



Synthesis, structure, photophysical, photochemical, and biological properties of hybrid macrocyclic structures containing two naphthalene moieties and a tri(ethylene glycol) tether

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ABSTRACT

The structure of some promising crown ether-paracyclophane hybrid macrocyclic derivatives showing remarkable cytotoxicity was modified by replacing the two phenyl rings with naphthalene units. The synthesis of the newly designed macrocyclic derivatives was accomplished by applying the previously developed CuAAC coupling-dimerization strategy to the previously published naphthalene MBHA derivative **5**. Then, the resulting tri(ethylene glycol)-tethered MBHA dimer **6** was reacted with n-butylamine to obtain the mixture of macrocyclic diastereomers **4** in good yields. These diastereomers were separated by flash chromatography and thoroughly characterized from the point of view of their structure, photophysical, photochemical, and biological features. The compounds showed interesting anti-proliferative and pro-apoptotic activities against cancer cells, paving the way to be further developed as antitumor/antiproliferative agents.

1. Introduction

Crown ethers represent macrocyclic oligomers of ethylene oxide and are structurally classified into aliphatic and aromatic variants. Their nomenclature and foundational characterization stem from the pioneering work of Charles J. Pedersen in 1967, who first described their ability to form host-guest complexes with metal cations [1]. Owing to their distinct conformational flexibility and electron-rich cavities, crown ethers exhibit high selectivity toward alkaline and alkaline-earth metal ions, as well as certain non-metallic species. These properties render them suitable for biological applications, particularly in the design of artificial intramembrane channels facilitating ion translocation across lipid bilayers [2]. Experimental evidence has demonstrated that hydrophile compounds incorporating crown ether moieties may exert cytotoxic effects on mammalian cells, likely due to uncontrolled ion flux across the plasma membrane [3]. Furthermore, Cai and Arenaz reported antiproliferative activity for specific crown ether derivatives [4]. These

findings, combined with the known antitumor potential of natural ionophores such as valinomycin, prompted Marjanović et al. to investigate the antiproliferative and antitumor properties of synthetic crown ethers [5]. Their results support the hypothesis that the pronounced cytotoxicity observed in several crown ether compounds is attributable to their function as membrane-active potassium ion transporters, promoting K⁺ efflux.

Targeting the cell membrane via cationic ionophores, including crown ethers, can disrupt physiological cation homeostasis, potentially inducing toxic effects even in non-malignant tissues. Previous studies have documented reversible toxicological manifestations in various species, including neurobehavioral alterations, ocular and dermal irritation, and testicular atrophy. Based on these observations, it has been proposed that crown ethers and similar membrane-active agents may serve as alternative anticancer therapeutics, offering distinct toxicity profiles compared to conventional drugs. Their ability to inhibit P-glycoprotein (P-gp)-mediated drug efflux further supports their

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potential as adjuvants in oncological treatment regimens.

The aforementioned study also revealed that the anticancer efficacy of crown ethers is closely linked to the structural features of their hydrophilic cavity and the surrounding hydrophobic framework. Notably, di-*tert*-butyldicyclohexano-18-crown-6 and di-*tert*-butyldibenzo-18-crown-6 exhibited marked cytotoxicity. These compounds significantly altered cell cycle progression, inducing G1 phase arrest followed by apoptotic events. The data underscore the relevance of molecular architecture, hydrophobic group orientation, and polarizable element distribution in mediating interactions with cellular membranes. Consequently, the authors posit that crown ethers may inhibit tumor cell proliferation by perturbing potassium ion homeostasis, thereby triggering cell cycle disruption and apoptosis. Nonetheless, further *in vitro* and *in vivo* investigations are warranted to validate these findings and assess their translational potential [5,6].

In parallel, the Copper(I)-Catalyzed Azide-Alkyne Cycloaddition (CuAAC) reaction, widely recognized as a prototypical “click” reaction, has been extensively employed in the synthesis of mechanically interlocked molecules (MIMs), including rotaxanes and catenanes [7,8]. Complementarily, the Morita–Baylis–Hillman (MBH) reaction offers a versatile synthetic route for constructing new C–C bonds, yielding allylic alcohol derivatives (MBH adducts, MBHAs) that can be acetylated to generate efficient leaving groups. This methodology exemplifies the utility of organocatalysis in the context of sustainable and stereoselective synthesis [9,10].

Within our laboratories, a series of MBHA derivatives have been rationally designed and synthesized to react with nucleophilic substrates such as imidazole, histidine analogs, *n*-butylamine, lysine derivatives, and proteins [11–20]. Reactivity assessments in protein models revealed that MBHA compounds can covalently modify hexahistidine tags on single-chain Fv antibodies, resulting in multi-PEGylated conjugates [13, 14]. Additionally, MBHA derivatives were shown to react with lysine residues located within hydrophobic pockets of retinoic acid-binding proteins [15], and with human serum albumin (HSA), inducing a notable fluorescence shift from blue to green-yellow, thereby generating green fluorescent albumin (GFA) derivatives [18]. When tested against model lysine substrates such as *n*-butylamine and α -acetyl-L-lysine methyl ester, MBHA compounds yielded both mono- and diadducts in variable ratios, contingent upon the reaction conditions [20].

Moreover, MBHA derivative **1** was used in the preparation of oligo (ethylene glycol)-tethered MBHA dimers **2a–d** (Fig. 1), which were found to react with *n*-butylamine leading to the formation of light-sensitive macrocyclic crown ether-paracyclophane hybrid structures **3a–d** [17,21].

Very interestingly, the assessment of the biological properties of the resulting crown ether-paracyclophane hybrid structures **3a–d** showed their remarkable cytotoxic activity in some cancer cell lines [21]. With the aim of developing novel macrocyclic architectures with enhanced anticancer potency, we designed compound **4** (Fig. 2) as a structurally optimized analogue of our lead scaffold **3a**.

In this design approach, the two core phenyl rings of **3a** were strategically replaced with naphthalene moieties. This structural modification was envisioned to improve biological activity by significantly increasing the overall lipophilicity (hydrophobicity) of the macrocycle, thereby promoting superior cell membrane permeability and target interaction. Furthermore, the extended aromatic system of the naphthalene units provides an expanded surface area for hydrophobic effects and π - π stacking, while concurrently imparting structural rigidity to counteract the flexibility of the tri(ethylene glycol) tether and pre-organize the macrocyclic cavity.

In the present study we report on the synthesis, the structural characterization, the photophysical, the photochemical, and the biological properties of hybrid macrocyclic structures **4** containing two naphthalene moieties and a tri(ethylene glycol) tether.

2. Results and discussion

2.1. Synthesis and structural characterization

The synthesis of macrocyclic naphthalene derivatives **4** (Scheme 1) was performed by following the same procedure previously developed for the preparation of phenyl analogue **3a** [17].

Reagents: (i) $N_3-(CH_2CH_2O)_2-CH_2CH_2-N_3$, CuBr(I), DIPEA, CH_3CN ; (ii) $CH_3CH_2CH_2CH_2NH_2$; $CHCl_3$.

The CuAAC coupling-dimerization reaction of the previously published naphthalene MBHA derivative **5** (see Ref. [12]) was carried out at room temperature for 3 h in acetonitrile in the presence of CuBr(I) as the catalyst and DIPEA as the base to obtain the tri(ethylene glycol)-tethered MBHA dimer **6**. Analogously to the procedure already used with **2a–d**, compound **6** was then made to react with *n*-butylamine to obtain the mixture of macrocyclic diastereomers **4** in good yields. Subsequently, the (*E,E*)- and (*E,Z*)-diastereomers of **4** were separated by flash chromatography and obtained in the ratio of ca. 3:1 in partial agreement with the results obtained with the corresponding phenyl derivatives **3a–d** [17,21].

To obtain information about the macrocyclic structure of the two diastereomers, the ^1H and ^{13}C NMR spectra of (*E,E*)-diastereomer were assigned and compared with those of (*E,Z*)-diastereomer of **4** (Figs. 3 and 4).

The comparisons shown in Figs. 3 and 4 highlight the symmetric structure of (*E,E*)-**4** with respect to the relative lack of symmetry observed for (*E,Z*)-**4**. In fact, this latter compound shows two distinct sets of signals in almost all the regions of its NMR spectra with the exception in the up-field region, where the signals of the butylamine moiety are located, in full agreement with the results obtained with the corresponding phenyl derivatives **3a** [17].

Interestingly, in the ^1H NMR spectrum of non-symmetric diastereomer (*E,Z*)-**4**, some signals assigned to some naphthalene protons appear to be more shielded than the corresponding ones of symmetric diastereomer (*E,E*)-**4** suggesting the existence of significant differences

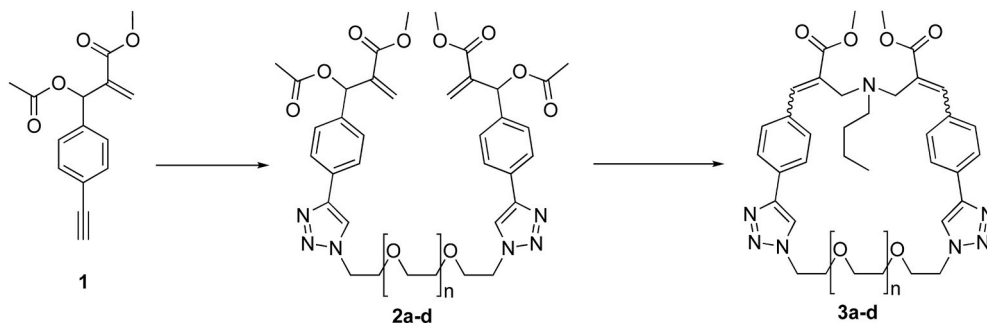


Fig. 1. Synthesis of MBHA dimers **2a–d** and their reaction with *n*-butylamine leading to light-sensitive macrocyclic crown ether-paracyclophane hybrid structures **3a–d** (i. e. **2a** and **3a**: $n = 1$; **2b** and **3b**: $n = 2$; **2c** and **3c**: $n = 3$; **2d** and **3d**: $n = 4$).

