TÜBİTAK

Turkish Journal of Chemistry

http://journals.tubitak.gov.tr/chem/

Research Article

Turk J Chem
(2018) 42: 482 – 492

doi:10.3906/kim-1705-28

Synthesis and biological evaluation of novel chalcones bearing morpholine moiety as antiproliferative agents

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Received: 16.05.2017 • **Accepted/Published Online:** 12.12.2017 • **Final Version:** 27.04.2018

Abstract: In this research, a new series of (*E*)-3-(4-substitutedphenyl)-1-(4'-morpholinophenyl)prop-2-en-1-one derivatives **1**–**7** was synthesized, aiming to develop effective antiproliferative agents. The antiproliferative activities of compounds **1**–**7** were examined against HeLa and C6 cell lines at eight different concentrations using the BrdU ELISA assay. The activity results were compared with reference anticancer drug 5-fluorouracil (5-FU). Compound **1** had almost the same antiproliferative activity as 5-FU. Compounds **1**–**7** were found to have greater effects against the C6 cell line than the HeLa cell line. These compounds were also tested in order to determine their effects on enzyme activities. Compounds **2** and **3** exhibited strong activator characteristics against pyruvate kinase isoenzyme M2 (PKM2) with AC_{50} values of 2.2 and 14.28 μM**.** Compounds **2**, **3**, and **7** acted as inhibitors with IC_{50} values in the range of 84.08–165.38 μM for carbonic anhydrase isoenzyme I (hCA I), and compounds **3** and **4** demonstrated inhibitory effects against carbonic anhydrase isoenzyme II (hCA II) with IC_{50} values of 108.11 and 112.52 μM, respectively. hCA II was activated by derivatives **1**, **2**, **6**, and **7** with AC_{50} values in the range of 85.53–146.59 μM.

Key words: Chalcone, antiproliferative activity, hCA I and II, PKM2, morpholine

1. Introduction

Cancer is one of the most deadly diseases. According to the GLOBOCAN 2012 report, a total of 14.1 million new cancer cases were recorded, causing 8.2 million deaths.[1,2] 1,3-Diaryl-2-propen-1-one, also known as chalcone, constitutes an important class of natural products. It exhibits a wide spectrum of pharmacological activities such as antimalarial,[3] antiinflammatory,[4] antioxidant,[5] anti-HIV,[6] and anticancer.[7] Additionally, the bielectrophilic character of the chalcone structure is used as an intermediate to prepare some heterocyclic rings such as pyrazolines,[8] isoxazoline,[9] pyrimidine,[10] thiazine,[11] oxazine,[12] and flavones[13] that are therapeutics. They react through a cyclocondensation reaction with binucleophiles. Therefore, synthesis is important to chemists for the discovery of new drugs, both organic and medicinal.

Cervical cancer is a malignant tumor arising from the cervix. It is the third most common cancer in women and the seventh overall, with an estimated 529,000 cases in 2008.[14] HeLa was the first human cell line established in culture[15] and since then it has become the most widely used human cell line in biological research.

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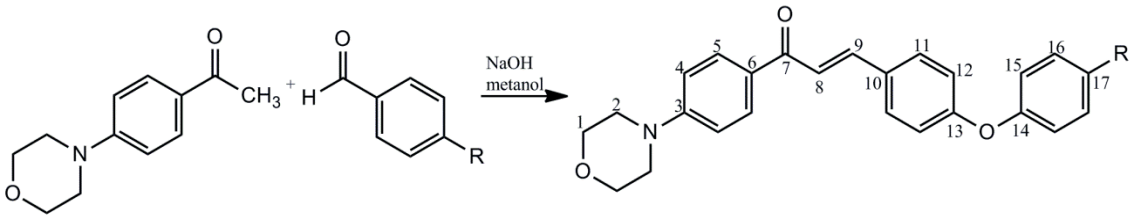
HeLa cells have contributed to the characterization of important biological processes in many publications (more than 70,000),[16] while the C6 glioma model continues to be used for a variety of studies related to brain tumor biology.[17] These have included several studies related to tumor growth, invasion, migration, blood–brain barrier disruption, neovascularization, growth factor regulation and production, and biochemical studies.[18−20]

Enzymes have a vital role in life because they catalyze all chemical reactions occurring in the living cells. Several diseases such as cancer have specific biochemical processes and different isoenzymes are overexpressed to contribute to the new metabolic state.[21,22] Hence, these critical enzymes are important targets to cure many diseases, and selective inhibitors or activators are used as medicinal agents. Pyruvate kinase isoenzyme M2, overexpressed in cancer cells, performs a regulatory role in the production of ATP and the synthesis of intermediate metabolites for the synthesis of biomolecules.[23,24] Many articles have reported that both its inhibitors and activators may be used to reduce the growth and proliferation of cancer cells.[25,26] Carbonic anhydrase inhibitors find applications in the clinic such as antiglaucoma agents, diuretics, or antiepileptics. It was also suggested that carbonic anhydrase activators might be employed as drugs to treat disease occurring with a considerable decline of carbonic anhydrase in the brain, such as in Alzheimer disease, or during aging.[27] According to these data, we designed new chalcones bearing morpholine moiety to investigate their pharmacological activities. In this study, antiproliferative activities of (*E*)-3-(4-substitutedphenyl)-1-(4'-morpholinophenyl)prop-2-en-1-ones **1**–**7** were examined against HeLa and C6 cell lines at eight concentrations using the BrdU ELISA assay. The results of activity were compared with 5-fluorouracil (5-FU) as a standard drug.

2. Results and discussion

2.1. Chemistry

(*E*)-3-(4-Substituted phenyl)-1-(4'-morpholinophenyl)prop-2-en-1-one derivatives **2**–**7** were obtained by Claisen–Schmidt condensation of 4-substituted benzaldehyde with 4'-morpholinoacetophenone by the route shown in the Scheme. The structure and purity of the synthesized compounds were determined by elemental analysis and spectroscopic methods such as FT-IR, ^{1}H NMR, ^{13}C NMR, and 2D NMR analysis. Comparison of all spectral data and results of elemental analysis showed evidence of the formation of the target products.



Scheme. General synthetic pathway.

Generally, the FT-IR spectra of (*E*)-3-(4-substituted phenyl)-1-(4'-morpholino phenyl)prop-2-en-1-one derivatives **1**–**7** showed a carbonyl (C=O) peak at 1644 to 1656 cm^{-1} and an (*E*) ethylenic (-CH=CH-) peak at 922 to 927 cm^{-1}. It also suggested morpholine -C-O-C- stretching at the 1185–1218 cm^{-1} and -C-N-C- stretching at the 1110–1122 cm^{-1} bands, as well as confirming the presence of phenoxy (Ar-O-Ar) at 1242–1289 cm^{-1}. In the ^{1}H NMR spectra of compounds **1**–**7**, H_1 and H_2 protons of the morpholine ring were detected at 3.31–3.34 ppm and 3.73–3.87 ppm as singlets, respectively. In the aromatic region, H_α and H_β protons of chalcones **1**–**7** resonated as sharp doublets at 7.45–7.68 ppm and 7.76–7.94 ppm, respectively. The coupling

constant (J) of these doublets, 15.6 Hz, confirmed the trans-configuration of the chalcones. The ^{13}C NMR spectra of compounds **1**–**7** displayed signals at 47.20–47.54 ppm and 66.30–66.57 ppm, which were assignable to C_1 and C_2 of the morpholine ring. The C_α and C_β signals of chalcone were detected at 120.38–121.97 ppm and 141.82–142.77 ppm as singlets, respectively.

