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New heterocyclic chalcones. Part 6. Synthesis and cytotoxic activities of 5- or 6-(3-aryl-2-propenoyl)-2(3*H*)-benzoxazolones

Abstract: A number of chalcones bearing an oxazole cycle were synthesized by Claisen-Schmidt condensation of 5-acetyl-2(3*H*)-benzoxazolone or 6-acetyl-2(3*H*)-benzoxazolone and the appropriate aldehydes. The chalcones were evaluated for cytotoxic activity against several tumor cell lines – BV-173 (human B cell precursor leukemia), MCF-7 and MDA-MB-231 (human breast adenocarcinoma) using the MTT-dye reduction assay. The tested compounds exhibit concentration-dependent cytotoxic effects at micromolar concentrations. Exposure of the BV-173 tumor cell line to compound **3f** results in strong mono- and oligonucleosomal fragmentation of genomic DNA, as evidenced by a 'cell death detection' ELISA kit, which unambiguously indicates that the induction of apoptosis is implicated in the cytotoxic mode of action of the tested compound.

Keywords: apoptosis; 2(3*H*)-benzoxazolone; chalcone; cytotoxicity; MTT.

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Introduction

Natural and synthetic chalcones have become important building blocks in medicinal chemistry and a number of derivatives endowed with anti-inflammatory, antimicrobial, antifungal, antioxidant, cytotoxic, antitumor, anticancer and chemopreventive effects have been synthesized [1–7]. These compounds are important intermediates for the synthesis of heterocyclic systems and play a role in organic syntheses as Michael acceptors [8].

Previous studies have indicated that chalcones and their derivatives demonstrate anticancer activity in various tumor cells – ovarian cancer cells [9], gastric cancer HGC-27 cells [10], HepG2 hepatocellular carcinoma cells [12], human melanoma cells A375 [13], KB human buccal carcinoma cells [14], PC-3 prostate cells, MCF-7 breast cells and KB nasopharyngeal cancer cells [15].

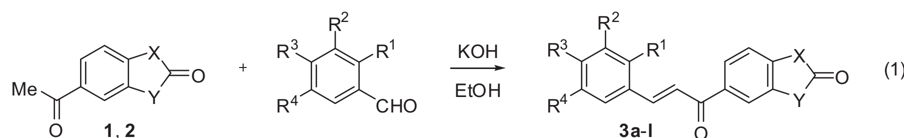
Our research group is interested in the synthesis and investigation of biological effects of chalcones. Recently, we have reported the synthesis of some chalcones substituted with a 2(3*H*)-benzoxazolone moiety that have showed good to excellent cytotoxic activity [16–18]. Our studies with BV-173 leukemic cells have suggested that the cytotoxic effect of these agents is at least partly mediated by induction of apoptotic cell death. We have also investigated the influence of an appended thiazole system in the chalcone scaffold [19] on the cytotoxic activity against the human chronic lymphoid leukemia SKW-3 tumor cells. In this report, we further extended our research on oxazole-bearing chalcone derivatives and evaluated their cytotoxic activity.

Results and discussion

Synthesis

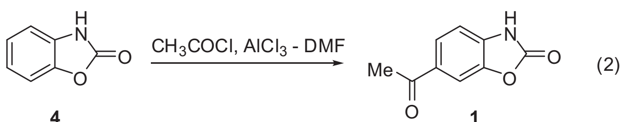
New 5- and 6-(3-aryl-2-propenoyl)-2(3*H*)-benzoxazolones **3a–l** (Equation 1) were prepared by treating 6-acetyl-2(3*H*)-benzoxazolone (**1**) or 5-acetyl-2(3*H*)-benzoxazolone (**2**) with different methoxy-substituted benzaldehydes. The general synthetic strategy employed to prepare the chalcones (**3a–l**) was based on Claisen-Schmidt condensation.

The reaction was performed under standard conditions by maintaining the mixture in aqueous ethanolic solution containing 10% KOH at room temperature for 24 h. The structures of the new compounds were confirmed by IR, ^{1}H NMR, ^{13}C NMR and elemental analysis. In particular, analysis of ^{1}H NMR spectra revealed that all structures are geometrically pure with the *E* configuration, as derived from coupling constant J = 15.6 Hz for vinyl protons.



