Journal of the Serbian Chemical Society
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ACCEPTED MANUSCRIPT

Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
*J. Serb. Chem. Soc.***00(0)**1-13 (2022)
JSCS–11747
Original scientific paper
PublishedDD MM,YYY

Synthesis and biological activity of novel zingerone-thiohydantoin hybrids

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(Received 4 April; Revised 28 May; Accepted 9 June 2022)

*Abstract:*A series of zingerone-thiohydantoin hybrids were synthesized from *O*-alkyl zingerone derivatives by cyclocondensation with thiosemicarbazide in a two-step reaction. Obtained new potentially bioactive compounds were structurally characterized by IR and NMR spectroscopy, as well as elemental and HRMS analysis. In addition, their antimicrobial and *in vitro* anticancer activities were tested. Tested compounds showed low to moderate antimicrobial activity. Zingerone-thiohydantoin hybrid with an *O*-buthyl substituent exerted the significant cytotoxic activity on colon HCT-116 cancer cells without toxicity on healthy MRC-5 cells.

Keywords: molecular hybrids; antimicrobial activity; cytotoxic activity

INTRODUCTION

The development of novel synthetic molecular hybrids is one of the main challenges in the drug discovery field. Hybrid drugs represent a combination of specific agents aimed to be more efficient than classic single synthesized compounds. In that way, the hybrid approach allows the connection of two distinct compounds in one molecule, increasing the biological potential of at least one of the compounds.[1] Many natural products play an important role in this field. For example, zingerone also called vanillylacetone, obtained from a ginger extract, is a natural compound that belongs to the methoxyphenol class along with its derivatives. Both natural and synthetic zingerone derivatives exhibit different biological and pharmacological activities such as anti-inflammatory, antimicrobial, anti-cancer, and hepatoprotective.[2] Furthermore, zingerone appears to be a potential agent for inhibiting colon cancer progression, as the number of

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https://doi.org/10.2298/JSC220404047V

larger foci was found to be significantly lower after zingerone treatment compared to dimethyl hydrazine-induced colon cancer cells.[2]

On the other hand, 2-thiohydantoin (2-thioxoimidazoline-4-one) is a non-aromatic five-membered heterocyclic compound with a cyclic ureid core.[3] Many synthesized thiohydantoin derivatives with various substituents attached to their nucleus exhibit a wide range of biological and pharmacological potentials, such as antimicrobial,[4] anti-convulsive,[5] anti-diabetes,[6] and anti-HIV.[7] However, novel studies showed that thiohydantoins and their synthesized derivatives could be used as promising anti-proliferative and anti-metastatic agents.[8] Taking into account that colon cancer is one of the most prominent tumors in the world and less sensitive to cytostatics, the search for new effective therapeutic drugs is crucial.

In this study, different zingerone derivatives were prepared as starting materials for obtaining a short series of new zingerone-thiohydantoin hybrids for evaluation of their potential biological activity.

EXPERIMENTAL

General methods

All reagents and chemicals were commercially available and used without additional purification. Solvents were distilled before use. Anhydrous methanol was prepared by standard drying methods. Zingerone, starting material for a preparation of *O*-alkyl zingerone derivatives, was obtained by condensation reaction of vanillin and acetone and subsequent reduction of yielded dehydrozingerone according to well known procedure.[9] IR spectra were recorded through KBr pellets on a Perkin-Elmer FT-IR spectrometer model Spectrum One in the 4000 to 450 cm^{-1} range. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 2000 NMR spectrometer using $CDCl_3$ as the solvent and TMS as the internal standard. Elemental analysis was done on an Elemental Vario ELIII CHNSO analyzer. HRMS were measured on an Agilent 6550 iFunnel Q-TOF LC/MS system. For biological assays microtitre plates and Multiskan SkyHigh Microplate spectrophotometer by Thermo Scientific were used.

*General procedure for the preparation of O-alkyl zingerone derivatives **1a-b***

Zingerone derivatives **1a** and **1b** were synthesized according to a procedure that utilizes dimethyl and diethyl sulfate, respectively.[10] A mixture of zingerone (0.971 g, 5 mmol) and 50 mL of boiling water is heated on a steam bath. A 2 mL portion of 20 % NaOH solution is heated to about 100 °C and added in one lot to the hot mixture of zingerone and water. Heating is continued and 6.25 mmol of methyl/ethyl sulfate is slowly added in portions. After the addition of all methyl/ethyl sulfate, which requires about 1.5 h, the reaction mixture is heated for 45 min longer and an additional portion of 1.1 mmol of methyl/ethyl sulfate is added at the same rate as the first portion. At the end of this addition the reaction mixture should show an acid reaction. The reaction mixture is rendered slightly alkaline with NaOH solution, and the addition of Me/Et sulfate and NaOH solution is done two more times until total amount of Me/Et sulfate (11.25 mmol) is added. The mixture is then made strongly alkaline by the addition of 1 mL NaOH solution and is heated another 20 minutes. The reaction mixture is rapidly cooled at ambient temperature with continued stirring and the product is extracted with diethyl ether (3×10 mL). The combined ether extracts are dried over anhydrous $MgSO_4$ and the ether is evaporated, giving yellow oil that soon solidifies.

*General procedure for the preparation of O-alkyl zingerone derivatives **1c-g***

Zingerone derivatives **1c-g** were synthesized according to a known procedure that uses alkyl halides with potassium carbonate in acetone.[11] The mixture of zingerone (0.971 g, 5 mmol), alkyl halide (12.5 mmol) and anhydrous K_2CO_3 (2.25 g, 16.3 mmol) in acetone (25 mL) were heated to reflux for 3 hours. The mixture was cooled at ambient temperature and then poured into cold water. The products were extracted from the mixture with ethyl acetate (3×10 mL). Combined extracts were rinsed with water and then dried over anhydrous Na_2SO_4. The solvent was removed by vacuum distillation and the product was separated from the residue by column chromatography with hexane/EtOAc (from 3:1 to 6:1).