Further examination of the HSQC, COSY, and HMBC spectra allowed the assignment of all remaining proton and carbon resonances for compound **7**. The HSQC spectrum provided the determination of all protonated carbons, as shown in Figure 1.

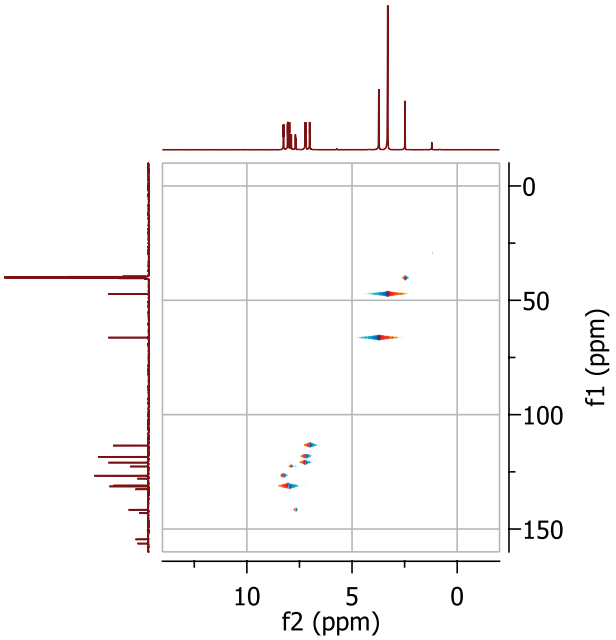


Figure 1. HSQC spectrum of compound **7**.

The five extended spin systems (H_1-H_2, H_4-H_5, H_8-H_9, H_{11}-H_{12}, and H_{15}-H_{16}) were determined in the ^{1}H, ^{1}H correlation spectra (COSY) of compound **7** (Figure 2). As a result of the signals of H_5, H_8, and H_9, the connection between the corresponding carbonyl group was determined by HMBC spectroscopy (Figure 3). Other C-C connectivities that were inferred from the HMBC spectrum included connections from C_9, C_{10}, and C_3 to the morpholine ring (Figure 3). Compound **7** was selected as a model compound to evaluate the NMR spectra, and the NMR data of other compounds are given in Section 3.

The mass spectra of all chalcones showed intensive molecular ions; the cleavage between the C_β atom and 4-substituted phenyl ring gave the fragment $C_{13}H_{14}NO_2^+$ at m/z 216.10 and also $C_{11}H_{12}NO_2^+$ at m/z 190. After leaving the aldehyde part, the fragment $C_6H_5^+$ (m/z 77.04) was detected.[28,29]

2.2. Antiproliferative activity results

Antiproliferative activities of compounds **1**–**7** and 5-FU were determined against C6 and HeLa cells. The IC_{50} and IC_{75} values of compounds **1**–**7** and 5-FU are given in Table 1.

For the antiproliferative activities of compounds **1**–**7,** a dose-dependent increase in activity against C6 cells was observed (Figure 4A). Compound **1** had almost the same antiproliferative activities as 5-FU, which is used as a standard compound against C6 cells at high concentrations (Figure 4A). However, compound **2** had

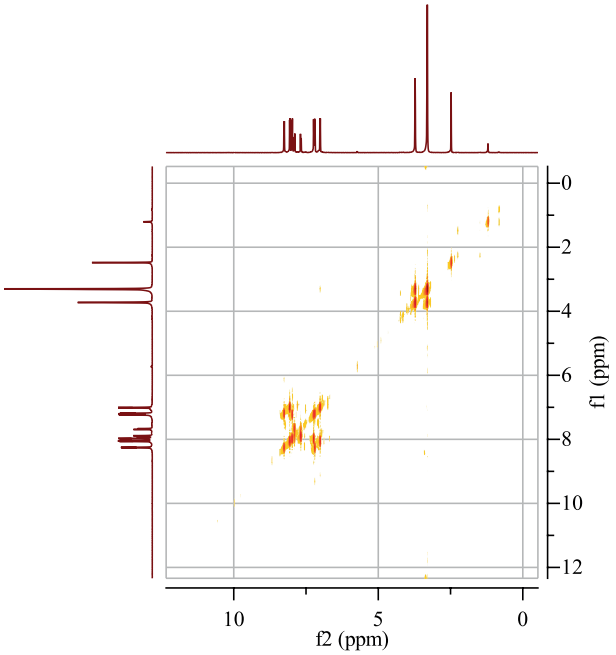


Figure 2. COSY spectrum of compound **7**.

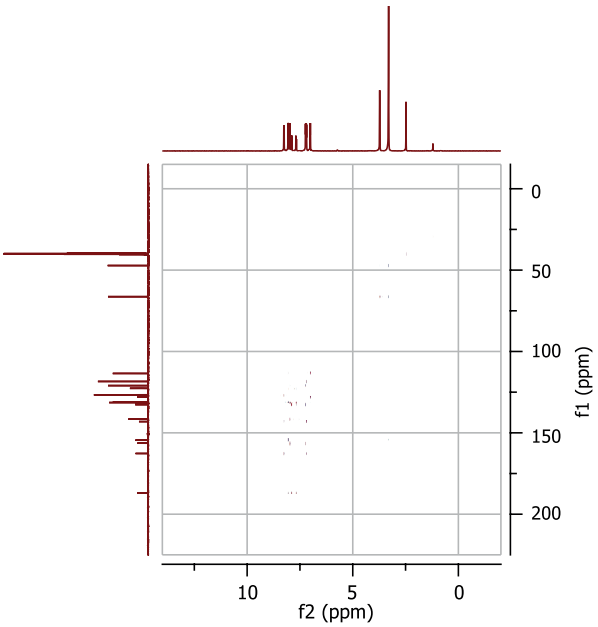


Figure 3. HMBC spectrum of compound **7**.

Table 1. IC_{50} and IC_{75} values of compounds **1**–**7** against C6 and HeLa cells.

		1	**2**	**3**	**4**	**5**	**6**	**7**	5-FU
C6	IC_{50}	7.36	35.65	10.60	26.54	30.13	21.80	61.78	5.80
	IC_{75}	45.32	68.48	54.63	65.89	61.37	56.26	78.73	nd
HeLa	IC_{50}	68.27	*	71.73	57.90	71.55	59.48	73.00	16.32
	IC_{75}	79.16	*	81.27	72.49	86.81	81.50	78.28	nd

*>100 μM, nd: not detected.

moderate antiproliferative activity compared with 5-FU. The potency of inhibitions at 100 μg/mL against C6 cells were as follows: compound **1** $\sim$ 5-FU $>$ **2** $>$ **4** $>$ **3** $>$ **7** $>$ **5** $>$ **6**. All the compounds were found to have greater effects against the C6 cell line than the HeLa cell line.