1: X = NH; Y = O
2: X = O; Y = NH
3a: X = NH; Y = O; $R^1 = OCH_3$; $R^2 = R^3 = R^4 = H$
3b: X = NH; Y = O; $R^1 = H$; $R^2 = OMe$; $R^3 = R^4 = H$
3c: X = NH; Y = O; $R^1 = R^2 = H$; $R^3 = OMe$; $R^4 = H$
3d: X = NH; Y = O; $R^1 = OMe$; $R^2 = H$; $R^3 = OMe$; $R^4 = H$
3e: X = NH; Y = O; $R^1 = H$; $R^2 = R^3 = OMe$; $R^4 = H$
3f: X = NH; Y = O; $R^1 = H$; $R^2 = R^3 = R^4 = OMe$
3g: X = O, Y = NH; $R^1 = OMe$; $R^2 = R^3 = R^4 = H$
3h: X = O; Y = NH; $R^1 = H$; $R^2 = OMe$; $R^3 = R^4 = H$
3i: X = O; Y = NH; $R^1 = R^2 = H$; $R^3 = OMe$; $R^4 = H$
3j: X = O; Y = NH; $R^1 = OMe$; $R^2 = H$; $R^3 = OMe$; $R^4 = H$
3k: X = O; Y = NH; $R^1 = H$; $R^2 = R^3 = OMe$; $R^4 = H$
3l: X = O; Y = NH; $R^1 = H$; $R^2 = R^3 = R^4 = OMe$

The lactam and ketone C=O stretching bands in the IR spectra are seen at approximately 1760 cm^{-1} and 1650 cm^{-1}, respectively.



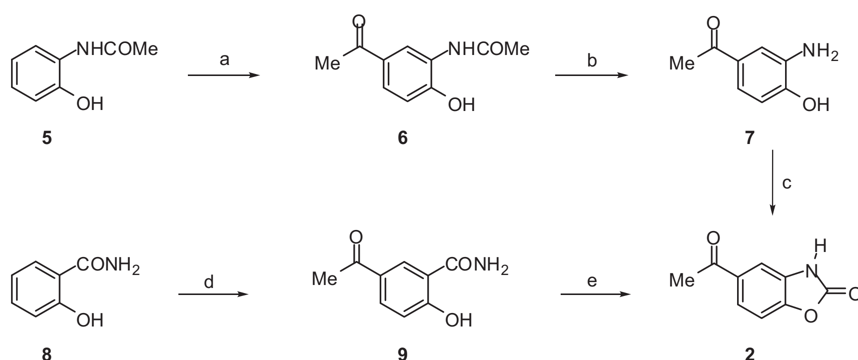
We aimed to synthesize 2(3*H*)-benzoxazolone derivatives bearing 3-phenyl-2-propenoyl substituents in positions 5 or 6, in order to study the influence of these functionalities on cytotoxicity. To meet this objective, the starting ketones were synthesized using different synthetic routes. As indicated in Equation 2, 6-acetyl-2(3*H*)-benzoxazolone (**1**) was synthesized by direct acetylation of 2(3*H*)-benzoxazolone (**4**) with acetyl chloride in the presence of the $AlCl_3$-DMF complex [16]. The product was obtained in good yield and purity. Because the *C*-acylation of 2(3*H*)-benzoxazolone is regioselective and always leads to a 6-acyl derivative, it was necessary to use another synthetic route for preparation of 5-acetyl-2(3*H*)-benzoxazolone (**2**). Aichaoui et al. [20] suggested a three-step synthesis for converting 2-acetamidophenol (**5**) to 5-acetyl-2(3*H*)-benzoxazolone (**2**), as shown in method A of Scheme 1. Following this procedure to prepare the starting ketone we modified the last step. Thus, the cyclization of 2-aminophenol to 2(3*H*)-benzoxazolone was conducted under mild conditions and with a higher yield than that reported by using 1,1′-carbonyldiimidazole [21].