Zingerone derivatives **1a**, **1b** and **1f** are known and commercially available chemicals, while the others are novel. The structure and purity of all new products were confirmed by IR and NMR spectroscopy.

*4-(3-methoxy-4-propoxyphenyl)butan-2-one (**1c**)*

Yield: 0.947 g (80 %). IR (KBr): 2963m, 2877m, 1714s, 1589w, 1514s, 1465m, 1419m, 1363m, 1258s, 1231s, 1158m, 1138s, 1036m, 978m, 800m cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$, δ / ppm): 6.74 (d, 1H, J = 8.0, H-9), 6.71 (s, 1H, H-6), 6.69 (d, 1H, J = 8.2 Hz, H-10), 3.94 (t, 2H, J = 6.8 Hz, CH_2-12), 3.84 (s, 3H, CH_3-11), 2.68-2.88 (m, 4H, CH_2-3, CH_2-4), 2.13 (s, 3H, CH_3-1), 1.84 (sext, 2H, J = 7.2 Hz, CH_2-13), 1.02 (t, 3H, J = 7.5 Hz, CH_3-14). ^{13}C NMR (50 MHz, $CDCl_3$, δ / ppm): 208.01 (C2), 149.14 (C7), 146.71 (C8), 133.44 (C5), 119.94 (C10), 112.99 (C9), 112.02 (C6), 70.42 (C12), 55.78 (C11), 45.24 (C4), 29.94 (C3), 29.20 (C1), 22.34 (C13), 10.28 (C14).

*4-(4-isopropoxy-3-methoxyphenyl)butan-2-one (**1d**)*

Yield: 0.926 g (78 %). IR (KBr): 2975m, 2933m, 1714s, 1587w, 1510s, 1465m, 1451m, 1419m, 1369m, 1259s, 1229m, 1157m, 1138m, 1110m, 1035m, 955w, 806w cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$, δ / ppm): 6.65-6.83 (m, 3H, H-6, H-9, H-10), 4.46 (sept, 1H, J = 6.0 Hz, CH-12), 3.83 (s, 3H, CH_3-11), 2.68-2.90 (m, 4H, CH_2-3, CH_2-4), 2.14 (s, 3H, CH_3-1), 1.33 (d, 6H, J = 6.2 Hz, CH_3-13, CH_3-14). ^{13}C NMR (50 MHz, $CDCl_3$, δ / ppm): 208.24 (C2), 150.24 (C7), 145.44 (C8), 134.02 (C5), 119.98 (C10), 116.01 (C9), 112.28 (C6), 71.45 (C12), 55.8 (C11), 45.31 (C4), 30.04 (C3), 29.30 (C1), 22.02 (C13, C14).

*4-(4-butoxy-3-methoxyphenyl)butan-2-one (**1e**)*

Yield: 1.088 g (87 %). IR (KBr): 2957m, 2935m, 2872w, 1715s, 1589m, 1514s, 1465m, 1419m, 1362m, 1257s, 1233s, 1158s, 1139s, 1034s, 972, 800m cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$, δ / ppm): 6.79 (d, 1H, J = 7.8 Hz, H-9), 6.71 (s, 1H, H-6), 6.69 (d, 1H, J = 8.0 Hz, H-10), 3.98 (t, 2H, J = 6.8 Hz, CH_2-12), 3.84 (s, 3H, CH_3-11), 2.68-2.88 (m, 4H, CH_2-3, CH_2-4), 2.13 (s, 3H, CH_3-1), 1.81 (quint, 2H, J = 3.6 Hz, CH_2-13), 1.48 (sext, 2H, J = 3.7 Hz, CH_2-14), 0.96 (t, 3H, J = 7.3 Hz, CH_3-15). ^{13}C NMR (50 MHz, $CDCl_3$, δ / ppm): 208.03 (C2), 149.17 (C7), 146.78 (C8), 133.44 (C5), 119.96 (C10), 112.97 (C9), 112.04 (C6), 68.65 (C12), 55.81 (C11), 45.28 (C4), 31.14 (C3), 29.98 (C1), 29.24 (C13), 19.07 (C14), 13.74 (C15).

*4-(3-methoxy-4-((2-methylallyl)oxy)phenyl)butan-2-one (**1g**)*

Yield: 0.942 g (76 %). IR (KBr): 2938w, 1714s, 1603w, 1515s, 1452m, 1430m, 1364m, 1268s, 1235m, 1158m, 1140m, 1035m, 906w, 860w, 809w, 630w cm^{-1}.^1H NMR (200 MHz, $CDCl_3$, δ / ppm): 6,83 (d, 1H, J = 7.8 Hz, H-9) 6.77 (s, 1H, H-6) 6.67 (d, 1H, J = 7.2 Hz, H-10), 5.07 (m, 1H, H-14), 4.96 (m, 1H, H-14), 3.86 (s, 3H, CH_3-11), 2.67-2.90 (m, 4H, CH_2-3, CH_2-4), 2.14 (s, 3H, CH_3-1), 1.82 (s, 3H, CH_3-15). ^{13}C NMR (50 MHz, $CDCl_3$, δ / ppm): 207.85 (C2), 148.99 (C7), 146.63 (C8), 143.89 (C13), 133.99 (C5), 120.03 (C10), 114.02 (C9), 112.46 (C14), 111.04 (C6), 72.95 (C12), 56.04 (C11), 45.52 (C4), 30.08 (C3), 29.44 (C1), 19.32 (C15).

