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Photochemistry of nitro-substituted (*E*)-2-azachalcones with theoretical calculations and biological activities

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ABSTRACT

Four new stereoselective dimerization products (**4a–c**, **5**) of *m*- and *p*-nitro-substituted (*E*)-2-azachalcones (**2** and **3**) were synthesized and tested for antimicrobial and antioxidant activities. Compounds **1–3** showed very good antimicrobial activities against all the tested microorganisms, *Escherichia coli*, *Yersinia pseudotuberculosis*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Candida albicans*. Five of the compounds tested were radical scavengers with 50% scavenging concentration (SC_{50}) values between 0.130 and 2.047 mg/mL. The monomeric *o*-nitro-substituted (*E*)-2-azachalcone (**1**) showed the highest 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity with the SC_{50} value of 0.130 mg/mL. Compound **3** was prooxidant and compound **4b** was inactive in DPPH test. The higher antimicrobial activity of compound **1** was paralleled with its higher antioxidant activity, which makes it potential agent for the cure of bacterial infections concurrent with oxidative stress.

The theoretical calculations based on transition state structures were made to obtain possible photochemical dimerization products of compounds **1–3**. The expected dimeric products from calculations coincided with those experimentally produced.

1. Introduction

Natural products and their homologues are the most challenging class of compounds for the total synthesis, due to their structural diversity and complexity as well as the interesting biological activity [1,2]. Azachalcones are unnatural analogues of naturally occurring chalcones with an annular nitrogen atom in the phenyl ring [3–7]. Azachalcones and their N-alkyl derivatives have been shown to posses a wide variety of biological activities, such as antituberculostatic, antimicrobial, anti-inflammatory, and antibacterial potentials [3,5–7].

In our previous work, (*E*)-3-, and 4-azachalcones, their N-alkyl derivatives, and photochemical dimerization products were synthesized and evaluated for their antimicrobial activities. They showed very good antibacterial activities especially against Gram (+) bacteria [5–7]. Thus, in the present work, we have focused on the synthesis of analogous nitro-substituted (*E*)-2-azachalcones, and their dimerization products. The attempts of N-alkylating the (*E*)-2-azachalcone were unsuccessful, probably due to the steric effects.

Several plant species including *Combretum albopunctatum* [8], *Goniothalamus thwaitesii* [9], *Agelas sceptrum* [10], and *Agelas conifera* [11] have been reported to contain cyclobutane derivatives of natural compounds. The most common type of reaction for azachalcones is dimerization to give cyclobutane containing unnatural compounds in organic photochemistry [6,7,12–23].

In the search for new bioactive compounds analogous to the natural ones, four new dimers of *m*- and *p*-nitro-substituted (*E*)-2-azachalcones (**4a–c**, **5**), were synthesized stereoselectively and investigated based on the photochemistry, spectral characterization, and antioxidant and antimicrobial activities (Scheme 1).

2. Experimental

2.1. General and instrumentation

NMR spectra were recorded on a Varian Mercury NMR at 200 MHz in $CDCl_3$. The mass spectral analyses were carried out on a Micromass Quattro LC–MS/MS spectrophotometer. The elemental analyses were performed on a Leco CHNS 932 instrument. Infrared spectra were obtained with a Perkin–Elmer 1600 FT-IR

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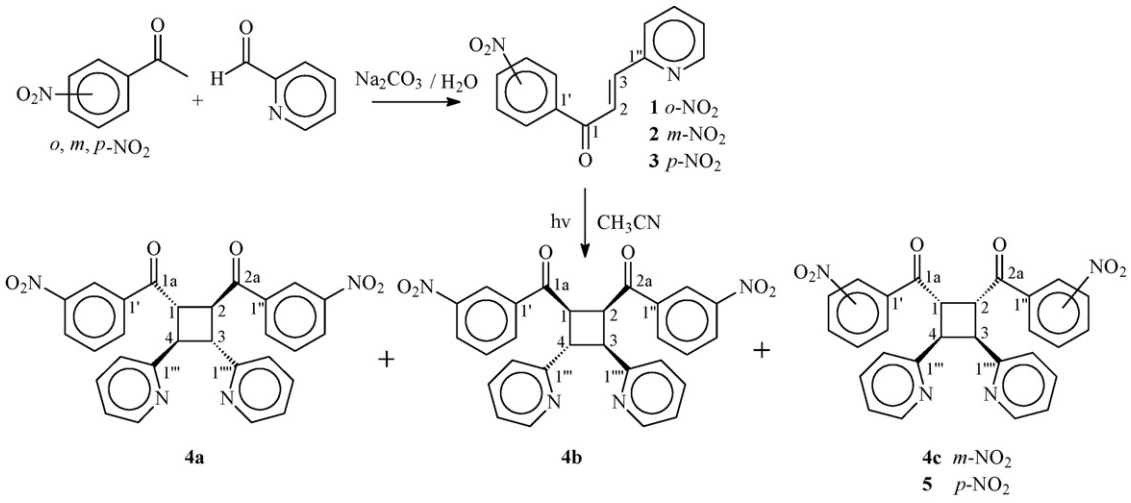


Scheme 1.

