heating). After that time, the solvent was removed under reduced pressure and the crude product (showing two major spots on TLC) loaded on silica for purification by column chromatography using EtOAc/petroleum ether (1:1, v/v) as an eluent system. Under these conditions, only the first-eluting compound (**7**) was purified successfully. For the second-eluting compound (**8**), three additional column chromatography purifications using EtOAc/petroleum ether (1:1, v/v) as an eluent system were needed.

*7-[Benzyl(methyl)amino]-1-oxo-1H-phenalene-2,3-dicarbonitrile (**7**)*

This compound eluted first from the column. Yield (36 mg, 41%); dark blue wax-like solid; mp: 244–248°C; $R_f$ = 0.36 (EtOAc/petroleum ether, 1:1); $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 3.35 (s, 3H, NC$H_3$), 4.99 (s, 2H, PhC$H_2$), 7.32–7.35 (m, 3H, Ar–H, H-2′, H-6′, H-4′), 7.38 (d, $J$ = 9.0 Hz, 1H, Ar–H, H-8), 7.39–7.42 (m, 2H, Ar–H, H-3′, H-5′), 7.75 (app t, $J$ = 7.9 Hz, 1H, Ar–H, H-5), 8.34 (d, $J$ = 7.3 Hz, 1H, Ar–H, H-4), 8.41 (d, $J$ = 9.0 Hz, 1H, Ar–H, H-9), 8.69 (d, $J$ = 8.2 Hz, 1H, Ar–H, H-6); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ $^{13}$C NMR (1125 MHz, DMSO-$d_6$) $\delta$ 43.1 ($CH_3$), 60.0 ($CH_2$), 113.9 (CN), 114.7 (CN), 115.6 (C8), 118.8 (C2), 119.4 (C9a), 121.9 (C3a), 122.1 (C6a), 124.5 (C5), 127.1 (C2′, C6′), 127.6 (C4′), 128.1 (C3), 128.8 (C3′, C5′), 129.1 (C3a$^1$), 134.0 (C4), 134.9 (C9), 135.4 (C6), 136.1 (C1′), 159.7 (C7), 174.0 (C1); LC-MS (ESI) (90% $H_2O$ to 100% MeCN in 10 min, then 100% MeCN to 20 min, DAD 220–400 nm), $t_R$ = 11.45 min, 98% purity, $m/z$ $[M+H]^+$ calcd. for $C_{23}H_{16}N_3O$: 350.12, found: 349.8; HRMS (ESI) $m/z$ $[M+H]^+$ calcd. for $C_{23}H_{16}N_3O$: 350.1288, found: 350.1281.

*6-[Benzyl(methyl)amino]-1-oxo-1H-phenalene-2,3-dicarbonitrile (**8**)*

This compound eluted second from the column. Yield (7 mg, 9%); dark violet wax-like solid; mp: 248–251°C; $R_f$ = 0.22 (EtOAc/petroleum ether, 1:1); $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 3.51 (s, 3H, NC$\underline{H}_3$), 5.13 (s, 2H, PhC$\underline{H}_2$), 7.31 (d, $J$ = 9.2 Hz, 1H, Ar–$\underline{H}$, H-5), 7.34–7.38 (m, 3H, Ar–$\underline{H}$, H-2′, H-6′, H-4′), 7.40–7.44 (m, 2H, Ar–$\underline{H}$, H-3′, H-5′), 7.89 (t, $J$ = 8.1 Hz, 1H, Ar–$\underline{H}$, H-8), 8.06 (d, $J$ = 9.2 Hz, 1H, Ar–$\underline{H}$, H-4), 8.67 (dd, $J$ = 7.5, 0.9 Hz, 1H, Ar–$\underline{H}$, H-9), 8.81 (dd, $J$ = 8.3, 0.9 Hz, 1H, Ar–$\underline{H}$, H-7); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 44.0 ($CH_3$), 59.8 ($CH_2$), 106.5 (C2*), 113.1 (C3a), 114.0 (C5), 114.4 (CN*), 115.9 (CN*), 123.4 (C6a), 126.3 (C3), 126.6 (C8), 127.1 (C2′, C6′), 127.8 (C4′), 129.0 (C3′, C5′), 129.2 (C3a$^1$), 129.6 (C9a), 132.8 (C9), 135.5 (C1′), 135.6 (C7), 136.6 (C4), 160.5 (C6), 176.9 (C1); LC-MS (ESI) (90% $H_2O$ to 100% MeCN in 10 min, then 100% MeCN to 20 min, DAD 220–400 nm), $t_R$ = 10.81 min, 97% purity, $m/z$ $[M+H]^+$ calcd. for $C_{23}H_{16}N_3O$: 350.12, found: 349.8; HRMS (ESI) $m/z$ $[M+H]^+$ calcd. for $C_{23}H_{16}N_3O$: 350.1288, found: 350.1279. *The assignations for these signals were obtained from predictions using ChemDraw Ultra 18.0.