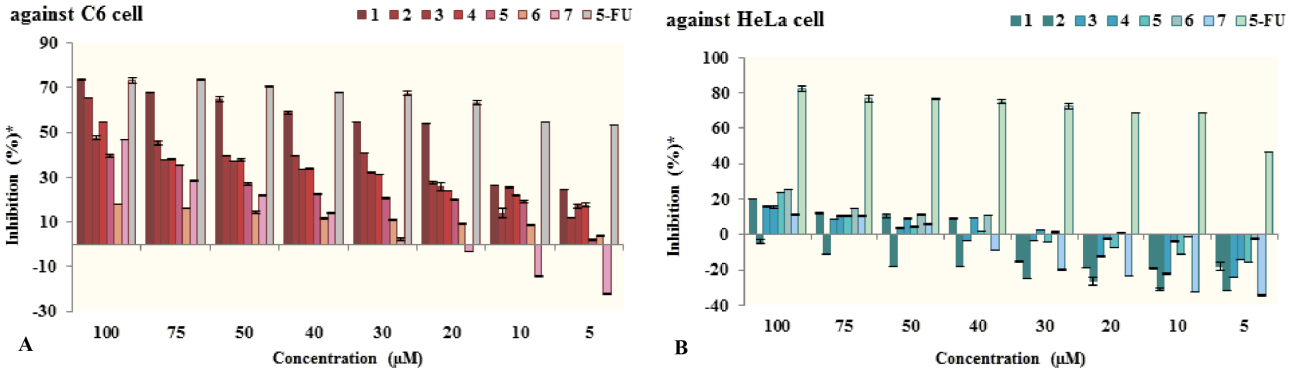


Figure 4. The antiproliferative activities of compounds **1**–**7** and 5-FU against C6 (A) and HeLa (B) cells. *: Each substance was tested twice in triplicate against the cell lines. Data show the average of two individual experiments ($P < 0.01$).

The antiproliferative activities of compounds **1**–**7** were observed to increase against HeLa cells in a dose-dependent manner (Figure 4B). All the compounds had weaker antiproliferative activities than 5-FU (Figure 4B). The potency of inhibitions at 100 μg/mL against HeLa cells were as follows: 5-FU $>$ **6** $>$ **5** $>$ **1** $>$ **3** $>$ **4** $>$ **7** $>$ **2**.

The structure–activity relationship is important to define the effects of groups. In this study, compound **1**, having a nonsubstituted phenyl ring, exhibited better activity with an IC_{50} value of 7.36 μg/mL against C6 cells than HeLa cells. When the 4-substituted phenoxy group was attached to the phenyl ring, the antiproliferative activities of other compounds decreased. Only compound **3** having 4-(4-chlorophenoxy)phenyl moiety showed moderate activity against the C6 cell line (IC_{50}: 10.60 μg/mL).

2.3. Enzyme results

Effects of the synthetic derivatives on PKM2, hCA I, and hCA II are presented in Table 2. Compounds **2**, **3**, and **7** exhibited inhibitory activities against hCA I with IC_{50} values in the range of 84.08–165.38 μM. hCA II was inhibited by compounds **3** and **4**; IC_{50} values were calculated as 108.11 and 112.52 μM, respectively. However, compounds **1**, **2**, **6**, and **7** showed hCA II activator effects, with AC_{50} values in the range of 85.53–146.59 μM. The PKM2 enzyme was activated by compounds **2** and **3** at low AC_{50} values calculated as 2.2 and 14.28 μM, respectively.

Table 2. Effect of compounds on the enzymes activities. Results are expressed as μM.

Compound	hCA I		hCA II		PKM2	
	IC_{50}	Effect	IC_{50}/AC_{50}	Effect	AC_{50}	Effect
1	-	-	146.59	Activation		
2	84.08	Inhibition	95.50	Activation	2.2052	Activation
3	165.38	Inhibition	108.11	Inhibition	14.2891	Activation
4	-	-	112.52	Inhibition	-	-
5	-	-	-	-	-	-
6	-	-	85.53	Activation	-	-
7	134.44	Inhibition	125.84	Activation	-	-
Acetazolamide	0.108	Inhibition	0.0053	Inhibition		
Myricetin					0.5	Activation

2.4. Druglikeness properties

According to Lipinski's rules of five,[30] our results showed that the molecular weights of compounds **1**–**7** were not greater than 500. The number of groups that accepted hydrogen atoms was less than 10 and the number of those that donated hydrogen atoms was less than 5. All chalcones **2**–**7** had poor aqueous solubility with values of log P between 5.47 and 6.19. All data for the calculation of absorption (% ABS) according to Zhao et al.[31] are shown in Table 3.

2.5. Conclusions

The synthesis and evaluation of the antiproliferative activity of chalcones carrying a morpholine ring were reported. The activity results showed that compound **1** was the most active against the C6 cell line among the

Table 3. Druglikeness properties of chalcone derivatives **1**–**7**.*

Compound	Log P	TPSA	% ABS	MW	nON	nOHNH	Druglikeness
1	3.76	29.54	98.807	293.366	3	0	–0.33
2	5.51	38.78	95.621	385.46	4	0	–0.31
3	6.19	38.78	95.621	419.91	4	0	–0.03
4	5.68	38.78	95.621	403.45	4	0	–0.18
5	5.57	48.01	92.437	415.49	5	0	–0.27
6	5.96	38.78	95.621	399.49	4	0	–0.21
7	5.47	84.60	79.813	430.46	7	0	–0.38

*These parameters were determined with Molinspiration calculation software and Molsoft software.

tested compounds. Compound **1** may be found to be an anticancer drug candidate against C6 cells after further investigations. The effects of the compounds on the PKM2, hCA I, and hCA II were determined. As a result, compounds **2**, **3**, and **7** displayed inhibitory activities against hCA I. hCA II was inhibited by compounds **3** and **4** and activated by **1**, **2**, **6**, and **7**. The PKM2 enzyme was activated by compounds **2** and **3**. When the results are taken into consideration, it can be suggested that compounds **2** and **3** may be ideal candidates for new molecule design in PKM2 activator investigations. Please see the Supplementary Material for more details.

3. Experimental

All chemicals and solvents were of analytical grade and were purchased from Sigma-Aldrich, Merck, or Roche. All chemical reactions were monitored with thin-layer chromatography using Merck silica gel 60 F_{254} plates. Melting points were determined with an EZ-Melt melting point apparatus and were uncorrected. FT-IR spectra were recorded on a PerkinElmer Frontier spectrometer by an attenuated total reflectance apparatus (Waltham, MA, USA). Elemental analyses (CHNS) were performed on a Thermo Scientific Flash 2000 elemental analyzer (Finnigan MAT, San Jose, CA, USA). By direct injection in an Ab-SciEx 3200 Q-Trap MSMS detector with an electron spray ionization probe (Framingham, MA, USA), mass spectra were recorded. ^{1}H, ^{13}C, ^{19}F, DEPT, COSY, HSQC, and HMBC NMR spectra were recorded on an Agilent Technologies apparatus with 600 MHz NMR. Cell proliferation ELISA and BrdU (colorimetric) kits were obtained from Roche (Germany). The antitumor drug 5-FU, recombinant enzymes (PKM2, hCA I, and hCA II), and reagents were obtained from Sigma-Aldrich Chemical Co. (Germany).