(4000–400 cm^{-1}) spectrometer. Melting points were determined by using a Thermo-var apparatus fitted with a microscope and are uncorrected. UV–vis spectral analyses were carried out on a Unicam UV2-100 spectrophotometer at 25 °C. Thin-layer chromatography (TLC) was carried out on Merck precoated 60 Kieselgel F_{254} analytical aluminum plates. Preparative TLC (PTLC) was carried out on Merck precoated 60 Kieselgel F_{254} (20 cm × 20 cm, 0.25 mm) silica gel plates.

3. Materials and methods

o-, *m*- and *p*-nitroacetophenone and 2-pyridinecarboxaldehyde were purchased from Aldrich/Merck and used without further purification. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical was obtained from Sigma. The solvents (chloroform, *n*-hexane, ethanol, methanol, acetonitrile, dimethyl sulfoxide, and diethyl ether) used were either of analytical grade or bulk solvents distilled before use. The compounds **1**–**3** were prepared according to the literature [24–26].

3.1. General procedure for the synthesis of compounds *4a–c*, *5*

3.1.1. Photodimerization of *2* and *3* in solution

Solutions of compounds **2** and **3** (0.150 g, each) in 40 mL of acetonitrile, kept in beakers, were exposed to UV light (400 W high-pressure Hg lamp). The progress of the reactions was followed by silica gel TLC (ethyl acetate–methanol, 10:1). The reactions were stopped after ~24 and 12 h, respectively. The solutions were evaporated, and the residues were purified by column chromatography (column length 30 cm, diameter 2 cm) on a silica gel (25 g, Merck, 230–400 mesh). The columns were eluted successively with the following solvent and solvent mixtures: hexane (20 mL), hexane–ethyl acetate (1:1, 30 mL, 1:2, 20 mL, 1:3, 20 mL, 1:4, 20 mL, 1:5, 20 mL); ethyl acetate–methanol (25:2, 30 mL; 20:7, 30 mL). Fractions (10–15 mL each) were collected and monitored by analytical TLC. The fractions 7–10 (87 mg) from the photochemical reaction of compound **2** were purified by PTLC (20 cm × 20 cm, 0.2 mm, two plates) to give compounds **4a** and **4c** (31.5 and 27.0 mg, 21% and 18% yield, R_f = 0.41 and 0.56, respectively, acetonitrile–dichloromethane, 1:7). The fraction 3 (40 mg) from the photochemical reaction of compound **2** was purified by PTLC (20 cm × 20 cm, 0.5 mm) to give compound **4b** (12.0 mg, 8% yield, R_f = 0.82, hexane–ethyl acetate, 1:1). The fractions 10–12 (45 mg) from the photochemical reaction of compound **3**, was purified by PTLC (20 cm × 20 cm, 0.5 mm) to give compound **5** (36.0 mg, 24% yield, R_f = 0.52, *n*-hexane–ethyl acetate, 1:1).

3.1.2. (1α,2β)-Di-(3-nitrobenzoyl)-(3α,4β)-di-(2-pyridyl)cyclobutane (*4a*)

Yellowish amorphous solid, mp 159–160 °C; UV λ_{max} ($CHCl_3$)/nm 243 (ε/dm^3 mol^{-1} cm^{-1} 77,411); ^{1}H NMR ($CDCl_3$, 200 MHz) and ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm) see Table 1; positive LC–MS/MS *m*/*z* (%); *m*/*z* = 509 (100) $[M+H]^+$, 493 (27), 284 (15), 270 (18), 192 (18), 164 (19), 148 (24). Calcd. for $C_{28}H_{20}N_4O_6$ (508.49): C 66.14, H 3.96, N 11.02; found (508.20): C 66.12, H 3.95, N 11.06; FT-IR (cm^{-1}): 3082, 3005, 2927, 2856, 1682, 1613, 1589, 1531, 1349, 1223, 1105, 813, 747, 714.