Amide, azide and hydroxamic acid derivatives of salicylic acid can be used for the synthesis of 2(3*H*)-benzoxazolones by Hofmann, Curtius and Lossen rearrangements. For the preparation of 5-acetyl-2(3*H*)-benzoxazolone (**2**), we developed an alternative two-step synthetic route using Hofmann rearrangement of 5-acetylsalicylamide (**9**) as shown in method B of Scheme 1. Commercially available salicylamide (**8**) was acetylated with acetyl chloride by the Friedel-Crafts method to obtain 5-acetylsalicylamide (**9**) exclusively. The product was isolated with high yield and purity. In this work, the Hofmann-type rearrangement of **9** to **2** was carried out in the presence of $C_6H_5I(OAc)_2$. The use of this hypervalent iodine reagent gave the desired product **2** in an 89% yield. The mechanism of this reaction apparently involves generation of an isocyanate from the amide function followed by an intramolecular nucleophilic attack on the isocyanate of the adjacent hydroxyl group. The advantages of method B are high total yield (89%), a two-step synthesis and the use of only 3 equiv of $AlCl_3$. By contrast, method A is less efficient (40%), involves three steps and requires the use of 8 equiv of $AlCl_3$.

Cytotoxic activity

The cytotoxic effects of compounds **3a–l** were examined against a panel of human tumor cell lines, namely BV-173 (chronic myeloid leukemia), MCF-7 (estrogen receptor-positive breast cancer) and MDA-MB-231 (estrogen receptor-negative breast cancer) following a 72-h exposure. The activity was assessed by the MTT-dye reduction assay as described by Mosmann, with minor modifications [22]. All compounds exhibited concentration-dependent cytotoxicity which enabled the construction of the concentration-response curves and the calculation of the corresponding IC_{50} values (Table 1). The clinically utilized anticancer drug cisplatin was used as a positive control throughout the cytotoxicity determination studies.

The data indicate that almost all compounds display certain cytotoxic activity against the three tumor cells, whereby invariably BV-173 cells are more sensitive, as compared with the breast cancer-derived cell lines. Within



Scheme 1 Method A: (a) MeCOCl, $AlCl_3$-DMF; (b) conc. HCl; (c) 1,1′-carbonyldiimidazole, THF; Method B: (d) MeCOCl, $AlCl_3$, $MeNO_2$, CH_2Cl_2; (e) $C_6H_5I(OCOMe)_2$, KOH, MeOH.

Table 1 Cytotoxic effects of compounds **3a–l** against the panel of human tumor cell lines as assessed by the MTT-dye reduction assay after 72-h exposure.

Compound	IC_{50} value (μM)±SD		
	BV-173	MCF-7	MDA-MB-231
3a	22.9±1.1	38.3±2.1	27.9±1.4
3b	151.9±11.1	288.9±12.0	192.5±10.2
3c	9.3±1.4	144.7±6.5	92.2±2.9
3d	27.4±1.8	282.9±11.9	179.3±5.1
3e	28.7±1.4	67.7±4.2	45.7±3.1
3f	8.3±1.5	131.2±5.1	15.5±1.1
3g	10.5±1.3	25.9±1.9	21.7±1.3
3h	12.1±2.7	41.2±2.2	33.8±1.8
3i	58.8±3.9	400	262.9±7.3
3j	71.1±2.4	204.2±9.8	38.9±2.6
3k	11.7±1.2	42.7±1.9	142.4±4.4
3l	4.9±0.7	34.8±1.9	16.7±0.9
Cisplatin	7.6±1.7	8.7±2.1	7.9±1.8

the BV-173 bioassay in both series of compounds, 3,4,5-trimethoxy analogs **3f** and **3l** displayed superior activity, causing 50% inhibition of cellular viability at low micromolar concentrations. Our data demonstrate that chalcone **3l** exerts the most pronounced cytotoxic activity with an IC_{50} value of 4.9 μM, whereas an IC_{50} value for cisplatin is 7.6 μM under similar conditions. This compound is also relatively active against the breast cancer cell lines. Among the compounds bearing one methoxy group in ring B, the *ortho*-position gives promising cytotoxic activity against MDA-MB-231 and MCF-7 cell lines. Derivatives with the *meta*-methoxy group show significantly less activity in the series of 6-substituted 2(3*H*)-benzoxazolones. This finding is in contrast within activity of the 5-substituted 2(3*H*)-benzoxazolone derivatives, where chalcone **3i** bearing the methoxy group in *para*-position shows diminished activity. Introduction of an additional methoxy group in B-ring imparts insignificant modulation of the biological activity. In general, the greatest chemosensitivity to the tested series was established in BV-173 cells followed by the two breast cancer cell lines MDA-MB-231 > MCF-7.