3.1. General method for synthesis

4-Morpholinoacetophenone (0.01 mol) 4-substituted benzaldehyde (0.01 mol), and solid NaOH (0.03 mol) were added in methanol. The reaction mixture was stirred for 2 days at room temperature and was kept in a refrigerator overnight. The mixture was extracted with dichloromethane. The organic phase was dried with anhydrous $MgSO_4$. The solvent was evaporated and the crude product was recrystallized from n-hexane /DCM.[32]

3.1.1. (*2E*)-1-[4-(Morpholin-4-yl)phenyl]-3-phenylprop-2-en-1-one (1)[33]

Green powder, yield: 67%, mp: 173–175 °C; FT-IR ν_{max} (cm^{-1}): 1643 (C=O); 1583, 1515, 1495, 1445 (C=C); 1220 (C-O-C morpholine); 1118 (C-N-C morpholine), 925 (ethylenic -CH=CH-). ^{1}H NMR (400 MHz, DMSO-d_6/TMS): δ 3.33 (t, J= 4.8 Hz, 4H, H_2), 3.75 (t, J= 4.8 Hz, 4H, H_1), 7.04 (d, J= 9.2 Hz, 2H, H_5), 7.44–7.48 (m, 3H, $H_{12,13}$), 7.68 (d, J = 15.6 Hz, 1H, H_8), 7.88 (dd, J_1 = J_2 = 2.4 Hz, 2H, H_{11}), 7.94 (d, J_1 = 15.6 Hz, 1H, H_9), 8.09 (d, J= 8.8 Hz, 2H, H_4). ^{13}C NMR (100 MHz, DMSO-d_6/TMS): δ 47.20 (s, C_2), 66.30 (s, C_1), 113.54, 122.6, 127.9, 129.14, 129.34, 130.7, 131.04, 135.5, 142.7, 154.6 (Ar-C), 187 (s, C_7). TOF/MS (m/z) 293.4150 $[M+H]^+$. Anal. calc. for $C_{19}H_{19}NO_2$: C, 77.79; H, 6.53; N, 4.77; found: C, 77.50; H, 6.55; N, 4.96.

3.1.2. (*E*)-3-[4-(Phenoxy)phenyl-1-[4-(morpholin-4-yl)phenyl]prop-2-en-1-one (2)

Light yellow crystal, yield: 45%, mp: 204–202 °C. FT-IR νmax (cm^{-1}): 1656 (C=O); 1591, 1552, 1520 (C=C); 1193 (C-O-C morpholine); 1113 (C-N-C morpholine), 923 (ethylenic -CH=CH-). ^{1}H NMR (600 MHz, $CDCl_3$,): δ 3.33 (s, 4H, H_2), 3.86 (s, 4H, H_1), 6.91 (d, 2H, J= 7.2 Hz, H_4), 7.01 (d, 2H, J= 7.2 Hz, H_{12}), 7.06 (d, 2H, J= 7.2 Hz, H_{15}), 7.16 (t, J= 7.2 Hz, H, H_{17}), 7.38 (t, J= 7.2 Hz, 2H, H_{16}), 7.48 (d, 1H, J= 15 Hz, H_8), 7.61 (d, 2H, J= 8.4 Hz, H_{11}), 7.77 (d, 1H, J= 15.6 Hz, H_9), 8,01 (d, 2H, J= 7.8 Hz, H_5). ^{13}C NMR (150 MHz, $CDCl_3$): δ 47.53 (s, C_2), 66.57 (s, C_1), 113.41 (s, C_4), 118.47 (s, C_{12}), 119.61 (s, C_{15}), 120.70 (s, C_8), 124.04 (s, C_{17}), 128.93 (s, C_6), 129.91 (s, C_{16}), 129.97 (s, C_{11}), 130.07 (s, C_{10}), 130.55 (s, C_5), 142.63 (s, C_9), 154.12 (s, C_3), 156.20 (s, C_{14}), 159.37 (s, C_{13}), 188.03 (s, C_7). COSY (150 MHz, $CDCl_3$): H_1-H_2, H_4-H_5, H_8-H_9, H_{11}-H_{12}, H_{15}-H_{16}. HSQC (150 MHz, $CDCl_3$): The resonances were 3.33, 47.53 ppm (C_2-H_2); 3.86, 66.57 ppm (C_1-H_1); 6.91, 113.41 ppm (C_4-H_4); 7.01, 118.47 ppm (C_{12}-H_{12}); 7.06, 119.61 ppm (C_{15}-H_{15}); 7.16, 124.04 ppm (C_{17}-H_{17}); 7.38, 129.91 ppm (C_{16}-H_{16}); 7.48, 120.70 ppm (C_8-H_8); 7.61, 129.97 ppm (C_{11}-H_{11}); 7.77, 142.63 ppm (C_9-H_9); 8.01, 130.55 (C_5-H_5) ppm. HMBC (150 MHz, $CDCl_3$): The correlations were C_1-H_2; C_2-H_1; C_3-H_5; C_4-H_5; C_5-H_4; C_7-H_5,H_8,H_9; C_8-H_9; C_9-H_{11}; C_{10}-H_8; C_{11}-H_9,H_{12}; C_{12}-H_{11}; C_{13}-H_{12}; C_{14}-H_{15},H_{16}; C_{16}-H_5; C_{17}-H_{15}. TOF/MS (m/z) = 386.1834 $[M+H]^+$. MS: m/z = $C_{25}H_{23}NO_3$ (385.1678); 386.57, 308.7, 264.8, 248.6, 230.6, 216.8, 190.8, 186.8, 172.8, 171, 126.9, 112.9, 105, 96.9 $[M+H]^+$. Anal. calc. for ($C_{25}H_{23}NO_3$): C, 77.90; H, 6.01; N, 3.63; found: C, 77.31; H, 5.94; N, 3.73.

3.1.3. (*E*)-3-[4-(4-Chlorophenoxy)phenyl-1-[4-(morpholin-4-yl)phenyl]prop-2-en-1-one (3)