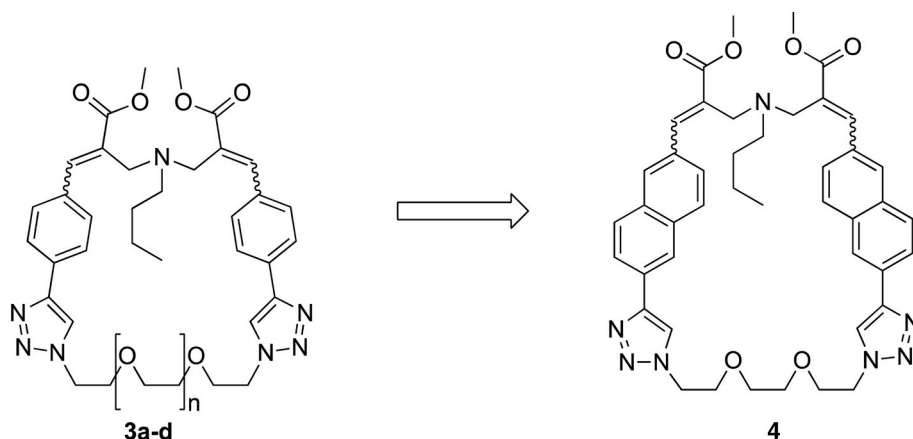
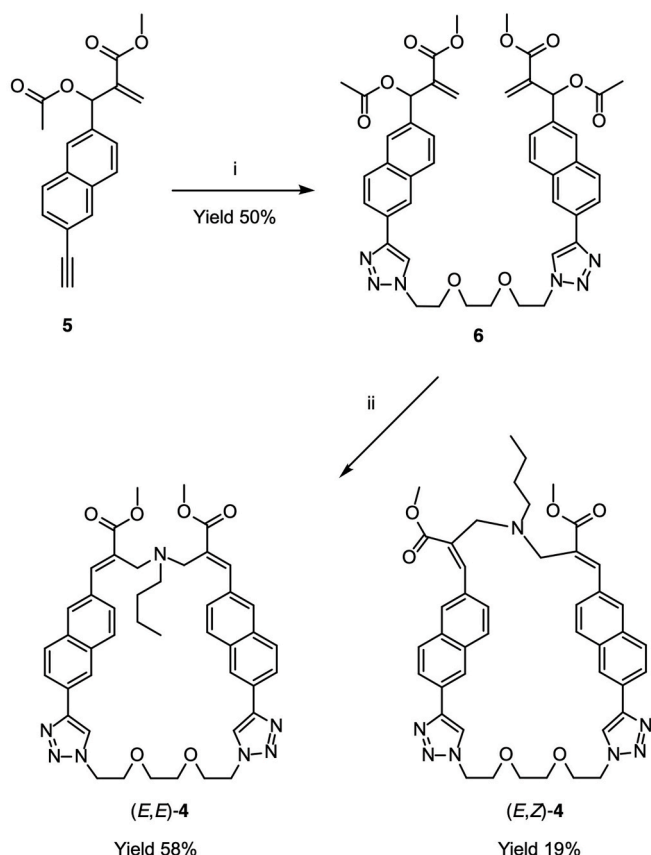


Fig. 2. Design of the naphthalene analogue **4** of the light-sensitive macrocyclic crown ether-paracyclophane hybrid structures **3a** (i. e. **3a**: $n = 1$; **3b**: $n = 2$; **3c**: $n = 3$; **3d**: $n = 4$).



Scheme 1. Synthetic procedure used in the preparation of macrocyclic naphthalene derivatives **4**.

in the conformational preferences in the two diastereomers of compound **4**.

2.2. Photophysical characterization

The photophysical properties of diastereomers (*E,E*)-**4** and (*E,Z*)-**4** were firstly investigated by recording their absorption spectra (Fig. 5 and SI-1).

The absorption spectra of both the diastereomers molecularly dissolved in methanol showed the presence of a low energy component centered at 313–318 nm and of a high energy component a ca. 257 nm

with a shoulder at higher energy. As expected, the two diastereomers showed very similar absorption spectra, with the one of symmetric and (*E,E*)-**4**, being red-shifted of about 5 nm in the lower energy component. Interestingly, the absorption spectra appeared to be reasonably insensitive to the concentration in the range evaluated (i. e. from 2×10^{-4} M to 1×10^{-5} M).

On the other hand, the emission features of the two diastereomers in solution in methanol showed a rather complex behavior. In particular, both the diastereomers showed weak emission features (PL quantum yield of about 0.2%) suggesting that the relaxation of the excited state occurred mainly through non-radiative mechanisms. The emission spectra (Fig. 6) were characterized by the presence of a main high energy component centered at 355 nm and a low energy component peaked at 410 nm. Very similar results were obtained for the compounds molecularly dissolved in DMSO solutions (see Fig. SI-2 in the Supporting Information).

It is noteworthy that the relative intensity of the low energy component at 410 nm was different in the spectra of the two diastereomers being more evident in the spectrum of (*E,Z*)-**4**, when excited at 313 nm, but was virtually absent when excited at 258 nm. Moreover, this low energy component appeared at 440 nm in the spectrum of (*E,Z*)-**4** when it was excited at 345 nm, probably associated to a component of the compounds not completely dissolved. In fact, the relative intensity of the low energy component was found to increase with concentration (Fig. SI-3) and appeared at 440–460 nm in the solid state (Fig. SI-4).

The results were tentatively explained by assuming that the low energy component (i. e. 410 nm) represented the true emission of both diastereomers and possible photochemical transformations were triggered by the excitation at the different wavelength values in the range 250–340 nm.

2.3. Photochemical characterization

Owing to the presence of two photo-isomerizable double bonds very similar to those present in the structure of phenyl analogue (*E,E*)-**3a**, a possible relaxation mechanism is represented by the double bond isomerization like that already observed for (*E,E*)-**3a** [17]. Another competing pathway is represented by the [2 + 2] photocycloaddition reaction already observed in other similar molecular systems [20,22].

Thus, the photochemical features of diastereomer (*E,E*)-**4** were investigated by ^1H NMR spectroscopy. In particular, (*E,E*)-**4** was dissolved in deuterated DMSO into a 5 mm NMR tube and the resulting solution was irradiated with monochromatic light centered at 320 nm and the reaction progress was followed via ^1H NMR spectroscopy by recording spectra at regular time intervals (Fig. 7).

The comparison of the ^1H NMR spectra suggested that (*E,E*)-**4**

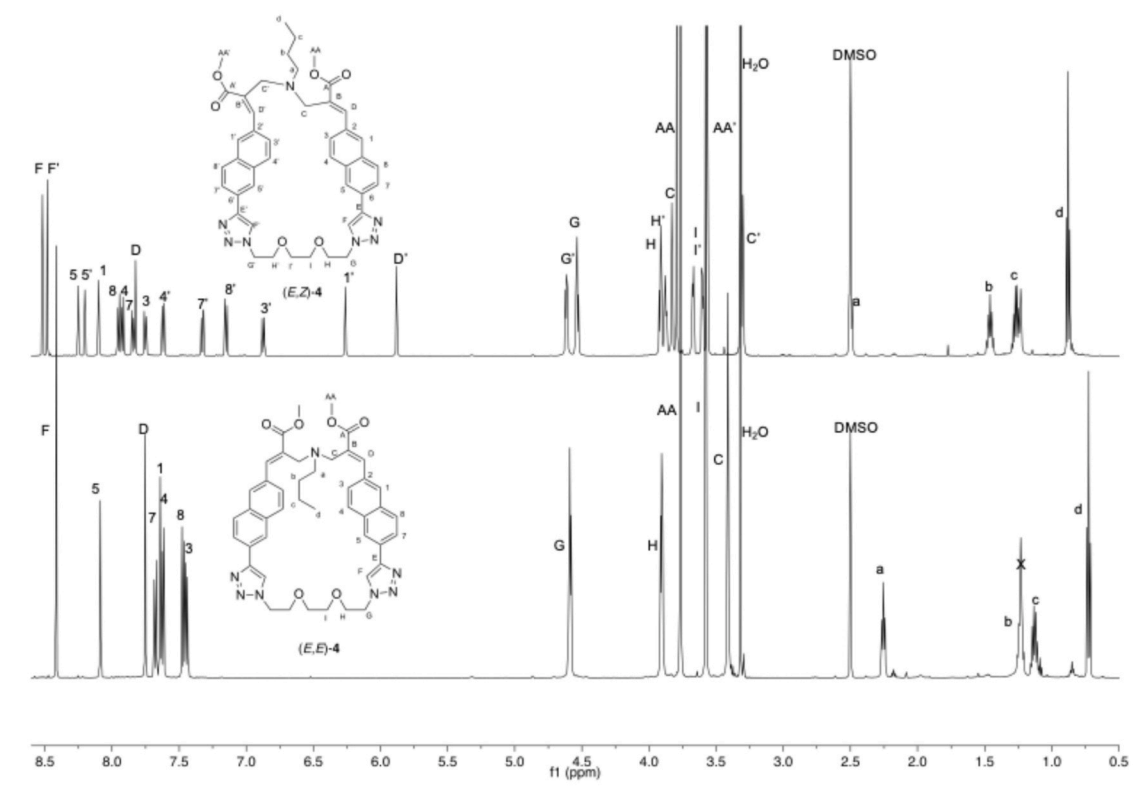


Fig. 3. Comparison of the ^1H NMR spectra recorded (600 MHz, DMSO-d_6) with crystalline samples of (E,E) -4 and (E,Z) -4.