To elucidate the mechanisms underlying the established cytotoxicity, we investigated the level of apoptotic fragmentation of genomic DNA, using a commercially available ‘cell death detection’ ELISA kit. This method allows semiquantitative determination of the histone-associated mono- and oligonucleosomal DNA fragments using ‘sandwich’ ELISA. The clinically applied anticancer drug cisplatin was used as a reference compound.

As indicated in Figure 1, chalcone **3f** treatment of BV-173 cells, even in concentrations lower than its IC_{50} value, leads to a significant elevation of the enrichment factor (corresponding to the level of histone-associated DNA fragments). The established proapoptotic activity is

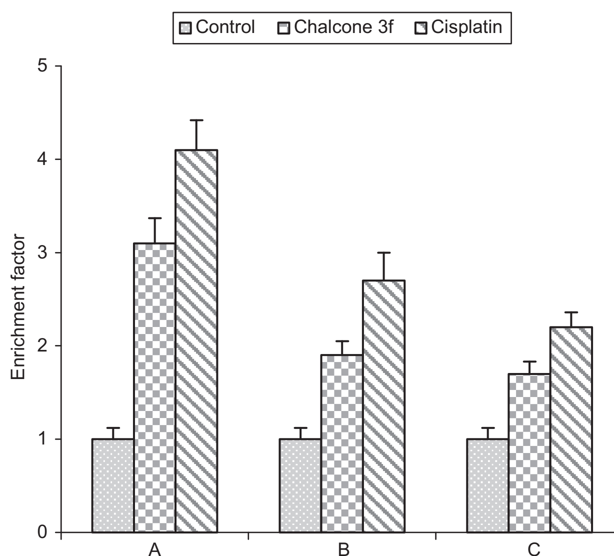


Figure 1 Increase in the levels of histone-associated DNA fragments (expressed as enrichment factor), following 24 h chalcone **3f** treatment in BV-173 [at IC_{50} (plot A), ½ IC_{50} μM (plot B), and ¼ IC_{50} (plot C)] as assessed by the ‘cell death detection’ ELISA.

comparable to that of the reference drug cisplatin. These findings suggest that cytotoxicity of these agents is mediated by induction of cell death through apoptosis, as already demonstrated for a series of previously described cytotoxic chalcones [23, 24].

Conclusions

Although the precise mode of action of tested compounds is yet to be determined, a possible mechanism may involve inhibition of tubulin polymerization, as firmly established for diverse structurally related chalcones. The SAR investigations failed to indicate the optimal substitution patterns governing cytotoxic potencies and it appears that compounds with multiple methoxy groups are characterized by superior activity.

Experimental section

All research chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solvents were dried and purified according to literature procedures, as necessary. Reactions were monitored by thin layer chromatography (TLC) on precoated silica gel plates from E. Merck (Darmstadt, Germany). Melting points were determined on a Boetius hot-stage microscope and were uncorrected. IR spectra (Nujol) were recorded on a Specord 71 spectrometer. ^{1}H NMR spectra were recorded in DMSO-d_6 on a Bruker DRX 250 operating at 250 MHz. ^{13}C NMR spectra were recorded in DMSO-d_6 at 62.5 MHz.

Synthesis of 5-acetyl-2(3*H*)-benzoxazolone (2)

A mixture of dichloromethane (25 mL), nitromethane (5 mL) and aluminum chloride (9.98 g, 75 mmol) was stirred briefly, and after cooling to 0°C, was treated with salicylamide (3.43 g, 250 mmol). Acetyl chloride (3.93 g, 50 mmol) was added dropwise with stirring after the suspension became a clear solution. The mixture was stirred at room temperature for an additional 4–5 h, and then poured on ice (100 g) with conc. HCl (20 mL). The crude 5-acetylsalicylamide (**9**) was filtered, washed with water, dried and crystallized from ethanol; yield 4.27 g (95%); mp 216–217°C.

To a solution of KOH (1.32 g, 20 mmol) in methanol (20 mL), 5-acetylsalicylamide (1.8 g, 10 mmol) was added. The resulting suspension was cooled to 0°C and iodobenzene diacetate (3.22 g, 10 mmol) was added within 5 min. The mixture was stirred for 1 h and acidified with 10% HCl. Water (20 mL) and petroleum ether (20 mL) were then added and the mixture stirred for 10 min. The product was filtered and washed with water and petroleum ether, and crystallized from ethanol; yield 1.58 g (89%); mp 230–232°C; IR: 3100–3300 (NH), 1780, 1660 (CO) cm^{-1}; ^{1}H NMR: δ 2.57 (s, 3H, CH_3), 7.37 (d, 1H, arom H, J = 8.4 Hz), 7.57 (d, 1H, arom H, J = 1.8 Hz), 7.78 (dd, 1H, arom H, J = 1.8 Hz, J = 8.4 Hz), 11.8 (br s, 1H, NH).