*Synthesis of zingerone-thiohydantoin derivatives **2a-g***

The zingerone-thiohydantoin derivatives were synthesized according to a previously published protocol for the synthesis of arylidene thiohydantoin derivatives.[12] A mixture of *O*-alkyl zingerone derivative **1a-g** (2 mmol) and thiosemicarbazide (0.182 g, 2 mmol) in 30 mL of methanol were heated to reflux for 3 h and then cooled to ambient temperature, resulting in the corresponding intermediate thiosemicarbazone without isolation. Ethyl chloroacetate (0.245 g, 2 mmol) and anhydrous sodium acetate (0.492 g, 6 mmol) were added *in situ* and the mixture was refluxed for another 6 h. The reaction mixture was cooled to room temperature at first and then poured into cold water. The resulting precipitate was filtered off, rinsed with hot water and re-crystallized from hot methanol giving white amorphous powder in all cases. The structure of the synthesized compounds was confirmed by IR and NMR spectroscopy, as well as elemental analysis and HRMS (Supplementary material). All compounds are obtained as an unseparable mixture of *Z* and *E* stereoisomers, as can be seen through the duplication of most signals in the ^{1}H NMR spectra. NMR spectral data are given for the major stereoisomer.

*3-((4-(3,4-dimethoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2a**)*

Yield: 0.439 g (68 %). IR (KBr): 3152w, 3079w, 2935m, 1722s, 1638s, 1604s, 1515s, 1452m, 1418m, 1346m, 1257s, 1158m, 1138m, 1035m, 897w, 844w, 799w, 702w, 632w, 516w cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$, δ / ppm): 9.98 (bs, NH, exchangeable with D_2O), 6.95 (d, 1H, J = 11.6 Hz, H-14), 6.86 (d, 1H, J = 8.4 Hz, H-15), 6.79 (s, 1H, H-11), 3.88 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 3.75 (s, 2H, CH_2-5), 2.57-2.96 (m, 4H, CH_2-8, CH_2-9), 2.03 (s, 3H, CH_3-6). ^{13}C NMR (50 MHz, $CDCl_3$, δ / ppm): 173.02 (C2), 167.29 (C4), 160.76 (C7), 149.26 (C12), 148.90 (C13), 135.70 (C10), 127.11 (C15), 120.16 (C14), 111.49 (C11), 55.96 (C16, C17), 40.55 (C9), 33.04 (C5), 31.91 (C8), 13.34 (C6). (+)LC-HRMS (*m*/*z*): calculated for $[C_{15}H_{19}O_3N_3S + H]^+$ 320.1074, observed 320.1220. Combustion analysis for $C_{15}H_{19}O_3N_3S$: Calculated. C 56.06, H 5.96, N 13.07; found C 56.10, H 5.98, N 13.04.

*3-((4-(4-ethoxy-3-methoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2b**)*

Yield: 0.465 g (69 %). IR (KBr): 3148m, 2983m, 1724s, 1694s, 1636s, 1602s, 1515s, 1449w, 1418w, 1344m, 1255m, 1233m, 1197m, 1154m, 1137m, 1032m, 896w, 792w, 701w, 516w cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$, δ / ppm): 9.68 (bs, NH, exchangeable with D_2O), 6.70-6.86 (m, 3H, H-11, H-14, H-15), 3.87 (s, 3H, CH_3-16), 3.75 (s, 2H, CH_2-5), 2.55-2.93 (m, 4H, CH_2-8, CH_2-9), 2.02 (s, 3H, CH_3-6), 1.45 (t, 3H, J = 7.0 Hz, CH_3-18). ^{13}C NMR (50 MHz, $CDCl_3$, δ / ppm): 172.95 (C2), 167.35 (C4), 160.40 (C7), 149.32 (C12), 143.84 (C13), 134.17 (C10), 120.94 (C15), 114.30 (C14), 111.11 (C11), 64.55 (C17), 56.01 (C16), 40.58 (C9), 32.99 (C5), 32.00 (C8), 17.86 (C6), 14.94 (C18). (+)LC-HRMS (*m*/*z*): calculated for $[C_{16}H_{21}O_3N_3S + H]^+$ 336.1376, observed 336.1378. Combustion analysis for $C_{16}H_{21}O_3N_3S$: Calculated. C 57.29, H 6.31, N 12.53; found C 57.33, H 6.33, N 14.49.

*3-((4-(3-methoxy-4-propoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2c**)*

Yield: 0.504 g (72 %). IR (KBr): 3142m, 2965m, 2933m, 2876m, 1709s, 1633s, 1598s, 1516s, 1470m, 1454m, 1418m, 1347m, 1256s, 1230s, 1160m, 1135m, 1034m, 1019m, 978w, 897w, 796m, 702w, 516w, 500w cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$, δ / ppm): 9.33 (bs, NH, exchangeable with D_2O), 6.65-6.85 (m, 3H, H-11, H-14, H-15), 3.95 (t, 2H, J = 6.8 Hz, CH_2-17), 3.86 (s, 3H, CH_3-16), 3.75 (s, 2H, CH_2-5), 2.56-2.94 (m, 4H, CH_2-8, CH_2-9), 2.02 (s, 3H, CH_3-6), 1.86 (sext, 2H, J = 7.2 Hz, CH_2-18), 1.03 (t, 3H, J = 7.4 Hz, CH_3-19). ^{13}C NMR (50 MHz, $CDCl_3$, δ / ppm): 172.66 (C2), 167.40 (C4), 159.90 (C7), 149.44 (C12), 146.94 (C13), 134.17 (C10), 120.28 (C15), 113.53 (C14), 112.59 (C11), 70.85 (C17), 56.13 (C16), 40.52 (C9), 32.93 (C5), 31.67 (C8), 22.64 (C18), 17.83 (C6), 10.48 (C19). (+)LC-HRMS (*m*/*z*): calculated

for $[C_{17}H_{23}O_3N_3S + H]^+$ 350.1533, observed 350.1532. Combustion analysis for $C_{17}H_{23}O_3N_3S$: Calculated. C 58.43, H 6.63, N 12.02; found C 58.40, H 6.30, N 12.06.