3.1.3. (1β,2β)-Di-(3-nitrobenzoyl)-(3β,4α)-di-(2-pyridyl)cyclobutane (*4b*)

Yellowish amorphous solid, mp 182-183 °C; UV λ_{max} ($CHCl_3$)/nm 244 (ε/dm^3 mol^{-1} cm^{-1} 33,102); ^{1}H NMR ($CDCl_3$, 200 MHz) and ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm) see Table 1; positive LC–MS/MS *m*/*z* (%); *m*/*z* = 509 (100) $[M+H]^+$, 493 (7), 284 (5), 270 (8), 192 (7), 164 (6), 148 (15). Calcd. for $C_{28}H_{20}N_4O_6$ (508.49): C 66.14, H 3.96, N 11.02; found: C 66.10, H 3.98, N 11.08; FT-IR (cm^{-1}): 3082, 3016, 2926, 2855, 1681, 1613, 1591, 1530, 1436, 1350, 1215, 1104, 812, 749, 712.

3.1.4. (1α,2α)-Di-(3-nitrobenzoyl)-(3β,4β)-di-(2-pyridyl)cyclobutane (*4c*)

Yellowish amorphous solid, mp 168–171 °C; UV λ_{max} ($CHCl_3$)/nm 244 (ε/dm^3 mol^{-1} cm^{-1} 75,381); ^{1}H NMR ($CDCl_3$, 200 MHz) and ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm) see Table 1; positive LC–MS/MS *m*/*z* (%); *m*/*z* = 531 (100) $[M+Na]^+$, 468 (55), 402 (17), 356 (20), 192 (32), 173 (38). Calcd. for $C_{28}H_{20}N_4O_6$ (508.49): C 66.14, H 3.96, N 11.02; found: C 66.03, H 3.95, N 11.09; FT-IR (cm^{-1}): 3083, 3014, 2928, 2870, 1693, 1613, 1591, 1531, 1474, 1350, 1221, 1096, 813, 751, 715.

3.1.5. (1α,2α)-Di-(4-nitrobenzoyl)-(3β,4β)-di-(2-pyridyl)cyclobutane (*5*)

Yellowish amorphous solid, mp 157–159 °C; UV λ_{max} ($CHCl_3$)/nm 267 (ε/dm^3 mol^{-1} cm^{-1} 41,015); ^{1}H NMR ($CDCl_3$, 200 MHz) and ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm) see Table 1; positive LC–MS/MS *m*/*z* (%); *m*/*z* = 531(75) $[M+Na]^+$, 468 (13), 402 (100), 356 (53), 173 (72). Calcd. for $C_{28}H_{20}N_4O_6$ (508.49): C 66.14, H 3.96, N 11.02; found: C 66.23, H 3.98, N 11.03; FT-IR (cm^{-1}): 3115, 3085, 3027, 2926, 2855, 1690, 1591, 1524, 1346, 1218, 837, 751.

Table 1
NMR data[a] of compounds **4a–c**, and **5** in $CDCl_3$.

Pos.	**4a**		**4b**		**4c**		**5**	
	δ_H (Hz)	δ_c	δ_H (Hz)	δ_c	δ_H (Hz)	δ_c	δ_H (Hz)	δ_c
1, 2	5.2, AA′BB′ 9.0, 5.8, 2.4	48.9	6.0, 1H, AA′BB′ 4.8, 2H, AA′BB′	49.4 46.7	5.6, AA′BB′ 6.0, 3.8, 1.4	45.9	5.6, AA′BB′, 5.8, 3.8, 2.0	46.2
3, 4	4.1, AA'BB' 9.0, 5.8, 2.4	44.2	4.5, 1H, AA′BB′	44.5 43.8	4.6, AA′BB′ 6.1, 3.8, 2.4	45.6	4.5, AA′BB′, 5.8, 3.8, 2.4	45.7
la, 2a	–	196.8	–	198.7 196.2	–	197.1	–	197.5
1′, 1″	–	ns	–	138.0 137.3	–	137.1	–	140.1
2′, 2″	8.8, dd, 2.0, 1.6	123.1	9.4, dd, 2.0, 1.6 8.5, t, 2.0	122.6 122.3	8.8, bs	123.2	8.1, d, 9.0	129.2
3′, 3″	–	148.1	–	148.4 147.9	–	148.3	8.2, d, 9.0	123.9
4′, 4″	8.3, dd, 8.2, 1.4	127.6	8.4, m 8.2, m	127.6 126.5	8.3, d, 8.2	127.3	–	150.2
5′, 5″	7.5, m	134.4	7.6, dd, 8.0, 7.8 7.5, dd, 8.2, 7.6	134.9 133.0	7.6, t, 8.0	133.7	8.2, d, 9.0	123.9
6′, 6″	8.3, dd, 7.8, 1.0	129.6	8.6, d, 7.8 8.1, d, 8.2	129.5 129.4	8.3, d, 8.0	129.9	8.1, d, 9.0	129.2
1‴, 1‴	–	158.1	–	156.1 155.7	–	156.5	–	156.5
3‴, 3‴	8.8, dd, 4.6, 1.0	150.5	8.4, d, 6.0 8.1, d, 5.6	149.1 148.1	8.5, d, 4.2	149.0	8.5, dd, 7.8, 1.0	149.0
4‴, 4‴	7.2, dd, 5.0, 1.2	122.6	6.9, dd, 4.6, 3.6 6.8, dd, 4.8, 5.0	121.6 121.5	7.0, dd, 5.6, 1.2	121.7	7.0, dd, 7.8 1.0	121.8
5‴, 5‴	7.5, m	136.5	7.4, dt, 7.8, 1.8 7.1, dt, 7.6, 2.0	135.8 135.6	7.4, dt, 7.8, 1.6	135.9	7.4, dt, 7.6, 1.8	136.0
6‴, 6‴	6.9, dd, 7.4, 1.0	124.2	6.9, d, 7.8 6.7, d, 7.6	124.9 123.9	6.9, d, 7.8	123.7	6.9, d, 7.6	123.7