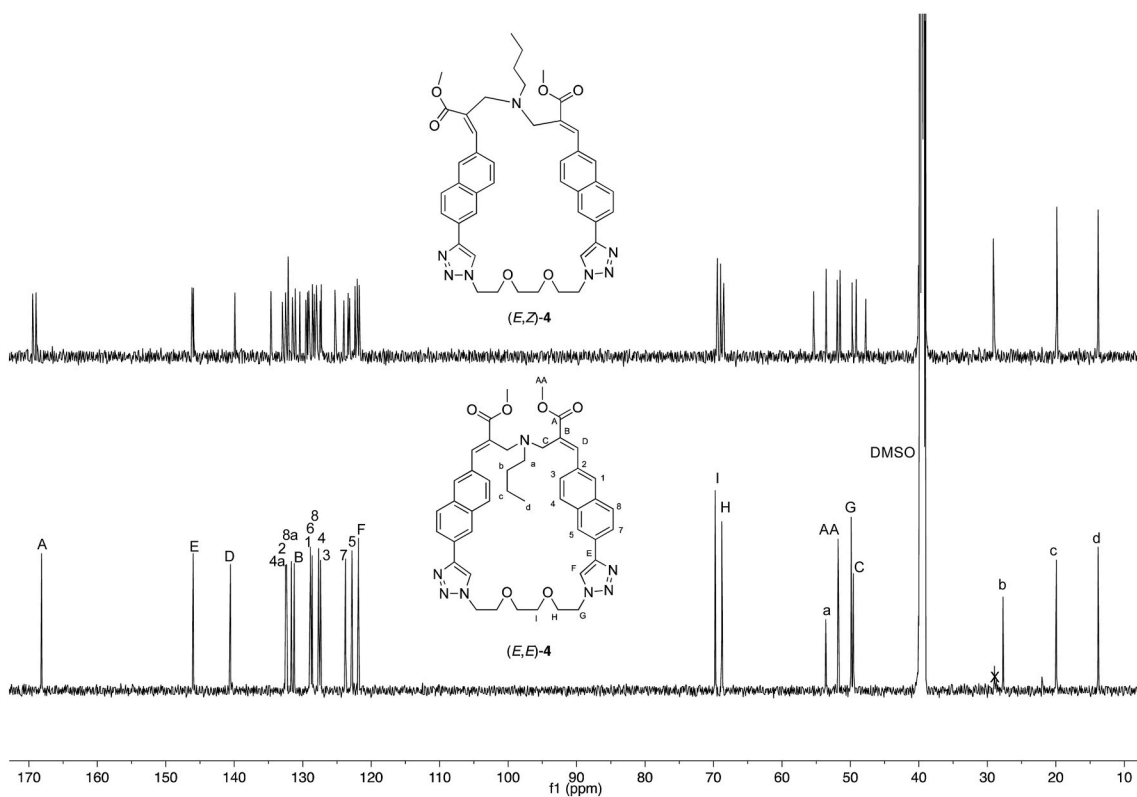


Fig. 4. Comparison of the ^{13}C NMR spectra recorded (150 MHz, DMSO-d_6) with crystalline samples of (E,E) -4 and (E,Z) -4.

appeared to be prone to undergo photoisomerization. In fact, after 1 h exposition to monochromatic light at 320 nm, some small peaks typical

of diastereomer (E,Z) -4 appeared in the ^1H NMR spectrum along with a set of new signals that increased in intensity during the irradiation much

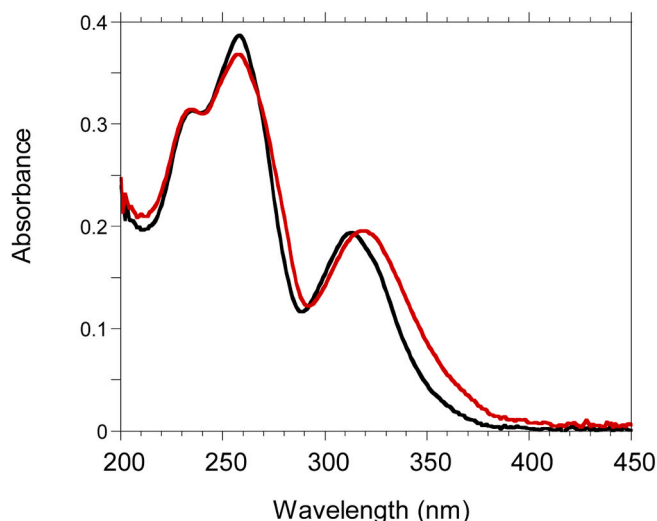


Fig. 5. Absorption spectra of (*E,E*)-4 (red line) and (*E,Z*)-4 (black line) in methanol solutions ($c \approx 10^{-5}$ M).

more than the ones attributed (*E,Z*)-4. Purification of the final reaction mixture by flash chromatography allowed the isolation of compound **7-M**, which was confirmed to be the major product of the [2 + 2] photocycloaddition reaction (Figs. 8 and 9 and Scheme 2).

Thus, compound (*E,E*)-4 can undergo the [2 + 2] photocycloaddition reaction with the formation of the corresponding cyclobutane derivative **7-M**. This reaction appears to be in competition with the double bond isomerization reaction leading to the corresponding (*E,Z*)-diastereomer [17], which appears to be unable to undergo the [2 + 2] photocycloaddition reaction, probably for ring strain reasons.

2.4. Computational modeling

The photochemical reaction in Scheme 2 yields a dominant cyclobutene stereoisomer **7-M** consistent with a $2\pi_s-2\pi_s$ (suprafacial-suprafacial) olefin cycloaddition - a selectivity forbidden by the ground-state Woodward-Hoffmann rules [23] but allowed upon photoexcitation, with the excited-state molecule returning to the ground-state through a conical intersection (CoIn). To support the experimental observation, we computed the minimum energy S_1/S_0 CoIn for the suprafacial-suprafacial attack and delivering the excited-state molecule to the ground-state using multiscale quantum mechanical/molecular mechanical (QM/MM) approach on a simplified gas-phase reactant model in which the butyl group attached to the nitrogen atom was replaced with a hydrogen atom (see Fig. 10A). Specifically, the S_1/S_0

CoIn was modeled by using the 2-roots state-averaged complete active space self-consistent field theory with a (4-electrons, 4- π -orbitals) active space and using the 6-31G* basis set (SA2-CASSCF(4,4)/6-31G*) [24] for the QM subsystem and the OPLS-AA force field [25] for the MM subsystem (see Fig. 10A). Details about the computational approach are reported in the Supporting Information.

The computed S_1/S_0 CoIn adopts the rhomboidal distortion characteristic of the conical intersection driving photochemical [2 + 2] cycloadditions [26,27], confirming the existence of this structural feature for the present system. Moreover, the analysis of the four active-space molecular orbitals (see Fig. SI-5 of the Supporting Information) reveals significant electron density and overlap between the two interacting π systems at the CoIn, consistent with suprafacial-suprafacial σ -bond formation with the $2\pi_s-2\pi_s$ stereochemistry. A more thorough investigation of the mechanistic details is currently being carried out and will be presented in a forthcoming study.

2.5. Preliminary biological evaluation in cytotoxicity assays

To evaluate the translational and therapeutic potential of the newly synthesized macrocyclic derivatives, MDA-MB-231 (triple-negative breast cancer) and A375 (metastatic melanoma) cell lines were selected as experimental models. Beyond their aggressive phenotype and well-known resistance to conventional therapies, both malignancies are characterized by a significant remodeling and overexpression of specific ion channels, particularly potassium and calcium channels, which actively drive tumor progression, invasiveness, and metastatic dissemination. Given that the design of our crown ether-based hybrids is inherently tailored to disrupt cellular ion homeostasis and dissipate membrane potential, these cell lines represent highly relevant biological targets to validate the efficacy of such synthetic ionophores.

Furthermore, because these macrocycles are hypothesized to act, at least in part, through modulation of cell membrane biophysical properties rather than by directly targeting a single mutated protein [21], they may represent a promising “receptor-independent” therapeutic strategy, potentially reducing the impact of secondary resistance mechanisms typically associated with targeted therapies.

The cytotoxic activity of the synthesized macrocyclic compounds (*E,E*)-4, (*E,Z*)-4, and **7-M** was evaluated in two human tumor cell lines, namely triple-negative breast cancer MDA-MB-231 and human melanoma A375, using the Neutral Red Uptake assay after 24 h of exposure. Doxorubicin, a widely used chemotherapeutic agent, was included as a reference compound to enable a direct comparison of cytotoxic potency. Cell viability was expressed as a percentage relative to untreated control cells, thereby providing a quantitative measure of compound-induced cytotoxicity.

As shown in Fig. 11, all the tested compounds produced a clear

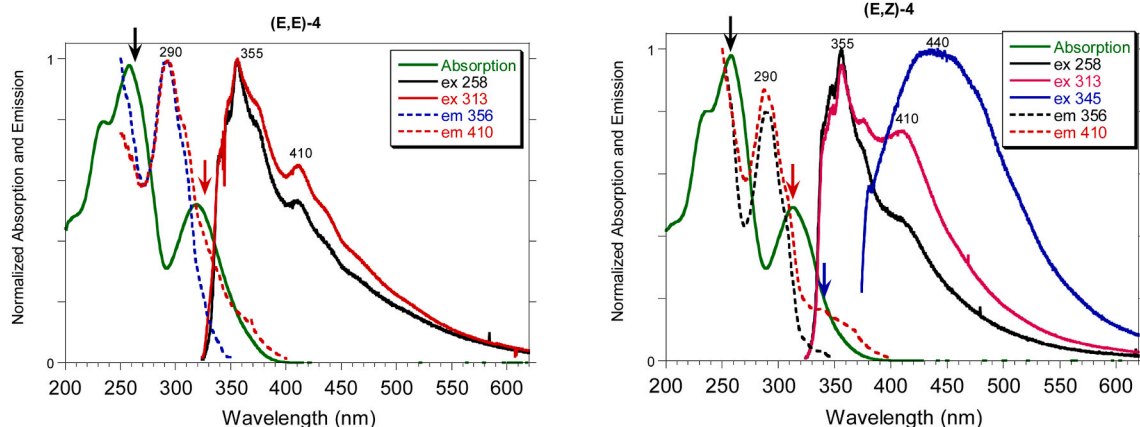


Fig. 6. Absorption (solid green line), emission (solid lines) and excitation spectra (dashed lines) of (*E,E*)-4 (left) and (*E,Z*)-4 (right) in methanol solutions.