General procedure for synthesis of chalcones 3a–l

To a solution of 5- or 6-acetyl-2(3*H*)-benzoxazolone [20] (2 mmol) in a mixture of 10% aq. KOH (2 mL) and ethanol (3 mL), aldehyde (2.2 mmol) was added. After stirring for 24 h at room temperature, the mixture precipitated, was poured on 30 mL water, warmed and acidified with 10% HCl. The crystalline product was filtered, washed to neutrality and dried.

6-[3-(2-Methoxyphenyl)-2-propenoyl]-2(3*H*)-benzoxazolone (3a) Yield 98%; mp 212–215°C; IR: 1779, 1643 (C=O) cm^{-1}; ^{1}H NMR: δ 3.90 (s, 3H, OCH_3); 7.04 (m, 1H, arom H); 7.12 (d, 1H, arom H, J = 8.0 Hz); 7.24 (d, 1H, arom H, J = 8.0 Hz); 7.43–7.48 (m, 1H, arom H); 7.91 (d 1H, =CHCO, J = 15.7 Hz); 8.00–8.08 (m, 4H, ArCH=, arom H); 12.09 (br s, 1H, NH); ^{13}C NMR: 55.7, 109.3, 109.5, 111.7, 120.6, 121.4, 122.9, 125.6, 128.3, 131.9, 132.3, 134.9, 138.1, 143.5, 154.5, 158.2, 187.4. Anal. Calcd for $C_{17}H_{13}NO_4$: C, 69.15; H, 4.44; N, 4.74. Found: C, 69.40; H, 4.54; N, 4.72.

6-[3-(3-Methoxyphenyl)-2-propenoyl]-2(3*H*)-benzoxazolone (3b) Yield 98%; mp 229–232°C; IR: 1743, 1657 (C=O) cm^{-1}; ^{1}H NMR: δ 3.84 (s, 3H, OCH_3); 7.03 (m, 1H, arom H); 7.24 (d, 1H, arom H, J = 8.2 Hz); 7.42 (m, 3H, arom H); 7.72 (d, 1H, =CHCO, J = 15.5 Hz); 7.99 (d, 1H, ArCH=, J = 15.5 Hz); 8.08 (dd, 1H, arom H, J_1 = 8.2 Hz, J_2 = 1.6 Hz); 8.13 (d, 1H, arom H, J = 1.6 Hz); 12.10 (br s, 1H, NH); ^{13}C NMR: 55.2, 109.5, 109.5, 113.3, 116.6, 121.7, 121.9, 125.7, 129.8, 131.7, 134.9, 136.1, 143.4, 143.7, 154.4, 159.6, 187.2. Anal. Calcd for $C_{17}H_{13}NO_4$: C, 69.15; H, 4.44; N, 4.74. Found: C, 68.76; H, 4.70; N, 4.77.

6-[3-(4-Methoxyphenyl)-2-propenoyl]-2(3*H*)-benzoxazolone (3c) Yield 89%; mp 209–211°C; IR: 1760, 1645 (C=O) cm^{-1}; ^{1}H NMR: δ 3.85 (s, 3H, OCH_3); 6.94 (d, 2H, arom H, J = 8.7 Hz); 7.24 (d, 1H, arom H, J = 8.2 Hz); 7.40 (d, 1H, =CHCO, J = 15.5 Hz); 7.60 (d, 2H, arom H, J = 8.7 Hz); 7.82 (d, 1H, ArCH=, J = 15.5 Hz); 7.98 (d, 1H, arom H, J = 1.5 Hz); 7.95 (dd, 1H, arom H, J_1 = 1.5 Hz, J_2 = 8.2 Hz), 11.14 (br s, 1H, NH); ^{13}C NMR: 55.3, 109.4, 114.3, 119.2, 125.5, 127.3, 130.8, 132.0, 134.7, 143.4, 143.7, 154.4, 161.3, 187.1. Anal. Calcd for $C_{17}H_{13}NO_4$: C, 69.15; H, 4.44; N, 4.74. Found: C, 69.33; H, 4.24; N, 4.62.