Light yellow crystal, yield: 34%, mp: 186–187 °C. FT-IR νmax (cm^{-1}): 1648 (C=O); 1584, 1548, 1502 (C=C); 1193 (C-O-C morpholine); 1120 (C-N-C morpholine, 926 (ethylenic -CH=CH-). ^{1}H NMR (600 MHz, $CDCl_3$,): δ 3.34 (s, 4H, H_2), 3.87 (s, 4H, H_1), 6.92 (d, 2H, J= 8.4 Hz, H_4), 6.99 (overlapped two t, J= 7.2 Hz, 4H, $H_{12,15}$), 7.33 (d, 2H, J= 7.8 Hz, H_{16}), 7.48 (d, 1H, J= 15.6 Hz, H_8), 7.62 (d, 2H, J= 7.8 Hz, H_{11}), 7.77 (d, 1H, J= 15.6 Hz, H_9), 8,01 (d, 2H, J= 8.4 Hz, H_5). ^{13}C NMR (150 MHz, $CDCl_3$): δ 47.50 (s, C_2), 66.55 (s, C_1), 113.40 (s, C_4), 118.57 (s, C_{12}), 120.75 (s, C_{15}), 120.76 (s, C_{16}), 120.98 (s, C_8), 127.87 (s, C_{10}), 128.85 (s, C_6), 129.91 (s, C_{11}), 130.03 (s, C_{17}), 130.56 (s, C_5), 142.39 (s, C_9), 154.16 (s, C_3), 154.92 (s, C_{14}), 158.85 (s, C_{13}), 187.95 (s, C_7). COSY (150 MHz, $CDCl_3$): H_1-H_2, H_4-H_5, H_8-H_9, H_{11}-H_{12}, H_{15}-H_{16}. HSQC (150 MHz, $CDCl_3$): The resonances were 3.34, 47.50 ppm (C_2-H_2); 3.87, 66.55 ppm (C_1-H_1); 6.92, 113.40 ppm (C_4-H_4); 6.99, 118.57, 120.75 (C_{12}-H_{12}), (C_{15}-H_{15}); 7.33, 120.76 ppm (C_{16}-H_{16}); 7.48, 120.98 ppm (C_8-H_8); 7.62, 129.91 ppm (C_{11}-H_{11}); 7.77, 142.39 ppm (C_9-H_9); 8.01, 130.56 ppm (C_5-H_5). HMBC (150

MHz, $CDCl_3$): The correlations were C_1-H_2; C_2-H_1; C_3-H_5; C_4-H_5; C_5-H_4,H_9; C_7 -H_5,H_8, H_9; C_8-H_9; C_9-H_{11}; C_{10}-H_{12}; C_{14}-H_{11},H_{16}; C_{15}-H_{16}; C_{16}-H_{15}; C_{17}-H_{15}. TOF/MS (m/z) = 420.1291 $[M+H]^+$. MS: m/z = $C_{25}H_{22}ClNO_3$ (419.1288); 421.18, 420.9, 257.9, 216, 204.2, 162, 165, 128.9, 111, 101, 99, 85, 75.1, 41 $[M+H]^+$. Anal. calc. for ($C_{25}H_{22}ClNO_3$): C, 71.51; H, 5.28; N, 3.34; found: C, 71.32; H, 5.22; N, 3.42.

3.1.4. (*E*)-3-[4-(4-Fluorophenoxy)phenyl-1-[4-(morpholin-4-yl)phenyl]prop-2-en-1-one (4)

Light yellow crystal, yield: 49%, mp: 162–164 °C. FT-IR νmax (cm^{-1}): 1647 (C=O); 1585, 1492, 1446 (C=C); 1185 (C-O-C morpholine); 1120 (C-N-C morpholine), 927 (ethylenic -CH=CH-). ^{1}H NMR (600 MHz, $CDCl_3$,): δ 3.33 (s, 4H, H_2), 3.87 (s, 4H, H_1), 6.91 (d, 2H, J= 7.8 Hz, H_4), 6.97 (d, 2H, J= 7.8 Hz, H_{12}), 7.07 (m, 4H, $H_{15,16}$), 7.47 (d, 1H, J= 15.6 Hz, H_8), 7.60 (d, 2H, J= 7.8 Hz, H_{11}), 7.77 (d, 1H, J= 15.6 Hz, H_9), 8,00 (d, 2H, J= 7.8 Hz, H_5). ^{13}C NMR (150 MHz, $CDCl_3$): δ 47.52 (s, C_2), 66.56 (s, C_1), 113.37 (s, C_4), 117.95 (s, C_{12}), 120.76 (s, C_8), 121.21 (s, C_{15}), 121.26 (s, C_{16}), 126.10 (s, C_6), 129.44 (s, C_{11}), 130.54 (s, C_5), 137.22 (s, C_{10}), 142.52 (s, C_9), 151.98 (s, C_{14}), 154.13 (s, C_3), 159.64 (s, C_{13}), 160.04 (s, C_{17}), 188 (s, C_7). COSY (150 MHz, $CDCl_3$): H_1-H_2, H_4-H_5, H_8-H_9, H_{11}-H_{12}, H_{15}-H_{16}. HSQC (150 MHz, $CDCl_3$): The resonances were 3.33, 47.52 ppm (C_2-H_2); 3.87, 66.56 ppm (C_1-H_1); 6.91, 113.37 ppm (C_4-H_4); 6.97, 117.95 ppm (C_{12}-H_{12}); 7.07, 121.21, 121.26 ppm (C_{15}-H_{15}), (C_{16}-H_{16}); 7.47, 120.76 ppm (C_8-H_8); 7.60, 129.44 ppm (C_{11}-H_{11}); 7.77, 142.52 ppm (C_9-H_9); 8.00, 130.54 ppm (C_5-H_5). HMBC (150 MHz, $CDCl_3$): The correlations were C_1-H_2; C_2-H_1; C_3-H_2, H_5; C_4-H_5; C_5-H_4; C_6-H_4; C_7-H_5,H_8,H_9; C_9-H_{11}; C_{10}-H_8; C_{12}-H_{11}; C_{13}-H_{11}; C_{14}-H_{15}; C_{17}-H_{16}. TOF/MS (m/z) = 404.1726 $[M+H]^+$. MS: m/z = $C_{25}H_{22}FNO_3$ (403.1584); 403.83, 317.6, 240.9, 215.9, 190, 165, 164, 111, 95, 89, 83, 75.1 $[M]^+$. Anal. calc. for ($C_{25}H_{22}FNO_3$): C, 74.43; H, 5.50; N, 3.47; found: C, 74.30; H, 5.63; N, 3.46.

3.1.5. (*E*)-3-[4-(4-Methoxyphenoxy)phenyl-1-[4-(morpholin-4-yl)phenyl]prop-2-en-1-one (5)