[a] Assignment based on ^1H, ^{13}C, APT, ^1H ^1H COSY, and comparison with ACD NMR program.

3.2. Theoretical calculations

Theoretical investigations were performed with HYPERCHEM 7.5 program on an IBM PC Pentium IV computer. The HOMO and LUMO energies in the ground state and the HSOMO and LSOMO energies in the exited state were calculated by using the PM3-RHF and PM3-RHF-CI semi-empirical methods [27,28].

3.3. Antimicrobial activity assessment

All test microorganisms were obtained from the Hıfzıssıhha Institute of Refik Saydam (Ankara, Turkey) and were as follows: *Escherichia coli* ATCC 25922, *Yersinia pseudotuberculosis* ATCC 911, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* 709 ROMA, *Listeria monocytogenes* ATCC 43251, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, and *Candida albicans* ATCC 60193. All the synthesized compounds were dissolved in DMSO for dilution to prepare compound stock solutions of 0.6–2.0 mg/mL for diffusion assay and of 500–12,600 μg/mL for dilution assay.

3.3.1. Agar well diffusion method

Simple susceptibility screening test using agar well diffusion method was used [29]. Each microorganism was suspended in Mueller Hinton (MH) (Difco, Detroit, MI) broth and diluted approximately 10^6 colony forming unit (cfu)/mL. They were "flood-inoculated" onto the surface of MH agar and Sabouraud Dextrose Agar (SDA) (Difco, Detriot, MI, USA) and then dried. For *C. albicans*, SDA was used. Five-millimeter diameter wells were cut from the agar using a sterile cork-borer, and 50 μL of the sample solutions was delivered into the wells. The plates were incubated for 18 h at 35 °C. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organism. Ampicillin (10 μg) and fluconazole (5 μg) were standard antibacterial and antifungal agents, respectively. DMSO was used as solvent control.

3.3.2. Dilution method

The antimicrobial effects of the compounds were also tested quantitatively in respective broth media by using double dilution method and the minimal inhibition concentration (MIC) values (μg/mL) were determined [29]. The antibacterial and antifungal assays were performed in Mueller-Hinton broth (MH) (Difco, Detroit, MI, USA) at pH: 7.3 and buffered Yeast Nitrogen Base (Difco, Detroit, MI, USA) at pH: 7.0, respectively. The MIC was defined as the lowest concentration that showed no growth. Ampicillin and fluconazole were used as standard antibacterial and antifungal drugs, respectively. DMSO with dilution of 1:10 was used as solvent control.

3.4. Antioxidant activity

The antioxidant activity of the compounds was tested by utilizing DPPH scavenging assay [30]. Briefly, 750 μL samples of various concentrations (0.015–1.00 mg/mL in DMSO) were mixed with 750 μL of 50 mM ethanolic DPPH solution. Following a 50 min incubation period at room temperature, the absorbance was measured against a blank at 517 nm. Lower absorbance of the reaction mixture shows higher DPPH radical scavenging activity. The results are given as SC_{50} (mg/mL), the compound concentration providing 50% scav-

Table 2
HOMO/LUMO and HSOMO/LSOMO energies and electron coefficients of compounds **1**–**3**.