6-[3-(2,4-Dimethoxyphenyl)-2-propenoyl]-2(3*H*)-benzoxazolone (3d) Yield 84%; mp 202–204°C; IR: 1785, 1643 (C=O) cm^{-1}; ^{1}H NMR: δ 3.85 (s, 3H, OCH_3); 3.91 (s, 3H, OCH_3); 6.62–6.65 (m, 2H, arom H); 7.22 (d, 1H, arom H, J = 8.1 Hz); 7.79 (d, 1H, =CHCO, J = 15.6 Hz); 7.96 (m, 4H, ArCH=, arom H); 12.04 (br s, 1H, NH); ^{13}C NMR: 55.5, 55.8, 98.2, 106.3, 109.2, 109.3, 115.9, 118.6, 125.3, 129.9, 132.2, 134.6, 138.3, 143.4, 154.4, 159.8, 163.0, 187.2. Anal. Calcd for $C_{18}H_{15}NO_5$: C, 66.46; H, 4.65; N, 4.31. Found: C, 66.78; H, 4.72; N, 4.49.

6-[3-(3,4-Dimethoxyphenyl)-2-propenoyl]-2(3*H*)-benzoxazolone (3e) Yield 83%; mp 210–212°C; IR: 1785, 1643 (C=O) cm^{-1}; ^{1}H NMR: δ 3.82 (s, 3H, OCH_3); 3.86 (s, 3H, OCH_3); 7.02 (d, 1H, arom H, J = 8.3 Hz); 7.24 (d, 1H, arom H, J = 8.2 Hz); 7.39 (dd, 1H, arom H, J_1 = 8.3 Hz, J_2 = 1.5 Hz); 7.55 (d, 1H, arom H, J = 1.5 Hz); 7.70 (d, 1H, =CHCO, J = 15.4 Hz); 7.86 (d, 1H, ArCH=, J = 15.4 Hz); 8.07–8.13 (m, 2H, arom H). Anal. Calcd for $C_{18}H_{15}NO_5$: C, 66.46; H, 4.65; N, 4.31. Found: C, 66.51; H, 4.46; N, 4.79.

6-[3-(3,4,5-Trimethoxyphenyl)-2-propenoyl]-2(3*H*)-benzoxazolone (3f) Yield 87%; mp 218–220°C; IR: 1650, 1780 (C=O) cm^{-1}; ^{1}H NMR: δ 3.72 (s, 3H, OCH_3); 3.86 (s, 6H, OCH_3); 7.24 (m, 2H, arom H);

7.26 (d, 1H, arom H, J = 8.2 Hz); 7.70 (d 1H, =CHCO, J = 15.5 Hz); 7.92 (d, 1H, ArCH=, J = 15.5 Hz); 8.08 (dd, 1H, arom H, J_1 = 8.2 Hz, J_2 = 1.5 Hz); 8.12 (d, 1H, arom H, J = 1.5 Hz); 12.09 (br s, 1H, NH); ^{13}C NMR: 56.1, 60.1, 106.5, 109.5, 120.8, 125.6, 130.2, 131.9, 134.9, 139.6, 143.4, 144.2, 153.0, 154.4, 187.1. Anal. Calcd for $C_{19}H_{17}NO_6$: C, 64.22; H, 4.82; N, 3.94. Found: C, 64.51; H, 5.05; N, 4.24.

5-[3-(2-Methoxyphenyl)-2-propenoyl]-2(3*H*)-benzoxazolone (3g) Yield 93%; mp 199–201°C; IR: 1790, 1643 (C=O) cm^{-1}; ^{1}H NMR: δ 3.90 (s, 3H, OCH_3); 7.04 (m, 1H, arom H); 7.12 (d, 1H, arom H, J = 7.9); 7.45 (dd, 2H, arom H, J_1 = 8.4, J_2 = 1.9 Hz) 7.74 (d, 1H, arom H, J = 1.7 Hz); 7.89 (d, 1H, =CHCO, J = 15.7 Hz); 7.99 (dd, 2H, arom H, J_1 = 8.4 Hz, J_2 = 1.7 Hz); 8.06 (d, 1H, ArCH=, J = 15.7 Hz); 11.94 (br s, 1H, NH); ^{13}C NMR: 55.7, 109.3, 109.4, 111.7, 120.6, 121.6, 123.8, 128.5, 130.9, 132.3, 133.7, 138.4, 146.7, 146.7, 158.2, 158.2, 187.8. Anal. Calcd for $C_{17}H_{13}NO_4$: C, 69.15; H, 4.44; N, 4.74. Found: C, 69.36; H, 4.57; N, 4.79.