*3-((4-(4-isopropoxy-3-methoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2d**)*

Yield: 0.228 g (33 %). IR (KBr): 2973m, 2931m, 2857w, 1717s, 1639s, 1610s, 1511s, 1465m, 1334m, 1262s, 1157w, 1139m, 1111m, 1037w, 956w, 850w, 809w, 736w, 710w, 514w cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$, δ / ppm): 9.39 (bs, NH, exchangeable with D_2O), 6.66-6.87 (m, 3H, H-11, H-14, H-15), 4.47 (sept, 1H, J = 6.2 Hz, CH-17), 3.85 (s, 3H, CH_3-16), 3.75 (s, 2H, CH_2-5), 2.56-2.94 (m, 4H, CH_2-8, CH_2-9), 2.02 (s, 3H, CH_3-6), 1.35 (d, 6H, J = 6.0 Hz, CH_3-18, CH_3-19). ^{13}C NMR (50 MHz, $CDCl_3$, δ / ppm): 172.82 (C2), 167.33 (C4), 160.97 (C7), 150.54 (C12), 145.63 (C13), 134.76 (C10), 120.28 (C15), 116.75 (C14), 112.81 (C11), 71.83 (C17), 56.08 (C16), 40.49 (C9), 32.97 (C5), 32.02 (C8), 22.24 (C18, C19), 17.84 (C6). (+)LC-HRMS (*m*/*z*): calculated for $[C_{17}H_{23}O_3N_3S + H]^+$ 350.1533, observed 350.1533. Combustion analysis for $C_{17}H_{23}O_3N_3S$: Calculated. C 58.43, H 6.63, N 12.02; found C 58.45, H 6.36, N 11.98.

*3-((4-(4-butoxy-3-methoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2e**)*

Yield: 0.637 g (88 %). IR (KBr): 3145m, 2958m, 2935m, 2871m, 1709s, 1636s, 1604s, 1517s, 1467m, 1419m, 1346m, 1257s, 1234s, 1161m, 1138m, 1034m, 1009w, 972w, 897w, 844w, 795w, 701w, 516w cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$, δ / ppm): 9.27 (bs, NH, exchangeable with D_2O), 6.65-6.85 (m, 3H, H-11, H-14, H-15), 3.99 (t, 2H, J = 6.8 Hz, CH_2-17), 3.86 (s, 3H, CH_3-16), 3.75 (s, 2H, CH_2-5), 2.56-2.94 (m, 4H, CH_2-8, CH_2-9), 2.02 (s, 3H, CH_3-6), 1.82 (quint, 2H, J = 7.3 Hz, CH_2-18), 1.48 (sext, 2H, J = 7.4 Hz, CH_2-19), 0.97 (t, 3H, J = 7.2 Hz, CH_3-20). ^{13}C NMR (50 MHz, $CDCl_3$, δ / ppm): 172.73 (C2), 167.37 (C4), 159.97 (C7), 149.46 (C12), 147.00 (C13), 134.15 (C10), 120.28 (C15), 113.50 (C14), 112.60 (C11), 69.03 (C17), 56.13 (C16), 40.51 (C9), 32.94 (C5), 31.96 (C8), 31.41 (C18), 19.27 (C19), 17.82 (C6), 13.88 (C20). (+)LC-HRMS (*m*/*z*): calculated for $[C_{18}H_{25}O_3N_3S + H]^+$ 364.1689, observed 364.1689. Combustion analysis for $C_{18}H_{25}O_3N_3S$: Calculated. C 59.48, H 6.93, N 11.56; found C 59.44, H 6.95, N 11.51.

*3-((4-(4-(benzyloxy)-3-methoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2f**)*

Yield: 0.686 g (86 %). IR (KBr): 3152w, 3035w, 3954w, 2870w, 1710s, 1639s, 1605s, 1515s, 1455w, 1418w, 1345m, 1256s, 1227s, 1161m, 1136m, 1034w, 1011w, 857w 806w, 745m, 698m, 515w cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$, δ / ppm): 8.97 (bs, NH, exchangeable with D_2O), 7.25-7.50 (m, 5H, H-19, H-20, H-21, H22, H-23), 6.65-6.85 (m, 3H, H-11, H-14, H-15), 5.13 (s, 2H, CH_2-17), 3.88 (s, 3H, CH_3-16), 3.74 (s, 2H, CH_2-5), 2.55-2.93 (m, 4H, CH_2-8, CH_2-9), 2.00 (s, 3H, CH_3-6). ^{13}C NMR (50 MHz, $CDCl_3$, δ / ppm): 172.52 (C2), 167.34 (C4), 160.12 (C7), 149.71 (C12), 146.58 (C13), 137.46 (C18), 134.86 (C10), 128.43 (C19, C23), 127.69 (C21), 127.26 (C20, C22), 120.28 (C15), 114.65 (C14), 112.65 (C11), 71.41 (C17), 56.13 (C16), 40.45 (C9), 32.90 (C5), 31.97 (C8), 17.83 (C6). (+)LC-HRMS (*m*/*z*): calculated for $[C_{21}H_{23}O_3N_3S + H]^+$ 398.1533, observed 398.1532. Combustion analysis for $C_{21}H_{23}O_3N_3S$: Calculated. C 63.45, H 5.83, N 10.57; found C 63.50, H 5.81, N 10.62.