	1[a]			**2**[a]			**3**[a]		
	S_0[b]	S_1[b]	T_1[b]	S_0[b]	S_1[b]	T_1[b]	S_0[b]	S_1[b]	T_1[b]
HOMO (eV)	−9.89			−9.865			−9.847		
C_α	−0.45			0.47			0.47		
C_β	−0.31			0.33			0.31		
LUMO (eV)	−1.61			−1.388			−1.384		
C_α	0.16			0.24			0.38		
C_β	−0.03			−0.29			0.33		
HSOMO (eV)		−4.386	−8.69		−4.336	−8.658		−4.346	−8.640
C_α		−0.47	−0.46		−0.50	0.48		−0.49	0.47
C_β		0.34	0.60		0.36	−0.63		0.36	−0.63
LSOMO (eV)		−1.366	−1.54		−1.172	−1.313		−0.962	−1.094
C_α		−0.16	−0.13		−0.10	0.09		0.17	0.16
C_β		−0.04	0.01		−0.04	−0.01		0.09	0.01

[a] Compounds.
[b] Electronic state.

enging of the DPPH radical present in the solution, and compared with those of Trolox® and Vitamin C.

4. Results and discussion

Scheme 1 illustrates the synthetic approach chosen for the preparation of dimerization products (**4a**–**c**, **5**) of known *m*- and *p*-nitro-substituted (*E*)-2-azachalcones (**2** and **3**) [5,6,12–23].

p-Nitro-(*E*)-2-azachalcone (**3**), when exposed to UV light (400 Watt high-pressure Hg lamp) in acetonitrile, was converted to the respective cyclobutane (**5**) with the yield (chromatographed product, PTLC) of 24%. The irradiation of *m*-nitro-(*E*)-2-azachalcone (**2**) under the same conditions gave a mixture of three dimers, **4a**–**c**, as major products, with the yields (chromatographed products, PTLC) of 21%, 8%, and 18%, respectively. However, the irradiation of *o*-nitro-(*E*)-2-azachalcone (**1**) in acetonitrile or diethyl ether, with or without benzophenone or benzoyl peroxide as sensitizer, in solution and solid state did not yield the anticipated products, as in the case with *o*-nitro-(*E*)-3- and 4-azachalcones observed earlier [5,6]. In fact, the substrate was also recovered unchanged after prolonged reaction time in this case, as in the literature [5,6,16,17].

The structures of the cyclobutyl rings of products **4a**–**c**, and **5** were elucidated from their ^{1}H NMR spectra, which show highly shielded CH proton signals at δ_H 5.2(H_{1-2})/4.1(H_{3-4}) for **4a**; at δ_H 6.0(H_1)/4.8(H_{2-3})/4.5(H_4) for **4b**; at δ_H 5.6(H_{1-2})/4.6(H_{3-4}) for **4c**, and at δ_H 5.6(H_{1-2})/4.5(H_{3-4}) for **5**, respectively. Stereochemistries of the dimers (**4a**–**c**, **5**) were established on the basis of the ^{1}H NMR spectra and by comparison with the literature data [4–7,12–23].

The obtained values for *J* are in agreement with a *cis* relationship between A and A′ and B and B′, because the values are 5.8 and 6.1 Hz for **4c** and **5**, respectively. Two symmetrical multiplets (AA′BB′ system) at δ_H 5.6 (δ_C 45.9)/δ_H 4.6 (δ_C 45.6) for compound **4c** and at δ_H 5.6 (δ_C 46.2)/δ_H 4.5 (δ_C 45.7) for compound **5** were observed for the cyclobutyl protons in ^{1}H NMR spectra. Simulation of these NMR patterns has allowed the calculation of the coupling constants of the cyclobutyl protons ($J_{AA'}$ = 6.0/5.8, J_{AB} = 3.8/3.8, $J_{AB'}$ = not detected, $J_{BB'}$ = 6.1/5.8). The values of these coupling constants and ^{1}H and ^{13}C NMR patterns of the cyclobutyl moieties of compounds **4c** and **5** suggest that the formation of cyclobutane ring occurs by *cis* head-to-head junction to give β-truxinic structure [6,12–23],

Simulation of the NMR patterns of compound **4a** has allowed the calculation of the coupling constants of the cyclobutyl protons ($J_{AA'}$ = 9.0, J_{AB} = 5.8, $J_{AB'}$ = not detected, $J_{BB'}$ = 9.0). The calculated values suggest that **4a** was formed by head-to-head coupling. The close similarity of the ^{1}H and ^{13}C NMR patterns of the cyclobutyl moieties with δ-truxinic structure strongly suggests that the formation of cyclobutane ring occurs by *anti*-head-to-head junction in compound **4a** [5,7]. The stereochemistry of cyclobutane ring in compound **4b** was in neotruxinic structure (rel.1β,2β,3β,4α) as in the literature [5,12–23], as evident from ^{1}H (δ_H 6.0, 1H; 4.8, 2H; 4.5, 1H) and ^{13}C (δ_c 49.4, 46.7, 44.5, 43.8) NMR data.