Light yellow crystal, yield: 33%, mp: 174–176 °C. FT-IR νmax (cm^{-1}): 1648 (C=O); 1588, 1571, 1496 (C=C); 1192 (C-O-C morpholine); 1122 (C-N-C morpholine), 925 (ethylenic -CH=CH-). ^{1}H NMR (600 MHz, $CDCl_3$,): δ 3.33 (s, 4H, H_2), 3.82 (s, 3H, H_{18}), 3.87 (s, 4H, H_1), 6.91 (d, 4H, J= 8.4 Hz, $H_{4,16}$), 6.92 (d, 2H, J= 6.6 Hz, H_{16}), 6.95 (d, 2H, J= 8.4 Hz, H_{12}), 7.01 (d, 2H, J= 8.4 Hz, H_{15}), 7.45 (d, 1H, J= 15.6 Hz, H_8), 7.58 (d, 2H, J= 8.4 Hz, H_{11}),7.76 (d, 1H, J= 15.6 Hz, H_9), 8,00 (d, 2H, J= 8.4 Hz, H_5). ^{13}C NMR (150 MHz, $CDCl_3$): δ 47.54 (s, C_2), 55.65 (s, C_{18}), 66.57 (s, C_1), 113.42 (s, C_4), 114.99 (s, C_{16}), 117.37 (s, C_{12}), 120.38 (s, C_8), 121.32 (s, C_{15}), 128.99 (s, C_6), 129.42 (s, C_{10}), 129.94 (s, C_{11}), 130.53 (s, C_5), 142.77 (s, C_9), 149.12 (s, C_{14}), 154.09 (s, C_3), 156.39 (s, C_{17}), 160.52 (s, C_{13}), 188.08 (s, C_7). COSY (150 MHz, $CDCl_3$): H_1-H_2, H_4-H_5, H_8-H_9, H_{11}-H_{12}, H_{15}-H_{16}. HSQC (150 MHz, $CDCl_3$): The resonances were 3.33, 47.53 ppm (C_2-H_2); 3.82, 55.65 ppm (C_1-H_1); 3.87, 66.57 ppm; 6.91 ppm, 113.42 ppm (C_4-H_4), 114.99; 6.95, 117.37 ppm (C_{12}-H_{12}); 7.01, 121.32 ppm (C_{15}-H_{15}); 7.45, 120.38 ppm (C_8-H_8); 7.58, 129.94 ppm (C_{11}-H_{11}); 7.76, 142.77 ppm (C_9-H_9); 8.00, 130.53 ppm (C_5-H_5). HMBC (150 MHz, $CDCl_3$): The correlations were C_1-H_2; C_2-H_1; C_3-H_5; C_4-H_5; C_5-H_4; C_6-H_4; C_7-H_5,H_8,H_9; C_8-H_9; C_9-H_{11}; C_{10}-H_9; C_{12}-H_{11}; C_{13}-H_{11}; C_{14}-H_{15}; C_{15}-H_{16}; C_{16}-H_{15}; C_{17}-H_{15}. TOF/MS (m/z) = 416.1995 $[M+H]^+$. MS: m/z = $C_{26}H_{25}NO_4$ (415.1784); 415.57, 252.9, 216, 189.9, 123, 117, 102, 95.1, 89, 77.1 $[M]^+$. Anal. calc. for ($C_{26}H_{25}NO_4$): C, 74.43; H, 5.50; N, 3.47; found: C, 75.07; H, 6.04; N, 3.49.

3.1.6. (*E*)-3-[4-(4-Methylphenoxy)phenyl-1-[4-(morpholin-4-yl)phenyl]prop-2-en-1-one (6)

Light yellow crystal, yield: 39%, mp: 194–196 °C. FT-IR νmax (cm^{-1}): 1645 (C=O); 1581, 1567, 1494 (C=C); 1218 (C-O-C morpholine); 1121 (C-N-C morpholine), 923 (ethylenic -CH=CH-). ^{1}H NMR (600 MHz, $CDCl_3$,): δ 2.36 (s, 3H, H_{18}), 3.32 (s, 4H, H_2), 3.86 (s, 4H, H_1), 6.91 (d, 2H, J= 8.4 Hz, H_4), 6.96 (d, 2H, J= 9.6 Hz, H_{12}), 6.98 (d, 2H, J= 9 Hz, H_{15}), 7.17 (d, 2H, J= 7.8 Hz, H_{16}), 7.46 (d, 1H, J= 15.6 Hz, H_8), 7.59 (d, 2H, J= 8.4 Hz, H_{11}), 7.77 (d, 1H, J= 15.6 Hz, H_9), 8.00 (d, 2H, J= 8.4 Hz, H_5). ^{13}C NMR (150 MHz, $CDCl_3$): δ 20.76 (s, C_{18}), 47.53 (s, C_2), 66.57 (s, C_1), 113.42 (s, C_4), 117.98 (s, C_{15}), 119.74 (s, C_{12}), 120.50 (s, C_8), 128.97 (s, C_6), 129.69 (s, C_{11}), 129.94 (s, C_{10}), 130.40 (s, C_5), 130.54 (s, C_{16}), 133.79 (s, C_{17}), 142.73 (s, C_9), 153.67 (s, C_{14}), 154.10 (s, C_3), 159.92 (s, C_{13}), 188.06 (s, C_7). COSY (150 MHz, $CDCl_3$): H_1-H_2, H_4-H_5, H_8-H_9, H_{11}-H_{12}, H_{15}-H_{16}. HSQC (150 MHz, $CDCl_3$): The resonances were 3.32, 47.53 ppm (C_2-H_2); 3.86, 66.57 ppm (C_1-H_1); 6.91, 113.42 ppm (C_4-H_4); 6.96, 119.74 ppm (C_{12}-H_{12}); 6.98, 117.98 ppm (C_{15}-H_{15}); 7.17, 130.54 ppm (C_{16}-H_{16}); 7.46, 120.50 ppm (C_8-H_8); 7.59, 129.69 ppm (C_{11}-H_{11}); 7.77, 142.73 ppm (C_9-H_9); 8.00, 130.40 ppm (C_5-H_5). HMBC (150 MHz, $CDCl_3$): The correlations were C_1-H_2; C_2-H_1; C_3-H_5; C_4-H_5; C_5-H_4; C_7-H_5, H_8, H_9; C_8-H_9; C_9-H_{11}; C_{10}-H_9,H_{12}; C_{13}-H_{12},H_{15}; C_{14}-H_{16}; C_{17}-H_{15}; C_{18}-H_{16}. TOF/MS (m/z) = 400.1932 $[M+H]^+$. MS: m/z = $C_{26}H_{25}NO_3$ (399.1834); 399.70, 237.1, 215.6, 190, 181, 117, 107, 91.1, 89, 77.1, 65.1 $[M]^+$. Anal. calc. for ($C_{26}H_{25}NO_3$): C, 78.17; H, 6.31; N, 3.51; found: C, 77.41; H, 6.23; N, 3.59.

3.1.7. (*E*)-3-[4-(4-Nitrophenoxy)phenyl-1-[4-(morpholin-4-yl)phenyl]prop-2-en-1-one (7)