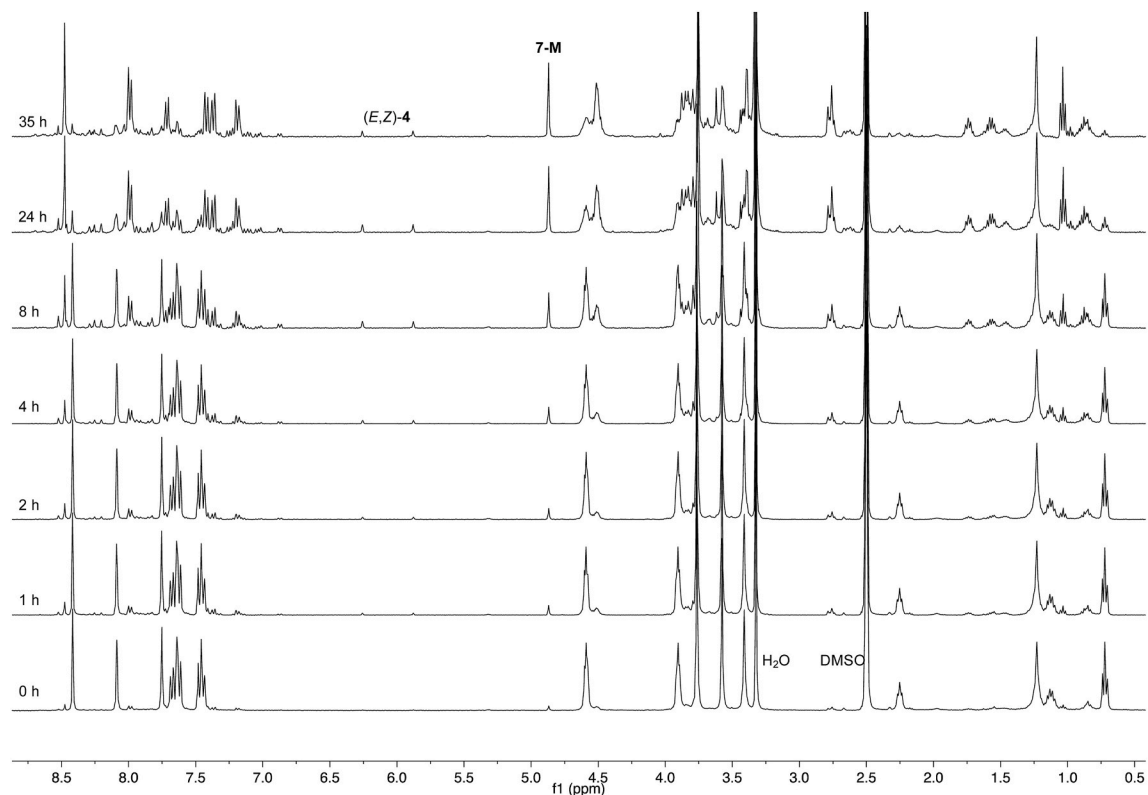


Fig. 7. Comparison of the ^1H NMR (400 MHz) spectra of the reaction mixtures obtained with (E,E)-4 (5.0 mg, 6.5 micromol) in DMSO- d_6 (0.75 mL) when irradiated with monochromatic light at 320 nm. The reaction mixtures contained into a 5 mm NMR tube were irradiated at room temperature and ^1H NMR spectra were recorded at regular time intervals. The marked peaks were considered to be diagnostic of either the major product (7-M) of the [2 + 2] photocycloaddition reaction or diastereomer (E,Z)-4.

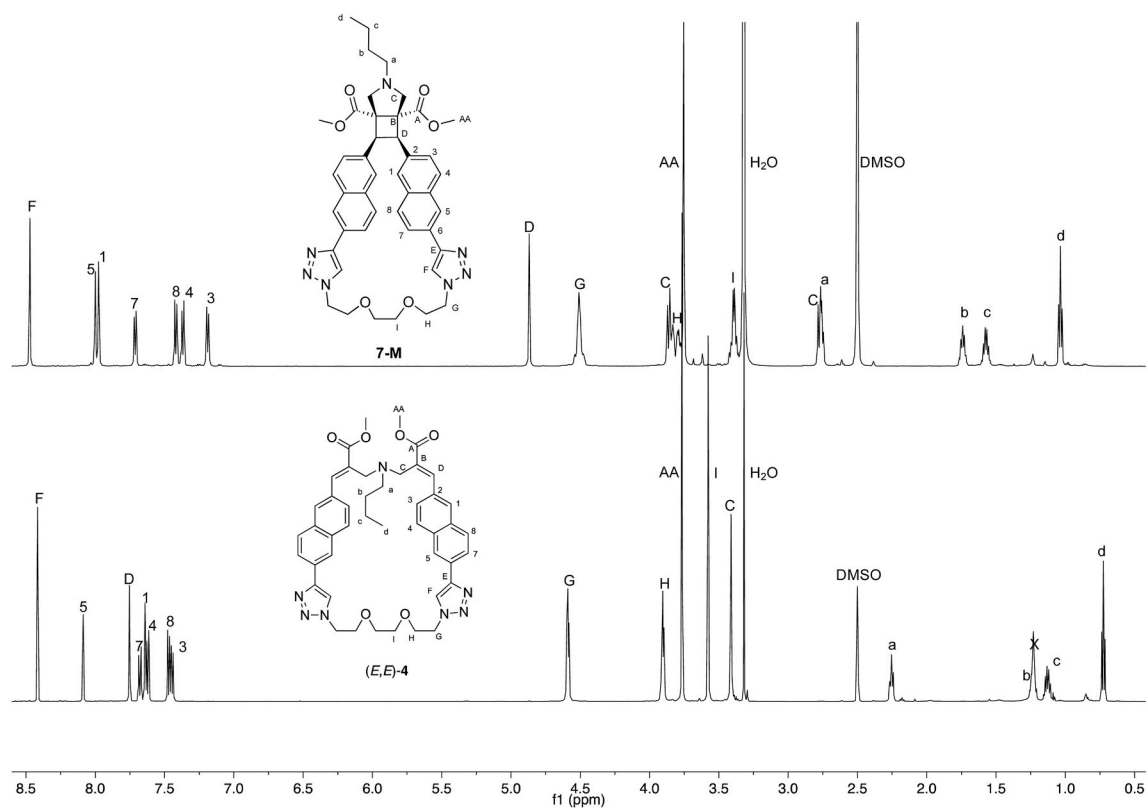


Fig. 8. Comparison of the ^1H NMR spectra recorded (600 MHz, DMSO- d_6) with crystalline samples of (E,E)-4 and 7-M.

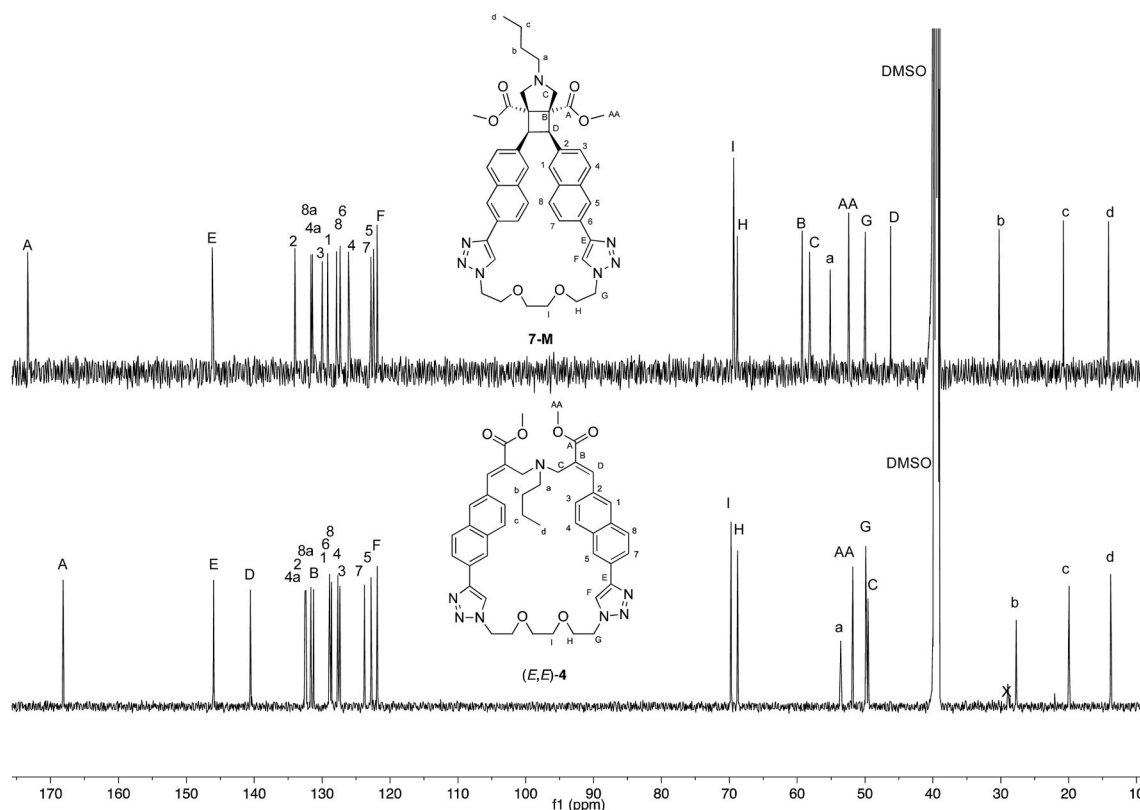
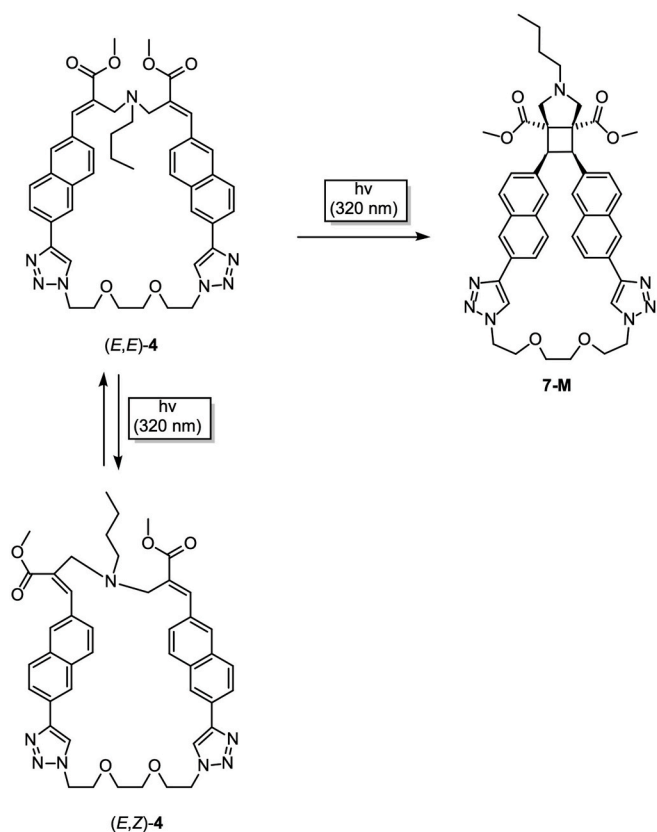


Fig. 9. Comparison of the ^{13}C NMR spectra recorded (150 MHz, DMSO-d_6) with crystalline samples of (E,E) -4 and 7-M.



Scheme 2. Possible reaction pathways produced by the irradiation of a solution of (E,E) -4 in DMSO with monochromatic light at 320 nm.

concentration-dependent decrease in cell viability in both cancer cell lines.

At lower concentrations, only modest reductions in cell viability were observed, whereas increasing concentrations resulted in a marked and progressive decline in the percentage of viable cells, ultimately leading to pronounced cytotoxic effects at the highest concentrations tested. In MDA-MB-231 cells, compounds (E,E) -4, (E,Z) -4, and 7-M exhibited the highest cytotoxic potency, with a mean IC_{50} value of $1.00\ \mu\text{M}$ (Table 1).