*3-((4-(3-methoxy-4-((2-methylallyl)oxy)phenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2g**)*

Yield: 0.441 g (61 %). IR (KBr): 3150m, 3079m, 2934m, 2852w, 1709s, 1634s, 1601s, 1514s, 1452m, 1418m, 1346m, 1256s, 1158m, 1137m, 1034m, 896w, 834w, 798w, 701w, 516w cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$, δ / ppm): 9.79 (bs, NH, exchangeable with D_2O), 6.65-6.85 (m, 3H, H-11, H-14, H-15), 5.08 (m, 1H, H-19), 4.97 (m, 1H, H-19), 4.49 (s, 2H, CH_2-17), 3.87

(s, 3H, CH_3-16), 3.75 (s, 2H, CH_2-5), 2.55-2.94 (m, 4H, CH_2-8, CH_2-9), 2.02 (s, 3H, CH_3-6), 1.82 (s, 3H, CH_3-20). ^{13}C NMR (50 MHz, $CDCl_3$, δ / ppm): 173.02 (C2), 167.27 (C4), 160.53 (C7), 149.50 (C12), 146.63 (C13), 141.06 (C18), 134.54 (C10), 120.21 (C15), 114.13 (C14), 112.64 (C11), 112.42 (C19), 73.05 (C17), 56.11 (C16), 40.47 (C9), 32.99 (C5), 31.94 (C8), 19.34 (C20), 17.84 (C6). (+)LC-HRMS (*m*/*z*): calculated for $[C_{18}H_{23}O_3N_3S + H]^+$ 362.1533, observed 362.1533. Combustion analysis for $C_{18}H_{23}O_3N_3S$: Calculated. C 59.81, H 6.41, N 11.63; found C 59.86, H 6.44, N 11.58.

Antimicrobial activity determination

In this preliminary testing, five microbial strains were used, four of which were bacteria, *Salmonella enteritidis* ATCC 13076, *Pseudomonas aeruginosa* ATCC 10145, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and the yeast *Candida albicans* ATCC 10259. The microorganisms were acquired from the Institute of Public Health Kragujevac, University of Kragujevac, Serbia, kept at 4 °C with subcultivation once a month. The broth used for bacteria cultivation was nutrient agar and yeast was cultured on Sabouraud dextrose agar. For antimicrobial evaluation of synthesized compounds, a standard microdilution method by Sarker *et al*. was used.[13,14]

Cytotoxic activity determination

All synthesized derivatives were dissolved in dimethyl sulfoxide (DMSO) in order to obtain stock solutions of 5 mM concentration, followed by further dilution in Dulbecco's Modified Eagle Medium (DMEM) to obtain working concentrations (0.1, 1, 10, 50, 100 and 250 μM), where at the highest applied concentration, the concentration of DMSO in the solution was lower than 0.05 %, previously confirmed as non-toxic to cancer cells.[15]

The healthy human lung fibroblasts (MRC-5) and colorectal carcinoma cell lines (HCT-116) were obtained from the European Collection of Authenticated Cell. Cells were cultured in a complete medium in humidified conditions, at 37 °C and 5 % CO_2. When cells reached 70 to 80 % of confluence, the detachment was done using 0.25 % trypsin–EDTA, followed by seeding (1×10^4 cells/well) in 96-well flat-bottomed microtitre plates. Treatment with 100 μL of synthesized compounds solution was done 24 h after cell seeding.

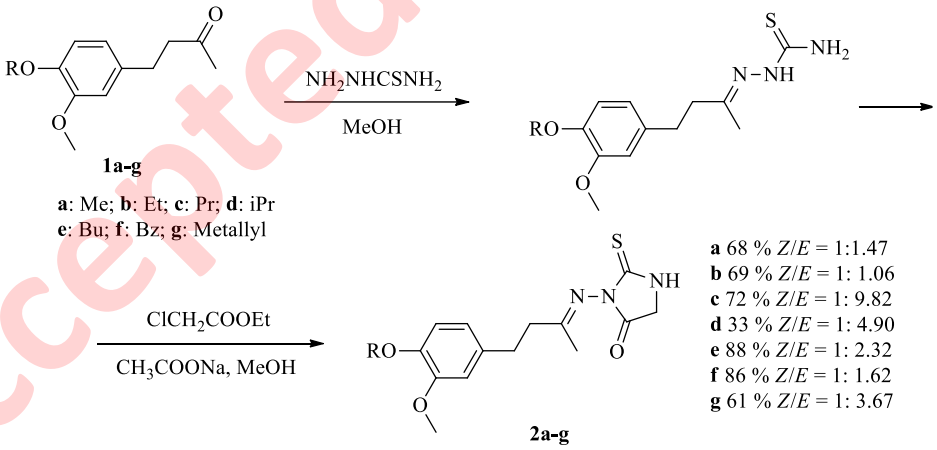
Effects of synthesized compounds on the viability of tested cell lines were assessed after 24 and 72 h using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay according to the previously described protocol.[16] At the end of the incubation period, 25 μL of MTT solution (from a 5 mg/mL stock) was added to each well, followed by incubation at 37 °C for 2 h, after which 100 μL of DMSO was added. The evaluation of cytotoxic activity was done by measuring the absorbances at 550 nm wavelength. The obtained results are presented as mean ± standard error (SE) expressed as percent of cell viability (%). IC_{50} values (minimal inhibitory treatment concentration that induces the death of 50 % of treated cells) were calculated from dose curves obtained by the MTT test. 5-Fluorouracil (5-FU) was used as positive control.

The magnitude of the correlation between variables was calculated using statistical software SPSS (SPSS for Windows, ver. 20, 2008, Chicago, IL) whereat the ANOVA test was applied, and for all comparisons $p < 0.05$ was considered as a statistically significant difference between control and tested compounds. The IC_{50} values were calculated by using the CalcuSyn software program.