Light yellow crystal, yield: 62%, mp: 180–182 °C. FT-IR νmax (cm^{-1}): 1644 (C=O); 1583, 1505, 1487 (C=C); 1193 (C-O-C morpholine); 1110 (C-N-C morpholine), 927 (ethylenic -CH=CH-). ^{1}H NMR (600 MHz, $CDCl_3$,): δ 3.31 (s, 4H, H_2), 3.73 (s, 4H, H_1), 7.01 (d, 2H,J= 8.4 Hz, H_4), 7.19 (d, 2H, J= 7.8 Hz, H_{15}), 7.23 (d, 2H, J= 7.8 Hz, H_{12}), 7.68 (d, 1H, J= 15.6 Hz, H_8), 7.98 (d, 2H, J= 8.4 Hz, H_{11}), 7.78 (d, 1H, J= 15.6 Hz, H_9), 8.06 (d, 2H, J= 8.4 Hz, H_5), 8.26 (d, 2H, J= 8.4 Hz, H_{16}). ^{13}C NMR (150 MHz, $CDCl_3$): δ 47.45 (s, C_2), 66.54 (s, C_1), 113.38 (s, C_4), 117.72 (s, C_{15}), 120.51 (s, C_{12}), 121.97 (s, C_8), 126 (s, C_{16}), 128.63 (s, C_6), 130.26 (s, C_{11}), 130.62 (s, C_5), 132.39 (s, C_{10}), 141.82 (s, C_9), 143.09 (s, C_{17}), 154.23 (s, C_3), 156.35 (s, C_{13}), 162.49 (s, C_{14}), 187.75 (s, C_7). COSY (150 MHz, $CDCl_3$): H_1-H_2, H_4-H_5, H_8-H_9, H_{11}-H_{12}, H_{15}-H_{16}. HSQC (150 MHz, $CDCl_3$): The resonances were 3.31, 47.45 ppm (C_2-H_2); 3.73, 66.54 ppm (C_1-H_1); 7.01, 113.38 ppm (C_4-H_4); 7.19, 117.72 ppm (C_{15}-15; 7.23, 120.51 ppm (C_{12}-H_{12}); 7.68, 121.97 ppm (C_8-H_8); 7.90, 141.82 ppm (C_9-H_9); 7.98, 130.26 ppm (C_{11}-H_{11}); 8.06, 130.62 ppm (C_5-H_5); 8.26, 126 ppm (C_{16}-H_{16}). HMBC (150 MHz, $CDCl_3$): The correlations were C_1-H_2; C_2-H_1; C_5-H_4; C_7-H_5,H_8,H_9; C_9-H_{11}; C_{12}-H_{11}; C_{13}-H_{11},H_{12}; C_{14}-H_{16},H_{15}; C_{17}-H_{15},H_{16}. TOF/MS (m/z) = 431.1747 $[M+H]^+$. MS: m/z = $C_{25}H_{22}N_2O_5$ (430.1529); 430.77, 268, 239.9, 221.9, 216.9, 193.9, 190, 186.8, 164.9, 126.9, 112.9, 103.2, 102.1, 97, 76.2, 65 $[M]^+$. Anal. calc. for ($C_{26}H_{25}NO_4$): C, 69.76; H, 5.15; N, 6.51; found: C, 69.30; H, 4.88; N, 6.61.

3.2. Biological activity

3.2.1. Cell culture and cell proliferation assay

HeLa and C6 cell lines in the cell culture conditions and antiproliferative activity in the experimental procedure were tested as described in the literature.[34,35] Cell proliferation was measured by using BrdU Cell Proliferation

ELISA according to the manufacturer's procedure.[34,35] The percentage of inhibition of cell proliferation was calculated as follows:

$$[1 - (A_{\text{treatments}}/A_{\text{vehicle control}})] \times 100.$$

IC_{50} was calculated by ED50 plus v1.0 and the results were given as means ± SD of six values. $P < 0.01$ was considered statistically significant. The statistical analysis was performed using SPSS 13.5.

3.2.2. Enzyme assays

Carbonic anhydrase activities were determined according to the electrometric methods described by Wilbur and Anderson following the pH change from 8.3 to 6.3.[36] PKM2 activity was measured by spectrophotometry at 340 nm using a quartz cuvette with a 1-cm path length.[37] Stock solutions of derivatives were prepared in DMSO (1 mg/mL) and diluted using distilled water for PKM2. To evaluate the effects on the enzyme activities of the compounds, hCA and PKM2 activities were tested with at least five different concentrations of each derivative and a constant concentration of substrates and coenzymes at 25 °C. IC_{50} or AC_{50} values were defined as the substance concentration that caused about a 50% reduction or increase in enzyme activities compared to the control enzyme activities without derivatives.[38] Acetazolamide in the carbonic anhydrase inhibitor studies and myricetin in the PKM2 activator studies were used as positive controls.

Acknowledgments

This work was supported by the Gaziantep University Scientific Research Projects Governing Unit (BAPYB) (Grant No: FEF.14.01, Gaziantep, Turkey). The authors are grateful to acknowledge Çankırı Karatekin University for supporting the NMR studies.

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Supplementary Table 1. NMR data in $CDCl_3$. Chemical shifts (d, ppm) and coupling constants (J, Hz) for compound **2**.

Carbon number	DEPT	Chemical shifts (ppm)		^{1}H-^{13}C HMBC	^{1}H-^{1}H COSY
		^{13}C	^{1}H		
C1	CH2	66.57	3.86 (s, 4H)	2	2
C2	CH2	47.53	3.33 (s, 4H)	1	1
C3	C	154.12		5	
C4	CH	113.41	6.91 (d, 2H, *J* = 7.2 Hz)	5	5
C5	CH	130.55	8.01 (d, 2H, *J* = 7.8 Hz)	4	4
C6	C	128.93			
C7	C	188.03		5, 8, 9	
C8	CH	120.70	7.48 (d, H, *J* = 15.6 Hz)	9	9
C9	CH	142.63	7.77 (d, H, *J* = 15.6 Hz)	11	8
C10	C	130.07		8	
C11	CH	129.97	7.61 (d, 2H, *J* = 8.4 Hz)	9, 12	12
C12	CH	118.47	7.01 (d, 2H, *J* = 7.2 Hz)	11	11
C13	C	159.37		12	
C14	C	156.20		15, 16	
C15	CH	119.61	7.06 (d, 2H, *J* = 7.2 Hz)		16
C16	CH	129.91	7.38 (t, 1H, *J* = 7.2 Hz)	15	15
C17	C	124.04	7.16 (t, 1H, *J* = 7.2 Hz)	15	

Supplementary Table 2. NMR data in $CDCl_3$. Chemical shifts (d, ppm) and coupling constants (J, Hz) for compound **3**.

Carbon number	DEPT	Chemical shifts (ppm)		^{1}H-^{13}C HMBC	^{1}H-^{1}H COSY
		^{13}C	^{1}H		
C1	CH2	66.55	3.87 (s, 4H)	2	2
C2	CH2	47.50	3.34 (s, 4H)	1	1
C3	C	154.16		5	
C4	CH	113.40	6.92 (d, 2H, *J* = 8.4 Hz)	5	5
C5	CH	130.56	8.01 (d, 2H, *J* = 8.4 Hz)	4, 9	4
C6	C	128.85			
C7	C	187.95		5, 8, 9	
C8	CH	120.98	7.48 (d, H, *J* = 15.6 Hz)	9	9
C9	CH	142.39	7.77 (d, H, *J* = 15.6 Hz)	11	8
C10	C	127.87		12	
C11	CH	129.91	7.62 (d, 2H, *J* = 7.8 Hz)		12
C12	CH	118.57	6.99 (t, 4H, *J* = 7.2 Hz)		11
C13	C	158.85			
C14	C	154.92		11, 16	
C15	CH	120.75	6.99 (t, 4H, *J* = 7.2 Hz)	16	16
C16	CH	120.76	7.33 (d, 2H, *J* = 7.8 Hz)	15	15
C17	CH	130.03		15	

Supplementary Table 3. NMR data in $CDCl_3$. Chemical shifts (d, ppm) and coupling constants (J, Hz) for compound **4**.