Interestingly, all three macrocyclic derivatives were slightly more potent than the reference drug doxorubicin, which displayed a mean IC_{50} value of $1.50\ \mu\text{M}$ in this aggressive breast cancer model. This finding is particularly noteworthy because triple-negative breast cancer is characterized by high aggressiveness, poor prognosis, and limited therapeutic options owing to the lack of well-defined molecular targets. Similarly, all three macrocyclic derivatives exhibited marked cytotoxic activity against A375 melanoma cells, with mean IC_{50} values of approximately $0.80\ \mu\text{M}$ (Table 1). Although doxorubicin remained the most potent compound in this model (mean $\text{IC}_{50} = 0.50\ \mu\text{M}$), the synthesized derivatives nevertheless showed strong and reproducible cytotoxic activity.

As already observed in the case of phenyl derivatives 3a-d [21], very similar IC_{50} values were obtained for compounds (E,E) -4, (E,Z) -4, and 7-M in both cell lines using the Neutral Red Uptake assay. These similarities appeared to suggest that the observed cytotoxic activity could be an intrinsic feature of this class of macrocyclic derivatives, which could exert their cytotoxic activity through a non-specific interaction with some key cellular components such as membrane [21]. Furthermore, the comparable activity observed in both breast cancer and melanoma cells indicates that these compounds may possess broad-spectrum antitumor potential. Overall, these findings highlight the potential of this class of macrocyclic derivatives as promising scaffolds for the development of novel anticancer agents and warrant further investigations to elucidate their molecular mechanisms of action.

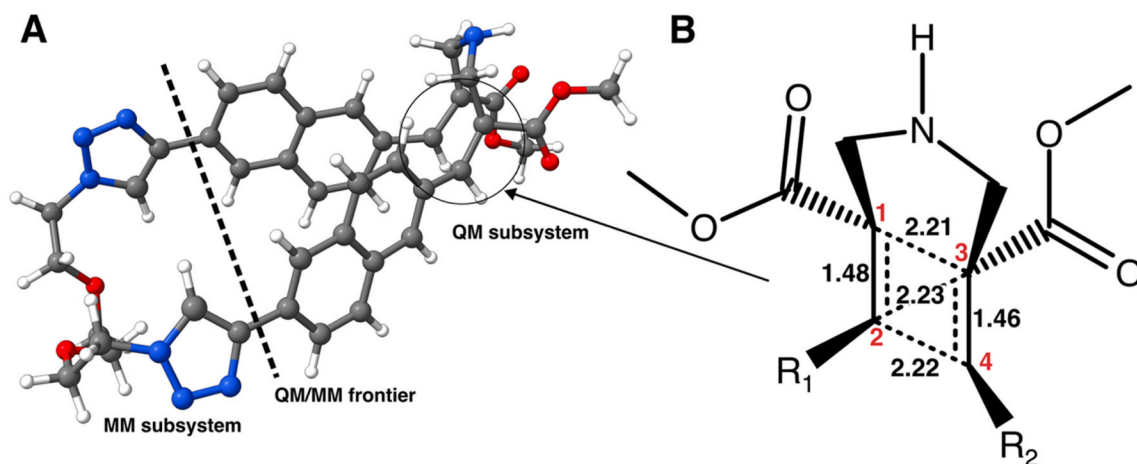


Fig. 10. (A) QM/MM model of the minimum energy S_1/S_0 CoIn optimized at the SA2-CASSCF(4,4)/6-31G*/OPLS-AA level of theory. Notice that the butyl group on the nitrogen has been replaced by a hydrogen atom. The QM subsystem encompasses the reactive moiety of the molecule, while the remaining fragment is treated at the cheaper MM level. (B) Geometrical features of the rhomboidal minimum energy S_1/S_0 CoIn leading toward the 7-M photoproduct.

2.6. Antiproliferative effects and mechanistic studies

To provide a more comprehensive biochemical characterization, the anti-proliferative results were corroborated by MTT assay as a complementary metabolic approach. The assay in fact is based on the mitochondrial reduction of MTT by metabolically active cells into insoluble formazan crystals. The amount of formazan produced is proportional to the number of viable cells. This second independent test was performed on the MDA-MB-231 cell line to confirm that the macrocyclic compounds impaired cellular metabolism further supporting the results of the initial Neutral Red screening.

Cells were treated for 24, 48, and 72 h with logarithmically increasing concentrations (0.1–300 μM) of (*E,E*)-4, and (*E,Z*)-4. The concentration range was selected to cover a broad interval for evaluating concentration-dependent biological activity.

Under growth-promoting conditions (medium supplemented with 10% FBS) (*E,E*)-4 exhibited the greatest potency after 24 h, with an IC_{50} of $2.73 \pm 0.31 \mu\text{M}$, (Fig. 12A and Table 2).

Diastereomer (*E,Z*)-4 was less effective in inhibiting the viability of MDA-MB-231 cells at 24 h, with an IC_{50} of $14.8 \pm 0.76 \mu\text{M}$ (Table 2). However, concentrations above 30 μM were found to be toxic for both tested compounds (Fig. 12A).

After 48 h of treatment, (*E,E*)-4 displayed an IC_{50} of $1.43 \pm 0.08 \mu\text{M}$ whereas the IC_{50} for (*E,Z*)-4 remained above 10 μM (Fig. 12B).

Finally, after 72 h of exposure, the cytotoxic effect remained comparable to that observed at earlier time points, indicating that the maximal cytotoxic response was already achieved within the first 24 h of treatment, with no further time-dependent increase in toxicity (Fig. 12C and Table 2). The parallel employment of the Neutral Red uptake and MTT assays provides an insightful screening of organelle-specific vulnerability. The pronounced difference in potency observed for the (*E,Z*)-isomer between the two assays ($\text{IC}_{50} = 1 \mu\text{M}$ in Neutral Red vs 14.8 μM in MTT at 24 h) strongly suggests a compartment-specific sensitivity. This indicates that while the (*E,Z*)-derivative efficiently triggers lysosomal membrane permeabilization or dysfunction at low micromolar concentrations, it preserves mitochondrial metabolic pathways up to significantly higher doses. Conversely, the (*E,E*)-isomer promotes a more uniform organelle collapse.

Based on these findings, three representative concentrations were selected for subsequent experiments: 1 μM , 10 μM , and the compound-specific IC_{50} value, allowing the assessment of cellular responses at sub-cytotoxic, effective, and high-dose conditions.

To investigate the mechanism of growth inhibition induced by the tested compounds, cell cycle distribution analysis was performed on

MDA-MB-231 breast cancer cells using flow cytometry. As shown in the figure, treatment with the compounds led to an increase in the Sub G0/G1 apoptotic fraction compared to the untreated cells (Ctr). Notably, compound (*E,E*)-4 induced a significant accumulation in this apoptotic population even at the lowest tested concentration (1 μM), suggesting that this compound triggers programmed cell death at early stages (Fig. 12D). In addition to the apoptotic induction, (*E,E*)-4 triggered a significant S-phase arrest at 10 μM , suggesting a direct interference with DNA synthesis or an early activation of DNA damage checkpoints. Conversely, compound (*E,Z*)-4 demonstrated a different redistribution of the cell cycle stages. At higher concentrations of 10 μM and 15 μM , (*E,Z*)-4 induced a substantial arrest in the G2/M phase, together with an increased fraction of cells in the sub-G0/G1 population, a behavior typically associated with the disruption of mitotic spindle dynamics or microtubule polymerization. Indeed, this shift was accompanied by a sharp reduction in the percentage of cells in the G0/G1 and S phases, indicating that (*E,Z*)-4 prevents cells from progressing through mitosis (Fig. 12D).

Taken together, these data show that while (*E,E*)-4 primarily modulates the S phase, (*E,Z*)-4 acts by blocking the cell cycle at the G2/M transition.

To further validate the induction of apoptosis, caspase-3 activation was evaluated by Western blot analysis after 14 h of treatment. Given that the analysis was performed at an early time point, prior to the onset of the cytotoxic effects observed at 24 h, representative sub-effective and biologically active concentrations were selected (1 and 3 μM for (*E,E*)-4; 1 and 10 μM for (*E,Z*)-4 to monitor the initiation of apoptotic signaling while avoiding extensive secondary cell damage.

As shown in Fig. 12E, treatment with (*E,E*)-4 resulted in a robust, concentration-dependent activation of caspase-3. Cleavage was already evident at 1 μM and became more pronounced at 3 μM . (*E,Z*)-4 induced a slight activation of caspase-3 at 1 μM , whereas clear cleavage was observed only at 10 μM . These findings corroborate the flow cytometric evidence of apoptosis and further demonstrate the higher pro-apoptotic potency of (*E,E*)-4 compared with its diastereomer (*E,Z*)-4, consistent with its lower IC_{50} values and earlier induction of cell death.

To complement the acute cytotoxicity data obtained from the MTT and Neutral Red assays, a clonogenic (colony formation) assay was performed in MDA-MB-231 cells. While short-term metabolic and lysosomal assays reflect immediate effects on cell viability, the clonogenic assay assesses long-term proliferative capacity and the ability of single cells to undergo sustained division, thereby providing a measure of reproductive cell death over an extended period (7 days). This approach allows discrimination between transient cytostatic effects and