RESULTS AND DISCUSSION

Synthesis of the zingerone-thiohydanoin molecular hybrids

The zingerone-thiohydantoin derivatives **2a-g** were obtained through a condensation reaction with thiosemicarbazide, utilizing a previously published two-step protocol (Scheme 1).[12] In the first step, synthesized *O*-alkyl zingerone derivatives **1a-g** reacted with thiosemicarbazone, giving corresponding thiosemicarbazides. The thiosemicarbazides then undergo intramolecular cyclocondenzation with ethyl chloroacetate in the presence of anhydrous sodium acetate, yielding the final thiohydantoin products **2a-g**. The structure and purity of the novel zingerone-thiohydantoin derivatives were confirmed by IR and NMR spectroscopy, as well as elemental and HRMS analysis. The compounds were obtained in medium to high yields, with the exception of **2d**, which was obtained in a modest yield. Naturally occurring zingerone itself did not react in this manner and the corresponding thiohydantoin derivative was not obtained. Similar to some Schiff-base derivatives,[17] all newly synthesized zingerone-thiohydantoin derivatives are obtained as a mixture of *Z* and *E* stereoisomers which could be found in corresponding NMR spectra. Most signals in the ^{1}H NMR spectra are duplicated, which indicates the presence of isomers. This is best seen through the singlets of CH_2-5, CH_3-6 and CH_3-16, which have the most pronounced difference of the chemical shifts and thus, less overlap. The ratios of *Z/E* isomers are obtained from relative integration of the most suitable and distinct pairs of singlets. Their ratios range from 1:1.06 to 1:9.82 (Scheme 1). In all cases the *E* isomer is favored.



Scheme 1. Synthesis of the zingerone-thiohydantoin hybrids **2a-g**

Furthermore, computational data for both possible configurations of **2a** were also calculated using the DFT method in order to perceive the more stable isomer as seen in Fig. 1. By neglecting any internal interconnection between molecules

for both isomers the less internal steric repulsion factor plays an important role in fixing the *E* isomer over *Z* isomer, as it is with less internal repulsion. The gaseous state DFT calculations also showed the energy difference value ΔE = 4.0 kJ mol^{-1} between *E* and *Z* isomer is very small.

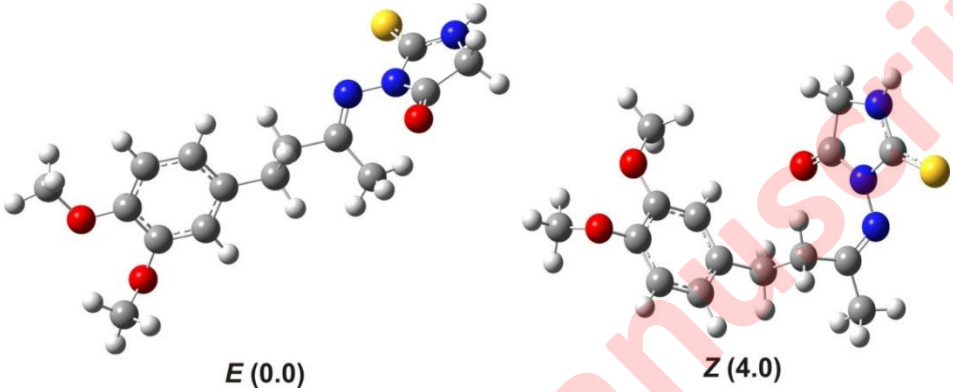


Fig. 1**.** The optimized geometries of *E* and *Z* isomers of **2a**, with relative free energy values indicated in kJ mol^{-1}

Antimicrobial activity

The results of antimicrobial effects of the zingerone-thiohydantoin hybrids are presented in Table 1. Compounds were tested against two Gram-negative (*S. enteritidis* and *P. aeruginosa*) and two Gram-positive (*S. aureus* and *E. coli*) bacterial species, as well as the yeast *C. albicans*. *S. aureus* and *C. albicans* were the most resistant to the action of **2a-g**, wherein the compounds had not shown any activity even at the highest applied concentration (*MIC* > 4 mg mL^{-1}). Moreover, compound **2c** was completely inactive towards all used microorganisms at the same concentration, while with other compounds *MIC* values were quite high, mostly around 2-4 mg mL^{-1}. **2b** was able to inhibit the growth of *S. enteritidis* in moderate concentration (*MIC* 0.5 mg mL^{-1}). Only *E. coli* was more susceptible to the action of the compounds, particularly **2a** and **2g** with *MIC* 0.25 mg mL^{-1}. Nevertheless, the quite high concentrations at which the compounds exhibit their activity cannot be easily compared with the activity of the reference standards, erythromycin and nystatin, where *MIC*s were expressed in micrograms per milliliter. This is the first study regarding the antimicrobial potential of zingerone-thiohydantoin hybrids. The literature data about similar compounds are scarce. There are some recently published results regarding the influence of some thiohydantoin derivatives on several bacterial species.[4] These compounds were the most active against Gram-positive bacteria such as *S. epidermidis*, *S. pyogenes*, *S. agalactiae*, *E. faecium* and *S. aureus* with *MIC*s below 1 mg mL^{-1}, but were less effective against Gram-negative bacteria like *K. pneumonia*, *P. mirabilis*, and *E. coli*.

TABLE I. Antimicrobial activity of the synthesized zingerone-thiohydantoin derivatives **2a-g**.

Compounds	*MIC* / mg mL^{-1}				
	Salmonella enteritidis	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Escherichia coli*	*Candida albicans*
2a	4	4	> 4	0.25	> 4
2b	0.5	4	> 4	2	> 4
2c	> 4	> 4	> 4	> 4	> 4
2d	-	-	-	-	-
2e	4	4	> 4	2	> 4
2f	> 4	4	> 4	4	> 4
2g	4	4	> 4	0.25	> 4
Antibiotic/ antimycotic	*MIC* / µg mL^{-1}				
Erythromycin	20	20	1.25	2.5	-
Nystatin	-	-	-	-	1.25

MIC: minimal inhibitory concentration; -: not tested

Cytotoxic activity

Thiohydantoin analogs have been already confirmed as potent anti-tumor agents. Furthermore, apoptosis-inducing activity of some thiohydantoin derivatives has been demonstrated.[18] Previous research has identified zingerone as a potential anti-cancer agent, an inhibitor of colon cancer progression,[2,19] with significant cytotoxicity (IC_{50} = 11.49 mM) when applied to mesothelioma cells. Su *et al*. showed that the zingerone-induced cytotoxic effect on colon cancer cells (HCT-116) was achieved through the mechanism of ROS-mediated apoptosis.[20] Besides that, the cytotoxic potential of thiohydantoin derivatives on HCT-116 was noticed when applied to colon cancer cells (with IC_{50} > 50 µM).[21]