Carbon number	DEPT	Chemical shifts (ppm)		^{1}H-^{13}C HMBC	^{1}H-^{1}H COSY
		^{13}C	^{1}H		
C1	CH2	66.56	3.87 (t, 4H, *J* = 4.8 Hz)	2	2
C2	CH2	47.52	3.33 (t, 4H, *J* = 4.8 Hz)	1	1
C3	C	154.13		2, 5	
C4	CH	113.37	6.91 (d, 2H, *J* = 7.8 Hz)	5	5
C5	CH	130.54	8 (d, 2H, *J* = 7.8 Hz)	4	4
C6	C	126.10		4	
C7	C	188		5, 8, 9	
C8	CH	120.76	7.47 (d, H, *J* = 15.6 Hz)		9
C9	CH	142.52	7.77 (d, H, *J* = 15.6 Hz)	11	8
C10	C	137.22		8	
C11	CH	129.44	7.60 (d, 2H, *J* = 7.8 Hz)		12
C12	CH	117.95	6.97 (d, 2H, *J* = 7.8 Hz)	11	11
C13	C	159.64		11	
C14	C	151.98		15	
C15	CH	121.21	7.07 (m, 4H)		16
C16	CH	121.26	7.07 (m, 4H)		15
C17	CH	160.04		16	

Supplementary Table 4. NMR data in $CDCl_3$. Chemical shifts (d, ppm) and coupling constants (J, Hz) for compound **5**.

Carbon number	DEPT	Chemical shifts (ppm)		^{1}H-^{13}C HMBC	^{1}H-^{1}H COSY
		^{13}C	^{1}H		
C1	CH2	66.57	3.87 (s, 4H)	2	2
C2	CH2	47.53	3.33 (s, 4H)	1	1
C3	C	154.09		5	
C4	CH	113.42	6.91 (d, 4H, *J* = 8.4 Hz)	5	5
C5	CH	130.53	8 (d, 2H, *J* = 8.4 Hz)	4	4
C6	C	128.99		4	
C7	C	188.08		5, 8, 9	
C8	CH	120.38	7.45 (d, H, *J* = 15.6 Hz)	9	9
C9	CH	142.77	7.76 (d, H, *J* = 15.6 Hz)	11	10
C10	C	129.42		9	
C11	CH	129.94	7.58 (d, 2H, *J* = 8.4 Hz)		12
C12	CH	117.37	6.95 (d, 2H, *J* = 8.4 Hz)	11	11
C13	C	160.52		11	
C14	C	149.12		15	
C15	CH	121.32	7.01 (d, 2H, *J* = 8.4 Hz)	16	16
C16	CH	114.99	6.91 (d, 4H, *J* = 8.4 Hz)	15	15
C17	C	156.39		15	
C18	CH3	55.65	3.82 (s, 3H)		

Supplementary Table 5. NMR data in $CDCl_3$. Chemical shifts (d, ppm) and coupling constants (J, Hz) for compound **6**.

Carbon number	DEPT	Chemical shifts (ppm)		^{1}H-^{13}C HMBC	^{1}H-^{1}H COSY
		^{13}C	^{1}H		
C1	CH2	66.57	3.86 (s, 4H)	2	2
C2	CH2	47.53	3.32 (s, 4H)	1	1
C3	C	154.10		5	
C4	CH	113.42	6.91 (d, 2H, *J* = 8.4 Hz)	5	5
C5	CH	130.40	8 (d, 2H, *J* = 8.4 Hz)	4	4
C6	C	128.97			
C7	C	188.06		5, 8, 9	
C8	CH	120.50	7.46 (d, H, *J* = 15.6 Hz)	9	9
C9	CH	142.73	7.77 (d, H, *J* = 15.6 Hz)	12	10
C10	C	129.94		9, 12	
C11	CH	129.69	7.59 (d, 2H, *J* = 8.4 Hz)		12
C12	CH	119.74	6.96 (d, 2H, *J* = 9.6 Hz)		11
C13	C	159.92		12, 15	
C14	C	153.67		16	
C15	CH	117.98	6.98 (d, 2H, *J* = 9 Hz)		16
C16	CH	130.54	7.17 (d, 2H, *J* = 7.8 Hz)		15
C17	C	133.79		15	
C18	CH3	20.76	2.36 (s, 3H)	16	

Supplementary Table 6. NMR data in $CDCl_3$. Chemical shifts (d, ppm) and coupling constants (J, Hz) for compound **7**.

Carbon number	DEPT	Chemical shifts (ppm)		^{1}H-^{13}C HMBC	^{1}H-^{1}H COSY
		^{13}C	^{1}H		
C1	CH2	66.54	3.73 (s, 4H)	2	2
C2	CH2	47.45	3.31 (s, 4H)	1	1
C3	C	154.23		2, 5	
C4	CH	113.38	7.01 (d, 2H, *J* = 8.4 Hz)		5
C5	CH	130.62	8.06 (d, 2H, *J* = 8.4 Hz)	4	4
C6	C	128.63		4	
C7	C	187.75		5, 8, 9	
C8	CH	121.97	7.68 (d, H, *J* = 15.6 Hz)		9
C9	CH	141.82	7.90 (d, H, *J* = 15.6 Hz)	11	8
C10	C	132.39			
C11	CH	130.26	7.98 (d, 2H, *J* = 8.4 Hz)		12
C12	CH	120.51	7.23 (d, 2H, *J* = 7.8 Hz)	11	11
C13	C	156.35		11, 12	
C14	C	162.39		15, 16	
C15	CH	117.72	7.19 (d, 2H, *J* = 7.8 Hz)		16
C16	CH	126	8.26 (d, 2H, *J* = 8.4 Hz)		15
C17	CH	143.09		15, 16	

Supplementary Figure 1. ^{1}H NMR spectrum of compound **2**.

Supplementary Figure 2. ^{13}C NMR spectrum of compound **2**.

Supplementary Figure 3. COSY NMR spectrum of compound **2**.

Supplementary Figure 4. HQSY NMR spectrum of compound **2**.

Supplementary Figure 5. HMBC NMR spectrum of compound **2**.

Supplementary Figure 6. ^{1}H NMR spectrum of compound **3**.

Supplementary Figure 7. ^{13}C NMR spectrum of compound **3**.

Supplementary Figure 8. COSY NMR spectrum of compound **3**.

Supplementary Figure 9. HSQC NMR spectrum of compound **3**.

Supplementary Figure 10. HMBC NMR spectrum of compound **3**.

Supplementary Figure 11. ^{1}H NMR spectrum of compound **4**.

Supplementary Figure 12. ^{13}C NMR spectrum of compound **4**.

Supplementary Figure 13. COSY NMR spectrum of compound **4**.

Supplementary Figure 14. HSQC NMR spectrum of compound **4**.

Supplementary Figure 15. HMBC NMR spectrum of compound **4**.

Supplementary Figure 16. ^{1}H NMR spectrum of compound **5**.

Supplementary Figure 17. ^{13}C NMR spectrum of compound **5**.

Supplementary Figure 18. COSY NMR spectrum of compound **5**.

Supplementary Figure 19. HSQC NMR spectrum of compound **5**.

Supplementary Figure 20. HMBC NMR spectrum of compound **5**.

Supplementary Figure 21. ^{1}H NMR spectrum of compound **6**.

Supplementary Figure 22. ^{13}C NMR spectrum of compound **6**.

Supplementary Figure 23. COSY NMR spectrum of compound **6**.

Supplementary Figure 24. HSQC NMR spectrum of compound **6**.

Supplementary Figure 25. HMBC NMR spectrum of compound **6**.

Supplementary Figure 26. ^{1}H NMR spectrum of compound **7**.

Supplementary Figure 27. ^{13}C NMR spectrum of compound **7**.