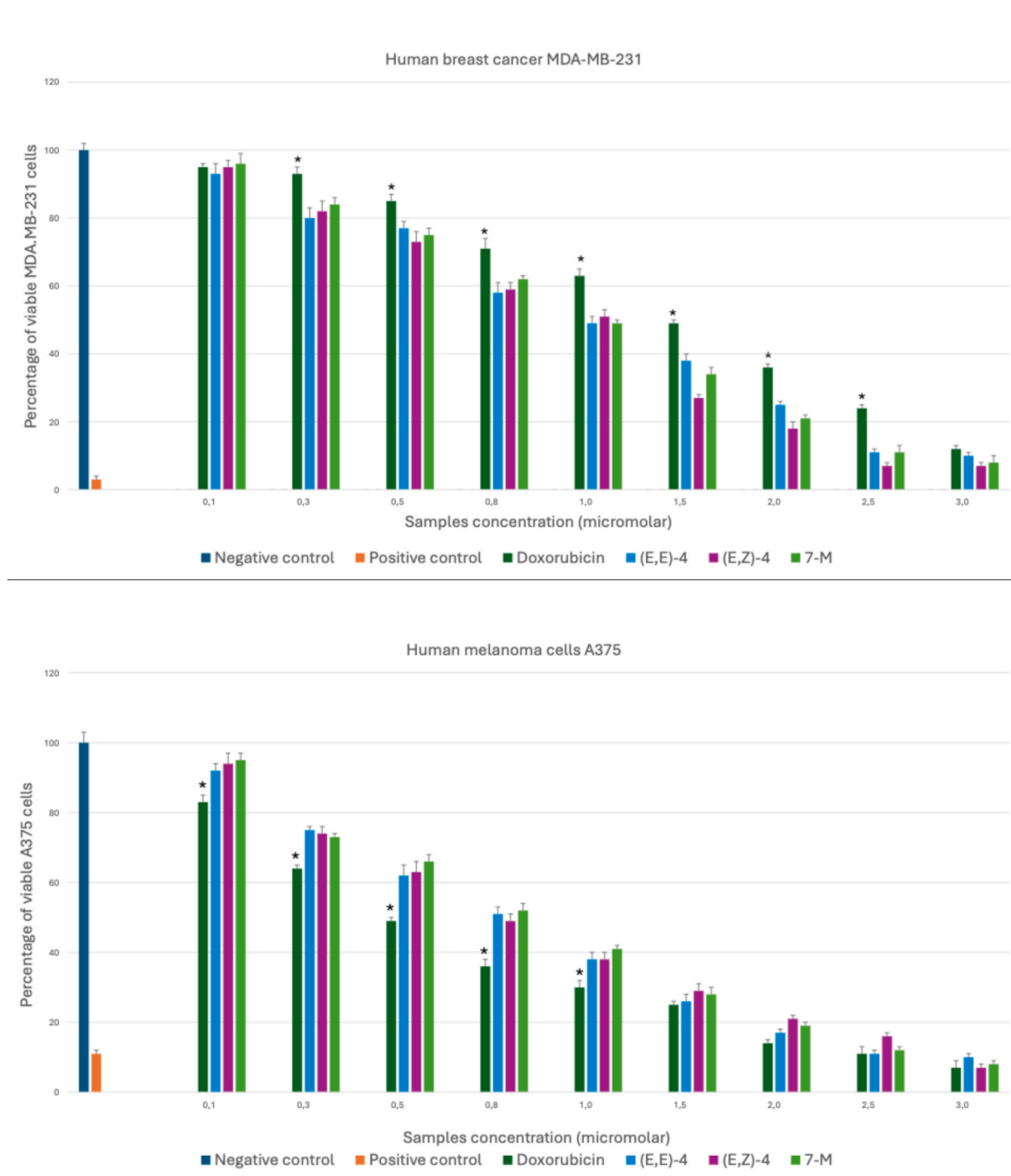


Fig. 11. Percentage of viable human triple-negative breast cancer MDA-MB-231 cells (top panel) and human melanoma A375 cells (bottom panel) after 24 h of exposure to increasing concentrations of the tested compounds, as determined by the Neutral Red Uptake assay. Untreated cells cultured in complete medium served as the negative control, whereas the positive control consisted of cells treated with 4 μ L of lysis solution (1:250 dilution of 9% w/v Triton X-100) per 100 μ L of culture medium. Data are expressed as the mean $\pm$ SD of three independent experiments, each performed in six technical replicates.

(Top panel) $*p < 0.05$ versus all tested compounds at concentrations ranging from 0.3 to 2.5 μ M (Bottom panel) $*p < 0.05$ versus compounds (E,E)-4, (E,Z)-4, and 7-M. Statistical analysis was performed as described in the Experimental section.

irreversible loss of clonogenic potential induced by the macrocyclic derivatives.

As illustrated in Fig. 12F and G, both macrocyclic derivatives impaired the clonogenic capacity of MDA-MB-231 cells following repeated treatment over seven days. Based on their distinct cytotoxicity profiles, concentrations of 1 and 3 μ M were selected for (E,E)-4, whereas 1 and 10 μ M were employed for (E,Z)-4, allowing the evaluation of long-term proliferative capacity under biologically relevant exposure

conditions.

Consistent with its higher antiproliferative activity, (E,E)-4 markedly reduced colony formation already at 1 μ M, with only minimal residual clonogenic growth observed at 3 μ M. In contrast, (E,Z)-4 produced a concentration-dependent inhibition of colony formation, with 1 μ M exerting only a modest effect and 10 μ M causing a pronounced reduction in the number and size of colonies. These findings indicate that sustained exposure to both compounds compromises the reproductive survival of

Table 1

Half-maximal inhibitory concentration (IC₅₀) values of doxorubicin and the synthesized macrocyclic derivatives against human triple-negative breast cancer MDA-MB-231 and human melanoma A375 cells after 24 h of exposure. Data are expressed as mean ± SD of three independent experiments.

Compound	MDA-MB-231 IC ₅₀ (μM) ± SD	A375 IC ₅₀ (μM) ± SD
Doxorubicin	1.50 ± 0.08	0.50 ± 0.03
(<i>E,E</i>)- 4	1.00 ± 0.06*	0.80 ± 0.02*
(<i>E,Z</i>)- 4	1.00 ± 0.05*	0.80 ± 0.03*
7-M	1.00 ± 0.05*	0.80 ± 0.03*

*Values are statistically different from doxorubicin ($p < 0.05$). Statistical analysis was performed as described in the Experimental section.

MDA-MB-231 cells, although substantially lower concentrations of (*E,E*)-**4** are sufficient to achieve this effect.

Overall, the clonogenic assay corroborates the results obtained from the short-term viability assays and demonstrates that the antiproliferative activity of the macrocyclic derivatives translates into a durable impairment of long-term proliferative capacity. Among the two diastereoisomers, (*E,E*)-**4** consistently exhibited greater potency across all biological endpoints evaluated, including metabolic activity, cell cycle perturbation, apoptosis induction, and clonogenic survival, identifying this compound as the lead candidate for further investigation in triple-negative breast cancer models.

In conclusion, the macrocyclic compounds exhibit antiproliferative effects in MDA-MB-231 cells, with (*E,E*)-**4** showing the lowest IC₅₀ values and retaining efficacy at low concentrations. Notably, (*E,E*)-**4** also significantly impairs clonogenic capacity highlighting a sustained effect on both short- and long-term cell survival. In contrast, (*E,Z*)-**4** is less potent and primarily induces G2/M arrest at higher doses. These findings identify (*E,E*)-**4** as the most promising candidate for further investigation in triple-negative breast cancer.

3. Conclusion

Based on the previously reported cytotoxic activity of some promising crown ether-paracyclophane hybrid macrocyclic derivatives, their structures were modified by replacing the phenyl rings with the two naphthalene units. The newly designed macrocyclic derivatives were synthesized in a straightforward manner by applying the previously published CuAAC coupling-dimerization strategy to naphthalene MBHA derivative **5**. The obtained tri(ethylene glycol)-tethered MBHA dimer **6** was made to react with *n*-butylamine to provide the mixture of macrocyclic diastereomers **4**, which were separated by flash chromatography and thoroughly characterized from the point of view of their structural, photophysical, photochemical, and biological features.

The potential cytotoxicity was preliminarily evaluated in human breast cancer MDA-MB-231 and human melanoma A375 cell lines. Very interestingly, all the newly synthesized macrocyclic compounds showed strong cytotoxic activity against both triple-negative breast cancer and melanoma models. Their slightly greater potency compared to doxorubicin in MDA-MB-231 cells highlights their potential therapeutic value against aggressive and difficult-to-treat breast cancers. Although slightly less potent than doxorubicin in A375 melanoma cells, the compounds still exhibited substantial cytotoxic activity, confirming their effectiveness in multiple tumor models. Both (*E,E*)-**4** and (*E,Z*)-**4** effectively impaired key in vitro hallmarks of cancer progression, including cell metabolism, cell cycle, apoptosis, and clonogenic capacity [28]. In MDA-MB-231 cells, the MTT assay revealed that (*E,E*)-**4** exhibited a slightly higher potency than (*E,Z*)-**4** after 24 h. This trend was mirrored by a marked impairment in clonogenic survival, which was substantially reduced at a concentration of 1 μM. Although a specific molecular target has not yet been identified, the rapid and non-specific effect observed within 24 h of exposure suggests potential damage to the cell membrane. Nevertheless, further studies are warranted to fully elucidate the exact

molecular mechanism(s) underlying this promising antitumor activity.

4. Experimental section

Synthesis. Melting points were determined in open capillaries in a Cole-Parmer Stuart apparatus and are uncorrected. Merck silica gel 60 (230-400 mesh) was used for column chromatography. Merck TLC plates, silica gel 60 F₂₅₄ were used for TLC. NMR spectra were recorded with a Bruker DRX-400 AVANCE III spectrometer or with a Bruker DRX-600 AVANCE spectrometer in the indicated solvents (TMS as internal standard): the values of the chemical shifts are expressed in ppm and the coupling constants (*J*) in Hz. An Agilent 1100 LC/MSD operating with an electrospray source was used in mass spectrometry experiments. A tunable light source Zolix (TLS2-X300PU-G, 300W UV Xenon Light Source with monochromator Omni-λ2047i) was used for the irradiation experiments.

4.1. Dimethyl 2,2'-(((1,1'-((ethane-1,2-diylbis(oxy))bis(ethane-2,1-diyl))bis(1*H*-1,2,3-triazole-4,1-diyl))bis(naphthalene-6,2-diyl))bis(acetoxymethylene))diacrylate (**6**)

In a two-neck round-bottom flask, compound **5** (271 mg, 0.88 mmol) and tri(ethylene glycol) diazide [17] (88 mg, 0.44 mmol) were solubilized in anhydrous CH₃CN (10 mL), and then DIPEA (60 μL, 0.35 mmol) and CuBr(I) (50 mg, 0.35 mmol) were added in sequence. The mixture was stirred at room temperature under a nitrogen atmosphere for 3 h, after which it was concentrated under reduced pressure. The resulting residue was partitioned between ethyl acetate and H₂O/NH₄OH (7:3). The organic layer was extracted, dried over MgSO₄, and concentrated under vacuum. The crude product was purified by flash chromatography with ethyl acetate and methanol (95:5) as the eluent to give homodimer **6** (181 mg, yield 50%) as an off-white glassy solid. ¹H NMR (400 MHz, CDCl₃): 2.12 (s, 6H), 3.59 (s, 4H), 3.69 (s, 6H), 3.87 (t, *J* = 5.0, 4H), 4.53 (t, *J* = 5.0, 4H), 5.93 (s, 2H), 6.44 (s, 2H), 6.83 (s, 2H), 7.47 (dd, *J* = 8.6, 1.5, 2H), 7.82 – 7.90 (m, 8H), 7.96 (s, 2H), 8.30 (s, 2H). MS (ESI): *m/z* 839.2 [*M* + Na⁺].