In this study, cytotoxic activity of newly synthesized zingerone-thiohydantoin derivatives was evaluated on healthy lung (MRC-5) and human colorectal carcinoma (HCT-116) cell lines by MTT assay. As positive control, commercial chemotherapeutic drug, 5-fluorouracil was used. 5-FU is widely used in the treatment of different types of cancer, such as gastric, pancreatic, breast, and ovarian cancers.[22]

In regard to the influence on the viability of the healthy MRC-5 cells, a moderate cytotoxic effect was observed only for compound **2a** after an extended time of exposure with $IC_{50}^{72\,h}$ = 184.15 µM (Fig. S-44) and control with $IC_{50}^{72\,h}$ = 181.71 µM (Fig. S-46). The tested compounds manifest their cytotoxicity potentials in a time and dose-dependent manner. The reduction of HCT-116 cells viability was obtained mainly after 72 h and in the highest applied concentration of investigated compounds (Fig. S-45). The effects of the synthesized compounds were expressed by dose curves (Fig. S-44 and S-45) and IC_{50} values (Fig. 2). Based on the results, **2e** exhibited the most prominent antiproliferative activity on the HCT-116 cell line with an $IC_{50}^{24\,h}$ = 209.08 µM and $IC_{50}^{72\,h}$ = 160.93 µM. 5-FU exerted a weaker cytotoxic

effect than **2e** on HCT-116 after 24 h and 72h with $IC_{50}^{24\ h}$ = >250 μM and $IC_{50}^{72\ h}$ = = 181.71 μM, respectively. (Fig. 2).

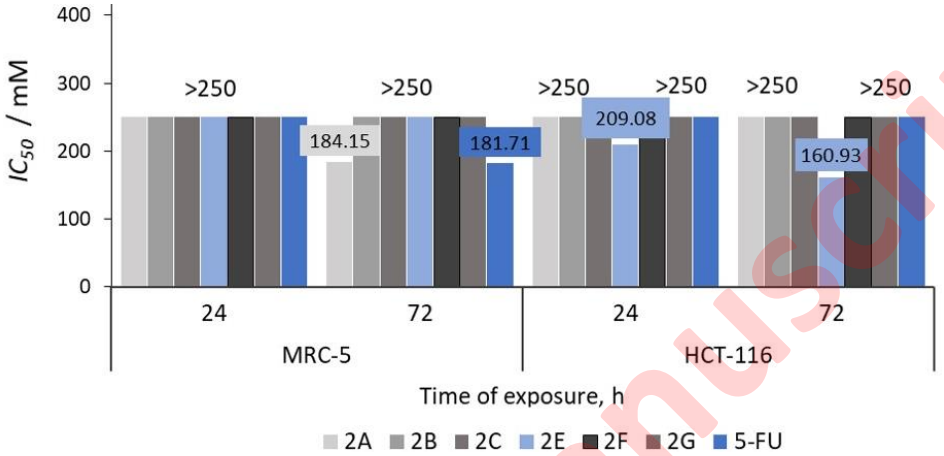


Fig. 2. The cytotoxic effects of zingerone-thiohydantoin derivatives and 5-FU after 24 h and 72 h exposure, represented as IC_{50} values.

Unlike other tested compounds, **2e** contains a butyl group. The result of its activity is probably related to the lipophilicity of the substituents in the structure of the tested molecule. The lipophilicity of molecules plays a major role in the transport of molecules across biological membranes.[23] As the length of hydrocarbon chain increases, the polarity of compounds decreases resulting in increased molecule permeability. Cell membranes are relatively impermeable to hydrophilic compounds, hence the permeability of molecules depends on the hydrophobic characteristics *i.e.* lipophilicity of the compound.[24] In addition, a previous study reported that the lipophilicity of the substituents (Ph > Allyl > Me) had a significant impact on the cytotoxic effect.[25]

Compound **2f**, with a benzyl group, did not exhibit a cytotoxic effect (IC_{50} > 250 μM). However, based on the obtained dose curves, it can be concluded that the wider range of concentrations could provide promising effects. Increasing the concentration range of the tested compounds would probably lead to an increase in the cytotoxic activity of some of them and also would allow the determination of the selectivity index on tested cell lines.

The presented results indicate the potential of tested compounds as anticancer agents with no significant toxicity on healthy cell lines. Compound **2e** exhibited the most promising bioactivity and is the leading candidate in the synthesized series. Its increased cytotoxicity compared to zingerone itself could be attributed to introduction of the thiohydantoin moiety.

CONCLUSION

This study presents the synthesis, characterization, and biological assessment of new zingerone-thiohydantoin molecular hybrids as potential anticancer agents. In addition to the zingerone-induced cytotoxic effect, it is known that the biological activity of thiohydantoin compounds depends largely on the nature of the substitution of the thiohydantoin ring. The difference in the cytotoxic activity of the tested compounds depends on the nature of the *O*-alkyl substituent of the benzene ring. Among the tested compounds, **2e** exerted significant cytotoxic potential without toxicity to healthy MRC-5 cells. However, all tested compounds showed low to moderate antimicrobial activity. Further toxicological testing is required to assess its therapeutic potential.

SUPPLEMENTARY MATERIAL

Supplementary Material is available electronically from https://www.shd-pub.org.rs/index.php/JSCS/article/view/11747, or from the corresponding author on request

Acknowledgements: The authors are grateful for financial support from the Ministry of Education, Science and Technological Development of the Republic of Serbia (Agreements No. 451-03-68/2022-14/200378 and 451-03-68/2022-14/200122).