## 4.2 | Biochemical assays

### 4.2.1 | BCL-2 competitive inhibition assay

A 0.06 µg/ml solution (1 mg/ml in 20% EtOH/$H_2O$) of biotinylated BIM peptide (residues 81–106: (Biotin)-β-Ala-β-Asp-Met-Arg-Pro-Glu-Ile-Trp-Ile-Ala-Gln-Glu-Leu-Arg-Arg-Ile-Gly-Asp-Glu-Phe-Asn-Ala-Tyr-Tyr-Ala-Arg-Arg-$NH_2$ [catalog no. 3526; Tocris Bioscience, Bio-Techne Ltd.]) was prepared in SuperBlock Blocking Buffer (Thermo Fisher Scientific). The solution (100 µl/well) was applied to a streptavidin-coated 96-well plate (Pierce® Streptavidin Coated High Binding Capacity Plates, catalog no. 15500; Thermo Fisher Scientific) and incubated at room temperature with shaking for 1.5 h. Different concentrations of inhibitors (prepared from a 20 mM stock DMSO solutions) in phosphate-buffered saline (PBS) were incubated with 20 nM purified His-tagged BCL-2 protein (catalog no. 10195-H08E; Sino Biological) for 1 h at room temperature. The BIM–biotin–streptavidin-coated plates were washed three times with 0.05% Tween-20 in PBS. Afterward, 100-µl aliquots of the inhibitor–protein solutions were transferred to the BIM–biotin–streptavidin-coated plate and incubated at room temperature for 2 h. The plate was washed three times with 0.05% Tween-20 in PBS, followed by applying 100 µl anti-His-tag mouse mAb–horseradish peroxidase conjugate (catalog no. BZ-652504; BioLegend), 1:1000 dilution in SuperBlock Blocking Buffer to the well, and incubating the plate for 1 h at room temperature. The plate was then washed five times with 0.05% Tween-20 in PBS, and 100 µl of *o*-phenylenediamine (prepared by dissolving one pre-packaged tablet [catalog no. P5412; Aldrich] in 10 ml of 0.1 M $Na_2HPO_4$/0.05 M citric acid buffer, pH 5, with added 6 µl of 30% $H_2O_2$ before use) was applied to the wells. The enzymatic activity was stopped after 5 min with the addition of 2 mM $H_2SO_4$. Absorbance was measured on BioTek Synergy HT microplate reader (BioTek Instruments Inc.) at 490 nm and $IC_{50}$ values were determined after fitting curves using GraphPad Prism (GraphPad Software). Three independent experiments were performed with each inhibitor to calculate average $IC_{50}$ values and standard deviation.

### 4.2.2 | Cell culture

A2780 (kind gift from Prof. Ulrich Jaehde, Pharmaceutical Institute, University of Bonn) and MV-3 (kind gift from Epo GmbH Berlin) cell lines were cultivated in RPMI-1640 medium (PAN Biotech) containing 10% fetal calf serum (FCS; Sigma-Aldrich), 1% penicillin/streptomycin (PAN Biotech), and 1.5% L-glutamine (all from PAN Biotech). U87 cells (kind gift from Prof. Christa E. Müller, Pharmaceutical Institute, University of Bonn) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 1% penicillin/streptomycin, and 1.5% L-glutamine. The same medium with the addition of 1 mM pyruvate was used for MDA-MB-231 cells (kind gift from Epo GmbH Berlin). All cells were grown in a humidified chamber at 37°C and with 5% $CO_2$.

### 4.2.3 | Metabolic activity assay

Cytotoxicity of BCL-2 inhibitors was determined with the MTT assay. Cells ($1 \times 10^4$ cells [A2780, U87, MV-3, or MDA-MB-231] per well) were seeded on a 96-well plate and left to adhere. Afterward, compounds were added at different concentrations ranging from 1 to 50 µM. After 24-h incubation, the MTT reagent was added and left for 1 h before adding DMSO. Next, absorbance was measured at 690 nm on the Tecan plate reader. $IC_{50}$ values were calculated from three independent experiments in GraphPad prism.

## ACKNOWLEDGMENTS

We thank Maja Frelih for HRMS measurements. This study was funded by the Slovenian Research Agency (research core funding No. P1-0208, Grant J1-2485 to Izidor Sosič). Izidor Sosič and Martina Gobec acknowledge short-term fellowships from FEBS and EMBO, respectively.

## CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interests.

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**How to cite this article:** I. Sosič, M. Gobec, C. Steinebach, M. Schlesinger, G. Bendas, M. Gütschow, *Arch. Pharm.* **2021**, e2100151. https://doi.org/10.1002/ardp.202100025