5-[3-(3-Methoxyphenyl)-2-propenoyl]-2(3*H*)-benzoxazolone (3h) Yield 95%; mp 180–181°C; IR: 1785, 1657 (C=O) cm^{-1}; ^{1}H NMR: δ 3.84 (s, 3H, OCH_3); 7.03 (m, 1H, arom H); 7.44–7.49 (m, 3H, arom H); 7.72 (d, 1H, =CHCO, J = 15.5 Hz); 7.77 (d, 1H, arom H, J = 1.7 Hz) 7.97 (d, 1H, ArCH=, J = 15.5 Hz); 8.05 (dd, 1H, arom H, J_1 = 8.4 Hz, J_2 = 1.7 Hz); 11.95 (br s, 1H, NH); ^{13}C NMR: 55.2, 109.3, 113.4, 116.6, 121.6, 122.1, 123.9, 129.8, 130.9, 133.6, 135.9, 143.9, 146.8, 154.2, 159.5, 159.6, 187.8. Anal. Calcd for $C_{17}H_{13}NO_4$: C, 69.15; H, 4.44; N, 4.74. Found: C, 68.86; H, 4.28; N, 4.88.

5-[3-(4-Methoxyphenyl)-2-propenoyl]-2(3*H*)-benzoxazolone (3i) Yield 81%; mp 222–225°C; IR: 1771, 1650 (C=O) cm^{-1}; ^{1}H NMR: δ 3.82 (s, 3H, OCH_3); 7.01–7.03 (m, 2H, arom H); 7.44 (d, 1H, arom H, J = 8.4 Hz); 7.71 (d, 1H, =CHCO, J = 15.5 Hz); 7.74–7.79 (m, 2H, arom H, ArCH=); 7.86 (d, 2H, arom H, J = 7.2 Hz); 8.01 (d, 1H, arom H, J = 8.4 Hz); 11.93 (br s, 1H, NH); ^{13}C NMR: 55.3, 109.2, 109.3, 114.3, 114.3, 119.2, 123.7, 127.2, 130.7, 130.8, 130.9, 133.9, 143.9, 146.6, 154.2, 161.3, 187.6. Anal. Calcd for $C_{17}H_{13}NO_4$: C, 69.15; H, 4.44; N, 4.74. Found: C, 69.55; H, 4.56; N, 4.62.

5-[3-(2,4-Dimethoxyphenyl)-2-propenoyl]-2(3*H*)-benzoxazolone (3j) Yield 85%; mp 239–243°C; IR: 1793, 1635 (C=O) cm^{-1}; ^{1}H NMR: δ 3.90 (s, 9H, OCH_3); 6.60–6.67 (m, 2H, arom H); 7.43 (d, 1H, arom H, J = 8.4 Hz); 7.71 (d, 1H, arom H, J = 1.4 Hz); 7.76 (d, 1H, =CHCO, J = 15.6 Hz); 7.91–8.02 (m, 3H, arom H, ArCH=); 11.91 (br s, 1H, NH); ^{13}C NMR: 55.5, 55.8, 98.2, 98.2, 106.3, 109.1, 109.3, 115.8, 118.7, 123.5, 130.0, 130.8, 134.1, 138.6, 146.5, 159.9, 163.0, 187.8. Anal. Calcd for $C_{18}H_{15}NO_5$: C, 66.46; H, 4.65; N, 4.31. Found: C, 66.52; H, 4.29; N, 4.47.