4.2. Reaction of homodimer **6** with *n*-butylamine

Compound **6** (62 mg, 0.076 mmol) was solubilized in CHCl₃ (30 mL), and subsequently *n*-butylamine (15 μL, 0.15 mmol) was added. The resulting mixture was refluxed for 24 h and then concentrated under vacuum. The organic residue was purified by flash chromatography with dichloromethane-methanol (97:3) as the eluent.

4.3. Dimethyl (1⁴*Z*,11⁴*Z*,3*E*,8*E*)-6-butyl-1¹*H*,11¹*H*-14,17-dioxo-6-aza-1,11(4,1)-ditriazole-2,10(2,6)-dinaphthalenacyclononadecaphane-3,8-diene-4,8-dicarboxylate ((*E,E*)-**4**)

Compound (*E,E*)-**4** was obtained as an off-white solid (34 mg, yield 58%, mp 165.1 – 166.3 °C) as the most polar fraction of the chromatographic purification. ¹H NMR (600 MHz, DMSO-*d*₆): 0.73 (t, *J* = 7.3, 3H), 1.08 – 1.17 (m, 2H), 1.19 – 1.28 (m, 2H), 2.25 (t, *J* = 7.1, 2H), 3.41 (s, 4H), 3.58 (s, 4H), 3.77 (s, 6H), 3.90 (t, *J* = 5.0, 4H), 4.59 (t, *J* = 5.0, 4H), 7.44 (dd, *J* = 8.5, 1.7, 2H), 7.47 (d, *J* = 8.5, 2H), 7.62 (d, *J* = 8.5, 2H), 7.64 (d, *J* = 1.6, 2H), 7.68 (dd, *J* = 8.5, 1.7, 2H), 7.75 (s, 2H), 8.09 (s, 2H), 8.42 (s, 2H); Fig. 3. ¹³C NMR (151 MHz, DMSO-*d*₆): 13.8, 19.9, 27.7, 49.6, 49.9, 51.8, 53.6, 68.8, 69.8, 121.9, 122.8, 123.7, 127.4, 127.7, 128.6, 128.8, 128.9, 131.3, 131.6, 132.4, 132.5, 140.6, 146.0, 168.2; Fig. 4. MS (ESI): *m/z* 770.3 [*M* + H⁺]

4.4. Dimethyl (1⁴*Z*,11⁴*Z*,3*Z*,8*E*)-6-butyl-1¹*H*,11¹*H*-14,17-dioxo-6-aza-1,11(4,1)-ditriazole-2,10(2,6)-dinaphthalenacyclononadecaphane-3,8-diene-4,8-dicarboxylate ((*E,Z*)-**4**)

Compound (*E,Z*)-**4** was obtained as a white solid (11 mg, yield 19%,

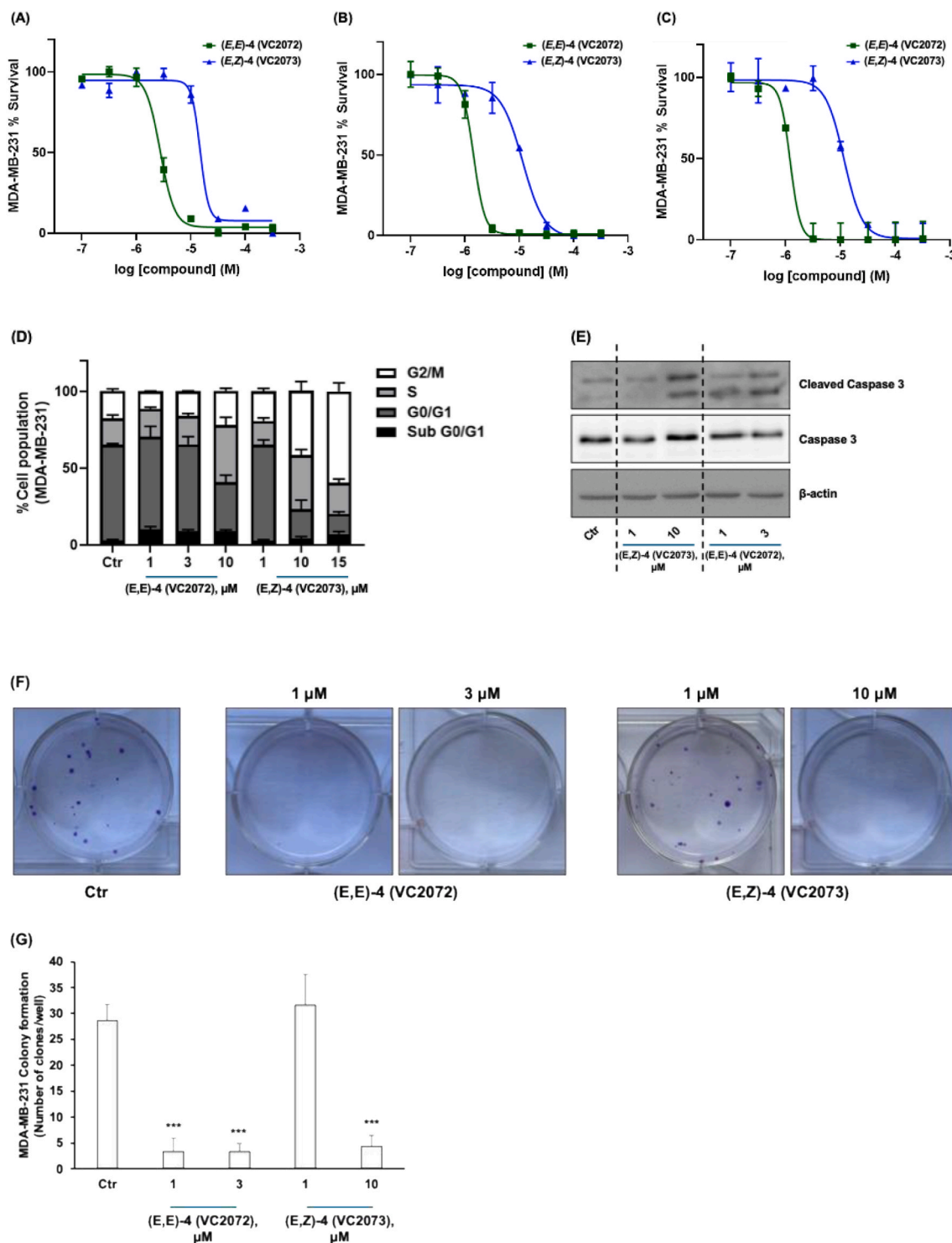


Fig. 12. Macrocyclic compounds inhibit cell viability, clonogenicity, and induce apoptosis in TNBC cells. (A-C). MDA-MB-231 viability evaluated by MTT assay. Cells were treated with increasing concentrations of macrocyclic compounds (0.1–300 μM) in medium with 10% FBS for 24 h (A), 48 h (B) and 72 h (C). Dose–response curves were generated using GraphPad Prism 8 software, fitting the data with a four-parameter logistic (4 PL) model. Data are expressed as percentage of cell viability relative to untreated controls, which were set at 100%, and are presented as mean ± SD (n = 3). (D). Percentage of cells treated with macrocyclic compounds at each stage of the cell cycle was analyzed by flow cytometry after DNA staining with propidium iodide. (E). Caspase 3 cleavage in A375 cells treated with (E,E)-4 (1 and 3 μM) and (E,Z)-4 (1 and 10 μM) for 14 h. Gel shown is representative of three experiments with similar results. (F). Representative images of the clonogenic assay in MDA-MB-231 cells treated with macrocyclic compounds every 48 h. After 7 days, cells were fixed, stained, and photographed. (G). Quantification of colony number of MDA-MB-231 treated with (E,E)-4 (1 and 3 μM) and (E,Z)-4 (1 and 10 μM) for 7 days. ***p < 0.001 vs untreated cells (Ctr). The graph represents the average of three independent experiments.

Table 2IC₅₀ values in MDA-MB-231 cells.

time	compound	IC ₅₀ ± SD (μM)
24 h	(<i>E,E</i>)-4	2.73 ± 0.31
	(<i>E,Z</i>)-4	14.8 ± 0.76
48 h	(<i>E,E</i>)-4	1.43 ± 0.08
	(<i>E,Z</i>)-4	11.44 ± 0.97
72 h	(<i>E,E</i>)-4	1.21 ± 0.06
	(<i>E,Z</i>)-4	11.58 ± 0.69

mp 177.7 – 179.2 °C) as the least polar fraction of the chromatographic purification. ¹H NMR (600 MHz, DMSO-*d*₆): 0.88 (t, *J* = 7.4, 3H), 1.20 – 1.32 (m, 2H), 1.41 – 1.51 (m, 2H), 2.50 (t, *J* = 7.0, 2H), 3.30 (s, 2H), 3.57 (s, 3H), 3.60 – 3.63 (m, 2H), 3.66 – 3.70 (m, 2H), 3.79 (s, 3H), 3.83 (s, 2H), 3.87 – 3.90 (m, 2H), 3.91 (t, *J* = 5.8, 2H), 4.54 (t, *J* = 5.8, 2H), 4.59 – 4.65 (m, 2H), 5.88 (s, 1H), 6.26 (s, 1H), 6.87 (dd, *J* = 8.6, 1.7, 1H), 7.15 (d, *J* = 8.5, 1H), 7.33 (dd, *J* = 8.5, 1.7, 1H), 7.62 (d, *J* = 8.6, 1H), 7.75 (dd, *J* = 8.5, 1.7, 1H), 7.83 (s, 1H), 7.84 (dd, *J* = 8.5, 1.7, 1H), 7.92 (d, *J* = 8.5, 1H), 7.95 (d, *J* = 8.6, 1H), 8.10 (s, 1H), 8.20 (s, 1H), 8.25 (s, 1H), 8.48 (s, 1H), 8.52 (s, 1H); Fig. 3. ¹³C NMR (151 MHz, DMSO): 13.8, 19.9, 29.1, 47.8, 49.1, 49.7, 51.5, 51.9, 53.6, 55.3, 69.0, 69.4, 69.5, 121.8, 122.0, 122.4, 123.2, 123.4, 124.0, 125.3, 127.3, 127.4, 128.0, 128.1, 128.3, 128.6, 129.1, 129.3, 129.5, 130.4, 131.1, 131.5, 132.1, 132.2, 132.5, 133.0, 134.6, 139.9, 145.9, 146.2, 169.0, 169.4; Fig. 4. MS (ESI): *m/z* 770.3 [*M* + *H*⁺].