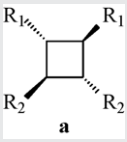
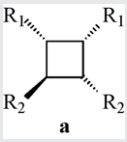
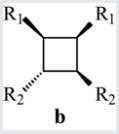
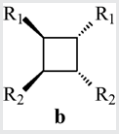
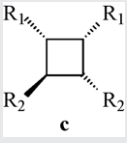
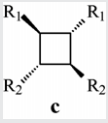
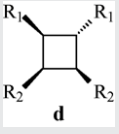
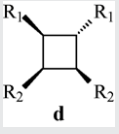
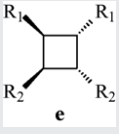
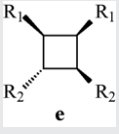
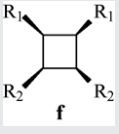
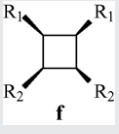
The compounds **4a**–**c**, and **5** were characterized on the basis of spectral data evaluations (^{1}H, ^{13}C, APT, attached proton test, ^{1}H–^{1}H COSY, ACD NMR, FT-IR, UV–vis, LC–MS/MS, and elemental analysis), whose results were in agreement with the proposed structures (Table 1).

The positive LC–MS/MS gave $[M+H]^+$ at *m*/*z* 509 (100) for **4a**,**b**, $[M+Na]^+$ at *m*/*z* 531 (75% and 100%) for **4c** and **5** and, respectively, which were consistent with the molecular formulas to be $C_{28}H_{20}N_4O_6$ for **4a**–**c**, and **5**.

The photochemical dimerization reactions of compounds **2** and **3** yielded compounds **4a**–**c**, **5** isomers. However, the dimerization of *o*-NO_2 substituted (*E*)-2-azachalcone (**1**) was unsuccessful, as were the case with (*E*)-3- and (*E*)-4-azachalcones [5,6]. The failure in the formation of any photoaddition product of compound **1** led us to the examination of the possibility of frontier orbital control in the stereochemical behavior. The optimized structures of compounds **1**–**3** were obtained from the theoretical calculations. The HOMO and LUMO energies on the ground state and HSOMO and LSOMO energies in the exited state were estimated by using the PM3-RHF and PM3-RHF-CI semi-empirical methods [27,28] (Table 2). To give a dimerization product, superposition of S_0–T_1 and S_0–S_1 HOMO-LSOMO orbitals of compound **1** are allowed but electron densities of ground state LUMO and excited state of S_1 and T_1 LSOMO orbital were too low to give any dimerization reaction. In fact, the best interaction occurs between the HSOMO of the excited singlet state and the LUMO of the ground singlet state because of low energy gap of 2.948 and 2.962 eV for compounds **2** and **3**, respectively. Results of the calculations are in agreement with the experimental findings. As expected from the total electronic energies of dimers and the transition state energies for the ring closure reactions for all possible dimers (Table 3), the products of the dimerization reactions of **2** and **3** are head-to-head dimers. From a synthetic point of view, the compound obtained in the highest yield (**4a**) showed the same stereochemistry as the naturally occurring cyclobutanes [8–11].

Based upon the above observations, the complete chemical shift assignments for **4a**–**c**, and **5** were deduced to be (1α,2β)-di-(3-nitrobenzoyl)-(3α,4β)-di-(2-pyridyl)cyclobutane (**4a**), (1β,2β)-di-(3-nitrobenzoyl)-(3β,4α)-di-(2-pyridyl)cyclobutane (**4b**), (1α,2α)-di-(3-nitro-benzoyl)-(3β,4β)-di-(2-pyridyl)cyclobutane (**4c**),

Table 3
The total electronic energy of dimers and the transition state energy for the ring closure reaction for isomers of **4** and **5** (kcal/mol).