5-[3-(3,4-Dimethoxyphenyl)-2-propenoyl]-2(3*H*)-benzoxazolone (3k) Yield 88%; mp 208–210°C; IR: 1785, 1664 (C=O) cm^{-1}; ^{1}H NMR: δ 3.82 (s, 3H, OCH_3); 3.87 (s, 3H, OCH_3); 7.03 (d, 1H, arom H, J = 8.4 Hz); 7.40 (dd, 1H, arom H, J_1 = 8.4, J_2 = 2.0 Hz); 7.46 (d, 1H, arom H, J = 8.4 Hz); 7.55 (d, 1H, arom H, J = 2.0 Hz); 7.70 (d, 1H, =CHCO, J = 15.4 Hz); 7.75 (d, 1H, arom H, J = 1.8 Hz); 7.84 (d, 1H, ArCH=, J = 15.4 Hz); 8.03 (dd, 1H, arom H, J_1 = 8.4 Hz, J_2 = 1.8 Hz); ^{13}C NMR: 55.6, 109.2, 110.7, 111.4, 119.3, 123.7, 123.9, 127.3, 130.8, 133.8, 142.6, 144.4, 145.7, 146.6, 148.9, 151.2, 154.2, 187.7. Anal. Calcd for $C_{18}H_{15}NO_5$: C, 66.46; H, 4.65; N, 4.31. Found: C, 66.70; H, 4.78; N, 4.07.

5-[3-(3,4,5-Trimethoxyphenyl)-2-propenoyl]-2(3*H*)-benzoxazolone (3l) Yield 98%; mp 210–212°C; IR: 1771, 1657 (C=O) cm^{-1}; ^{1}H NMR: δ 3.73 (s, 3H, OCH_3); 3.87 (s, 6H, OCH_3); 7.23–7.26 (m, 2H, arom H); 7.48 (d, 1H, arom H, J = 8.4 Hz); 7.70 (d, 1H, =CHCO, J = 15.5 Hz); 7.75 (d, 1H, arom H, J = 1.7 Hz); 7.91 (d, 1H, ArCH=, J = 15.5 Hz); 8.05 (dd, 1H, arom H, J_1 = 8.4 Hz, J_2 = 1.7 Hz); ^{13}C NMR: 56.0, 60.0, 106.4, 109.4, 120.9, 123.9, 130.1, 130.9, 133.7, 133.7, 139.7, 144.4, 146.7, 153.0, 154.2, 187.7. Anal. Calcd for $C_{19}H_{17}NO_6$: C, 64.22; H, 4.82; N, 3.94. Found: C, 64.33; H, 4.61; N, 3.94.

Biological activities

Cytotoxic activity (MTT-dye reduction assay)

The cytotoxic activity of the tested compounds was assessed by the MTT-dye reduction assay as described by Mosmann, with minor modifications [22]. Briefly, exponentially growing cells were seeded into 96-well plates (100 μL aliquots/well at a density of 1 × 10^5 cells/mL). Following a 24-h adaptation period, they were exposed to various concentrations of the tested compounds for 72 h. After the treatment period, MTT solution (10 mg/mL in PBS) was added (10 μL/well). Plates were further incubated for 4 h at 37°C and the MTT-formazan crystals formed were dissolved by adding 100 μL/well of 5% formic acid in 2-propanol. Absorption was measured on an ELISA reader (Uniscan® Titertek, Helsinki, Finland) at 540 nm. For each concentration at least eight wells were used. A mixture of 100 μL RPMI-1640 medium with 10 μL MTT stock and 100 μL 5% formic acid in 2-propanol served as a blank solution. The cell viability (*% of untreated control*) for each treatment group was calculated using the formula:

$$\%\ of\ untreated\ control = \frac{A_T}{A_C} \times 100$$

where A_T denotes MTT-formazan absorption of the test sample and A_C denotes MTT-formazan absorption of the control (solvent treated) sample.

Concentration response curves were generated and the corresponding IC_{50} values were extrapolated using Origin plot Software for PC.

Cell-death detection

The characteristic for apoptosis oligonucleosomal DNA fragmentation was examined using a commercially available ‘cell death detection’ ELISA kit (Roche Applied Science). This method allows semiquantitative determination of the characteristic for the apoptotic process of histone-associated mono- and oligonucleosomal DNA fragments using ‘sandwich’ ELISA. Exponentially growing cells were exposed to varying concentrations of

the tested compounds and thereafter cytosolic fractions of 1×10^4 cells per group (treated or untreated) served as an antigen source in a 'sandwich' ELISA, utilizing a primary antihistone antibody-coated microplate and a secondary peroxidase-conjugated anti-DNA antibody. The photometric immunoassay for histone-associated DNA fragments was executed according to the manufacturer's instructions at 405 nm, using an ELISA reader (Labexim LMR-1). The results are expressed as the oligonucleosomal enrichment factor (representing a ratio between the absorption in the treated vs. the solvent-treated control samples).

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