4.5. Photochemical features of (*E,E*)-4

4.5.1. Dimethyl (14*Z*,36*R*,37*S*,54*Z*)-33-butyl-11*H*,51*H*-8,11-dioxo-33-aza-1,5(4,1)-ditriazole-2,4(2,6)-dinaphthalena-3(6,7)-bicyclo[3.2.0]heptanacyclotridecaphane-31,35-dicarboxylate (7-*M*)

The solution of compound (*E,E*)-4 (5.0 mg, 6.5 μmol) in DMSO-*d*₆ (0.75 mL) contained into 5 mm NMR Pyrex tubes was irradiated with a monochromatic light centered at 320 nm through a tunable light source Zolix (TLS2-X300PU-G, 300W UV Xenon Light Source with monochromator Omni-λ2047i) at room temperature for 35 h ¹H NMR spectra were recorded at regular time intervals to follow the reaction. The reaction mixture was then concentrated under a N₂ stream, and the resulting crude product was purified by flash chromatography with dichloromethane–methanol (95:5) as eluent to obtain compound 7-*M* (2.5 mg, yield 50%) as an off-white solid. ¹H NMR (600 MHz, DMSO-*d*₆): 1.04 (t, *J* = 7.4, 3H), 1.54 – 1.60 (m, 2H), 1.72 – 1.76 (m, 2H), 2.75 – 2.78 (m, 4H), 3.37 – 3.41 (m, 4H), 3.75 (s, 6H), 3.76 – 3.87 (m, 6H), 4.47 – 4.55 (m, 4H), 4.87 (s, 2H), 7.19 (d, *J* = 8.5, 2H), 7.37 (d, *J* = 8.5, 2H), 7.42 (d, *J* = 8.5, 2H), 7.71 (dd, *J* = 8.4, 1.8, 2H), 7.98 (s, 2H), 8.00 (s, 2H), 8.47 (s, 2H); Fig. 8. ¹³C NMR (151 MHz, DMSO-*d*₆): 14.1, 20.8, 30.2, 46.2, 50.0, 52.4, 55.1, 58.2, 59.3, 68.8, 69.4, 121.9, 122.4, 122.8, 126.1, 127.4, 127.9, 129.2, 130.0, 131.4, 131.7, 134.0, 146.2, 173.4; Fig. 9. MS (ESI): *m/z* 770.4 (*M* + *H*⁺).

Photophysical properties. UV-vis absorption spectra were obtained with a PerkinElmer Lambda 900 spectrometer. Photoluminescence (PL) spectra were obtained with a NanoLog composed by a iH320 spectrograph equipped with a Synapse QExtra charge-coupled device by exciting with a monochromated 450W Xe lamp. The spectra were corrected for the instrument response. PL QY values of solutions were obtained by using quinine sulfate as the reference.

4.6. Biological evaluation of the synthesized macrocyclic derivatives (*E,E*)-4, (*E,Z*)-4, and 7-*M*

Materials. Dulbecco's Modified Eagle's Medium (DMEM), trypsin-EDTA solution, phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), Triton X-100, and all other reagents used for cell culture were purchased from Merck (Darmstadt, Germany).

Cell culture. Human triple-negative breast cancer MDA-MB-

231 cells and human melanoma A375 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured according to the supplier's recommended protocols. Cultures were maintained at a 1:4 split ratio twice weekly and used until passage 20. Mycoplasma screening was conducted on all cryopreserved stocks prior to use.

Neutral Red Uptake assay. Cells (1.5 × 10⁴ cells per well) suspended in 1 mL of complete culture medium were seeded into 24-well tissue culture plates and incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO₂ to allow cell attachment, reaching approximately 50% confluence. The culture medium was then removed and replaced with fresh complete medium containing the appropriate concentrations of the test compounds. Stock solutions of compounds (*E,E*)-4, (*E,Z*)-4, 7-*M*, and doxorubicin were prepared in DMSO and diluted in complete culture medium immediately before use. Cells were treated with final concentrations of 0.1, 0.3, 0.5, 0.8, 1.0, 1.5, 2.0, 2.5, and 3.0 μM. Complete culture medium served as the negative control, whereas cells treated with 4 μL of lysis solution (1:250 dilution of 9% w/v Triton X-100) per 100 μL of culture medium served as the positive control [29]. After 24 h of treatment, cell viability was determined using the Neutral Red Uptake assay, as previously described [30]. Each experiment was performed independently three times, with six technical replicates for each experimental condition.

MTT assay. Cell survival was assessed via the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Merck KGaA, Germany). Briefly, 3 × 10³ cells per well were plated in 96-well formats in 10% FBS-supplemented medium. Following an overnight attachment period, cells were exposed to a logarithmic range of (*E,E*)-4 or (*E,Z*)-4 (0–300 μM) for 24, 48, or 72 h. After each incubation period, the supernatant was substituted with 0.5 mg/mL MTT solution for 4 h at 37 °C. The resulting formazan precipitates were solubilized in DMSO (Merck KGaA), and optical density was recorded at 540 nm using a Tecan F200 Pro microplate reader. Viability was calculated as a percentage relative to the untreated control (fixed at 100%). Half-maximal inhibitory concentrations (IC₅₀) were determined by non-linear regression using a four-parameter variable-slope model (log[inhibitor] vs. response) in GraphPad Prism 8 (San Diego, CA, USA), as previously described [31].

Cell cycle assessment. Cell cycle analysis was performed by flow cytometry following propidium iodide staining. MDA-MB-231 cells (7 × 10⁵) were plated in six-well plates in complete growth medium containing 10% FBS and allowed to adhere for 24 h. Cells were subsequently serum-starved in medium supplemented with 0.1% FBS for an additional 24 h, then exposed to (*E,E*)-4 or (*E,Z*)-4 at three concentrations (1 μM, 10 μM, and the compound-specific IC₅₀) for 24 h. After treatment, cells were rinsed three times with PBS, detached using trypsin, and harvested by centrifugation at 0.3 × *g* for 5 min. The cell pellets were fixed in 80% ethanol at –20 °C overnight. Fixed cells were washed twice with PBS and incubated for 30 min at 37 °C in 0.5 mL PBS containing RNase (100 μg/mL; Thermo Fisher Scientific, Waltham, MA, USA) and propidium iodide (50 μg/mL; Merck KGaA, Darmstadt, Germany).

DNA content was quantified using a BD Biosciences flow cytometer (Milan, Italy), and cell cycle profiles were evaluated using CellQuest Pro software [31]. All experiments were conducted in triplicate.

Clonogenic assay. For the clonogenic assay, MDA-MB-231 cells were plated in six-well plates at a density of 100 cells per well in growth medium supplemented with 10% FBS and allowed to adhere for 24 h at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were then treated with (*E,E*)-4 or (*E,Z*)-4 at three concentrations (1 μM, 10 μM, and the compound-specific IC₅₀) with treatments renewed every 48 h and maintained in culture for a total of seven days. At the end of the incubation period, colonies were fixed with a rapid-staining fixative (Panoptic No.1) for 15 min at room temperature, followed by staining with eosin (Panoptic No.2) for 15 min and a final staining step using a blue dye (Panoptic No.3, Azur B-based; PanReac AppliChem ITW Reagents, Darmstadt, Germany) for 15 min at room temperature [32].

Colony images were acquired using a phase-contrast microscope (Nikon Eclipse TE 300, Nikon, Tokyo, Japan).

Western blot. Western blot analysis was performed on total cell lysates. MDA-MB-231 cells (3×10^5 cells per dish) were seeded in 60-mm culture dishes and allowed to adhere, then serum-starved for 18 h before treatment with (*E,E*)-4 or (*E,Z*)-4 at different concentrations for 24 h in medium containing 10% FBS. At the end of treatment, cells were washed twice with ice-cold Dulbecco's phosphate-buffered saline (PBS; Merck KGaA, Darmstadt, Germany) and lysed on ice using RIPA lysis buffer supplemented with 2 mM Na_3VO_4 and $1\times$ protease inhibitor cocktail for mammalian cells (Merck KGaA), as previously described [33]. Lysates were clarified by centrifugation at maximum speed for 20 min at 4 °C, and the supernatants were collected. Protein concentrations were determined using the Bradford assay. Equal amounts of protein (50 µg per sample) were resolved by SDS-PAGE using 4–15% Mini-PROTEAN® TGX™ Precast Protein Gels (Biorad, Hercules, CA, USA) and subsequently transferred onto nitrocellulose membranes (Biorad, Hercules, CA, USA). Membranes were blocked with 5% non-fat dry milk for 1 h at room temperature and incubated overnight at 4 °C with primary antibodies against Cleaved Caspase-3 (Asp175) (5A1E) Rabbit Monoclonal Antibody #9664 (Cell Signaling Technology, Danvers, MA, USA) (rabbit, 1:1000). Caspase-3 Antibody #9662 (Cell Signaling Technology, Danvers, MA, USA) (rabbit, 1:1000). β -Actin (mouse, 1:10,000; Merck KGaA, Darmstadt, Germany) was used as a loading control. After three washes with PBS containing 0.5% Tween-20, membranes were incubated for 1 h at room temperature with HRP-conjugated secondary antibodies (anti-rabbit, 1:2500, cat. no. W401B; anti-mouse, 1:2500, cat. no. W402B; Promega Corporation, Madison, WI, USA). Membranes were washed three additional times, and immunoreactive bands were visualized using an enhanced chemiluminescence substrate and detected with a ChemiDoc XRS imaging system (Bio-Rad Laboratories, Milan, Italy).

Statistical analysis. Multiple comparison was performed by one-way ANOVA. Differences with $p < 0.05$ were considered significant.

CRediT authorship contribution statement

Marco Paolino: Investigation. **Jacopo Venditti:** Investigation. **Gabriele Manenti:** Investigation. **Mario Saletti:** Investigation. **Alessandro Donati:** Investigation. **Claudia Bonechi:** Investigation. **Germano Giuliani:** Investigation. **Chiara Botta:** Investigation. **Stefania Lamponi:** Investigation. **Valerio Ciccone:** Investigation. **Maria Frösini:** Investigation, Formal analysis. **Sandra Donnini:** Investigation, Formal analysis. **Lucia Morbidelli:** Investigation, Formal analysis. **Riccardo Carletti:** Investigation. **Filippo Sacchetta:** Investigation. **Andrea Cappelli:** Writing – original draft, Supervision, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejmc.2026.100374>.

Data availability

Data will be made available on request.

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