4			**5**		
Isomers (head-to-head)	$-E$	Biradicals $\Delta H^{\neq}$	Isomers (head-to-head)	$-E$	Biradicals $\Delta H^{\neq}$
 **a**	138974.83	87.16	 **a**	138969.75	92.23
 **b**	138969.33	92.66	 **b**	138969.13	92.86
 **c**	138967.62	94.37	 **c**	138967.94	94.05
 **d**	138966.24	95.75	 **d**	138967.93	94.06
 **e**	138961.34	100.64	 **e**	138967.80	94.18
 **f**	138959.60	102.38	 **f**	138957.95	104.03

R_1 = *m*- and *p*-$O_2NC_6H_4CO$-; R_2 = 2-pyridyl.

and (1β,2β)-di-(4-nitro-benzoyl)-(3β,4β)-di-(2-pyridyl)cyclobutane (**5**).

The antimicrobial activity of all the compounds (**1–5**) was determined based on inhibition zone diameters in agar well diffusion test (Table 4) and minimum inhibitory concentrations in dilution test (Table 5) [29]. All the compounds showed from slight to pronounced antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, and the yeast-like fungus. The monomeric (*E*)-2-azachalcones **1**–**3** were more effective than the dimers as was the case in (*E*)-4-azachalcones [5]. Similarly, the antimicrobial activity was more pronounced against Gram-positive bacteria compared to Gram-negative ones. Compound **1** was the most active azachalcone tested in the diffusion assay, with even greater inhibition zones than that of the standard antibacterial Ampicilline. However, its effect in the dilution assay was lower than Ampicilline and fluconazole, though it still appeared as quite effective against the Gram (+) bacteria and the fungus tested. *m*-Nitro-substituted compound **2** was the most active among the monomers against Gram-positive bacteria in dilution test, similar to that observed with (*E*)-4-azachalcones [5]. Monomeric compounds **1**–**3** were bet-

Table 4
Screening results for antimicrobial activity of the compounds **1**–**5** based on agar well diffusion assay.

Comp. No.	Stock conc. (μg/50 μL)	Microorganisms[a] and inhibition zones (mm)							
		Ec	*Yp*	*Pa*	*Bc*	*Li*	*Sa*	*Ef*	*Ca*
1	100	10	7	25	22	15	23	25	25
2	100	6	6	9	13	9	14	9	16
3	50	7	6	8	16	9	14	8	10
4a	75	7	–	6	6	6	6	6	8
4b	30	9	7	6	6	8	9	6	10
4c	100	6	–	7	–	6	–	7	8
5	100	–	–	7	6	6	–	7	10
Amp.	10	10	18	18	15	10	35	10	nt
Flu.	5	nt	nt	nt	nt	nt	nt	nt	25

Amp.: Ampicilline, Flu.: Fluconazole, (–): no activity, nt: not tested.

[a] *Ec*: *Escherichia coli* ATCC 25922, *Yp*: *Yersinia pseudotuberculosis* ATCC 911, *Pa*: *Pseudomonas aeruginosa* ATCC 27853, *Bc*: *Bacillus cereus* Roma 709, *Li*: *Listeria monocytogenes* ATCC 43251, *Sa*: *Staphylococcus aureus* ATCC 25923, *Ef*: *Enterococcus faecalis* ATCC 29212, *Ca*: *Candida albicans* ATCC 60193.

Table 5
Minimum inhibitory concentrations (MIC, μg/mL) of the compounds **1**–**5** based on dilution assay.

Comp. No.	Stock conc. (μg/mL)	Microorganisms[a] and minimum inhibitory concentrations (MIC, μg/mL)							
		Ec	*Yp*	*Pa*	*Bc*	*Li*	*Sa*	*Ef*	*Ca*
1	12,600	630	630	630	4.9	4.9	4.9	4.9	4.9
2	4,900	245	245	245	3.8	3.8	3.8	7.6	15.3
3	8,200	205	205	205	12.8	12.8	25.6	25.6	25.6
4a	6,000	600	300	300	300	300	300	600	>600
4b	500	50	50	50	50	50	50	50	50
4c	5,600	280	280	280	280	140	280	280	>560
5	5,000	250	250	250	250	250	250	250	125
Amp.		8	32	32	>128	2	2	<1	nt
Flu.		nt	nt	nt	nt	nt	nt	nt	<1

[a] *Ec*: *Escherichia coli* ATCC 25922, *Yp*: *Yersinia pseudotuberculosis* ATCC 911, *Pa*: *Pseudomonas aeruginosa* ATCC 27843, *Bc*: *Bacillus cereus* Roma 709, *Li*: *Listeria monocytogenes* ATCC 43251, *Sa*: *Staphylococcus aureus* ATCC 25923, *Ef*: *Enterococcus faecalis* ATCC 29212, *Ca*: *Candida albicans* ATCC 60193. Amp.: Ampicilline, Flu.: Fluconazole, nt: not tested.

ter effective compared with Ampicilline in the dilution assay against *B. cereus*, a foodborne pathogen causing severe nausea, vomiting, and diarrhea. All test compounds were effective against the yeast-like fungus *C. albicans*. The fungus growth inhibitory activity of compound **1** was equivalent to the standard antifungal fluconazole in the diffusion assay. Compounds **4c** and **5**, which have similar substituent orientation with respect to the cyclobutane ring, were the least active. The solvent control DMSO showed no inhibition effect on all test microorganisms.

When the antimicrobial activities of (*E*)-2-azachalcones tested in the current study are compared with those of (*E*)-4-azachalcones [5] and (*E*)-3-azachalcones [6], it is clearly seen that (*E*)-2-azachalcones have better activity in both monomeric and dimeric forms. (*E*)-3-azachalcone series monomers showed the lowest activity.

The effect of nitro substitution on antimicrobial activity does not show a uniform trend. While the *p*-nitro substitution has been shown to increase antimicrobial activity of pyrazolines [31], mixed results have been reported with *p*- and *o*-nitrosubstituted flavones [32]. Nitro substitution also affects other biological activities of chalcones and flavonoids including their antitumor potentials [33].

The antioxidant activity of the synthesized monomeric and dimeric compounds of nitro-substituted (*E*)-2-azachalcones (**1**–**5**) were also tested based on their ability to scavenge the stable radical DPPH [30]. The antioxidant activities are expressed as the compound concentration providing 50% scavenging of the available radicals (SC_{50}, mg/mL) (Fig. 1). Compounds **1**, **2**, **4a**, **4c**, and **5** showed antioxidant activities with SC_{50} values in the range of 0.130–2.047 mg/mL. The five active azachalcones showed three to ten fold better radical scavenging activity as compared to the heteroaryl chalcones containing thiophenyl ring tested in our earlier study [22]. The nitro-substitution has earlier been shown to result in better radical scavenging activity, compared to ethyl substitution, in monomers and dimers of chalcones [23]. Among the monomeric forms **1**–**3**, compound **1** with *ortho*-substitution exhibited the best radical scavenging potential (Fig. 1), with the lowest SC_{50} value, forming a general trend of the activity in the order of substitution *o*- > *m*- > *p*- among nitro-substituted monomeric azachalcones [5,6]. The dimerization product **4c** showed lower radical scavenging activity among the five active compounds tested, while compound **4b** was inactive and compound **3** was prooxidant in DPPH test. The dimers obtained from (*E*)-2-azachalcone in this study showed better scavenging activity compared to dimers of (*E*)-3-azachalcones and (*E*)-4-azachalcones [5,6]. The antioxidant potentials of the five active radical scavenger compounds were lower than those of standard antioxidants Trolox® and Vitamin C. The monomeric azachalcone **1** showed high antimicrobial and antioxidant activities and, thus, deserves further in vitro and in vivo investigations to explore its potential use for the cure of bacterial infections concurrent with oxidative stress.

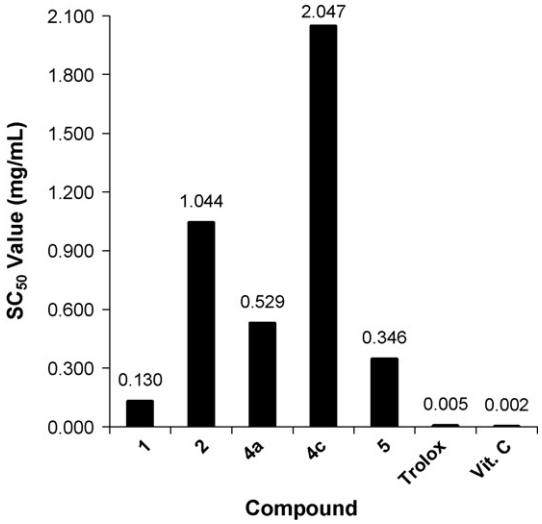


Fig. 1. The antioxidant capacities of the synthesized compounds and the reference antioxidants Trolox® and Vitamin C based on DPPH radical scavenging activities. The results are expressed as SC_{50} (mg/mL), the concentration providing 50% scavenging of the DPPH radicals already available in the test mixture. Compound **3** was prooxidant, and compound **4b** was inactive.

Acknowledgements

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