ИЗВОД

СИНТЕЗА И БИОЛОШКА АКТИВНОСТ НОВИХ ЗИНГЕРОН-ТИОХИДАНТОИНСКИХ ХИБРИДА

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Серија зингерон-тиохидантоинских хибрида је синтетисана из деривата *O*-алкил зингерона циклокондензацијом са тиосемикарбазидом у двостепеној реакцији. Добијена нова потенцијално биоактивна једињења структурно су окарактерисана ИЦ и НМР спектроскопијом, као и елементалном анализом. Поред тога, тестиране су њихове антимикробне и *in vitro* антиканцерогене активности. Испитана једињења су показала ниску до умерену антимикробну активност. Зингерон-тиохидантоински хибрид са *O*-бутил супституентом је показао значајну цитотоксичну активност на ћелије рака дебелог црева HCT-116 без токсичности на здраве ћелије MRC-5.

(Примљено 4. априла; ревидирано 28. маја; прихваћено 9.јуна 2022.)

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J. Serb. Chem. Soc. **00(0)**S1-S27 (2022) *Supplementary material*

SUPPLEMENTARY MATERIAL TO

Synthesis and biological activity of novel zingerone-thiohydantoin hybrids

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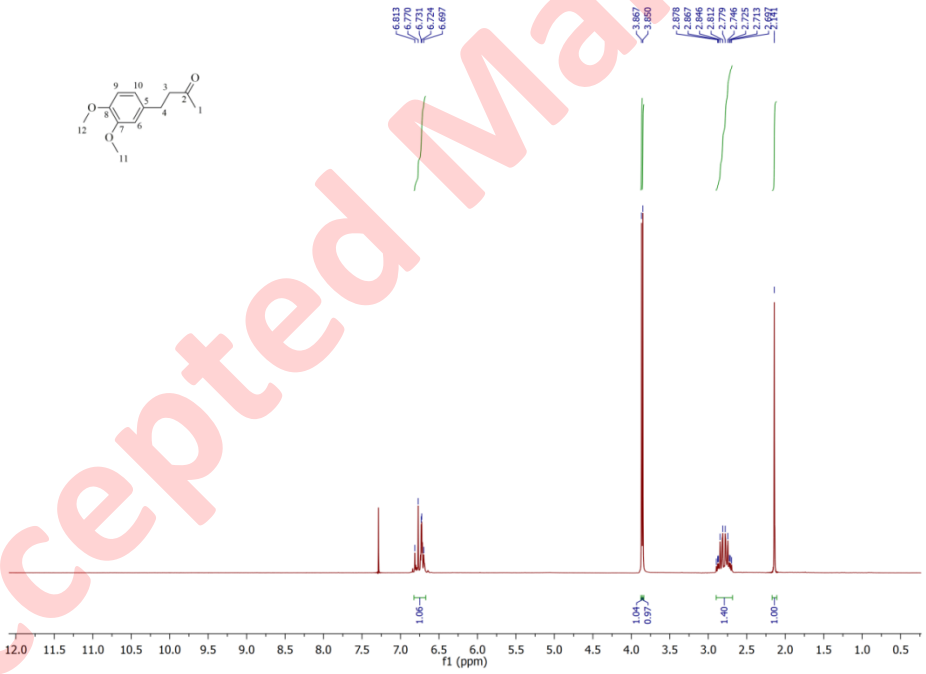

Fig. S-1. [1]H-NMR spectra of 4-(3,4-dimethoxyphenyl)butan-2-one (**1a**)

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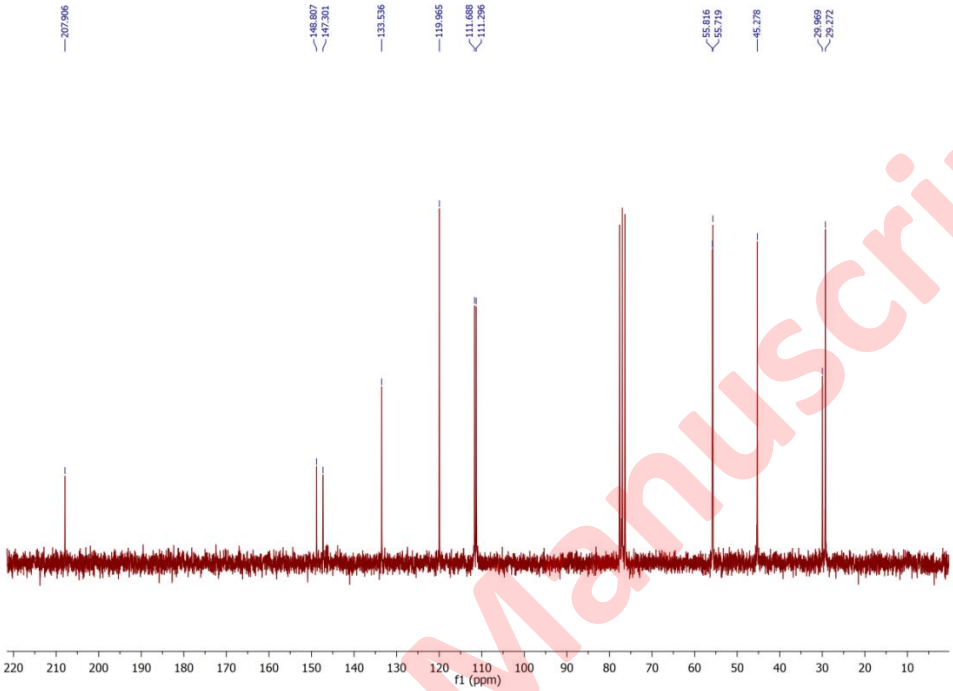


Fig. S-2. ^{13}C-NMR spectra of4-(3,4-dimethoxyphenyl)butan-2-one (**1a**)

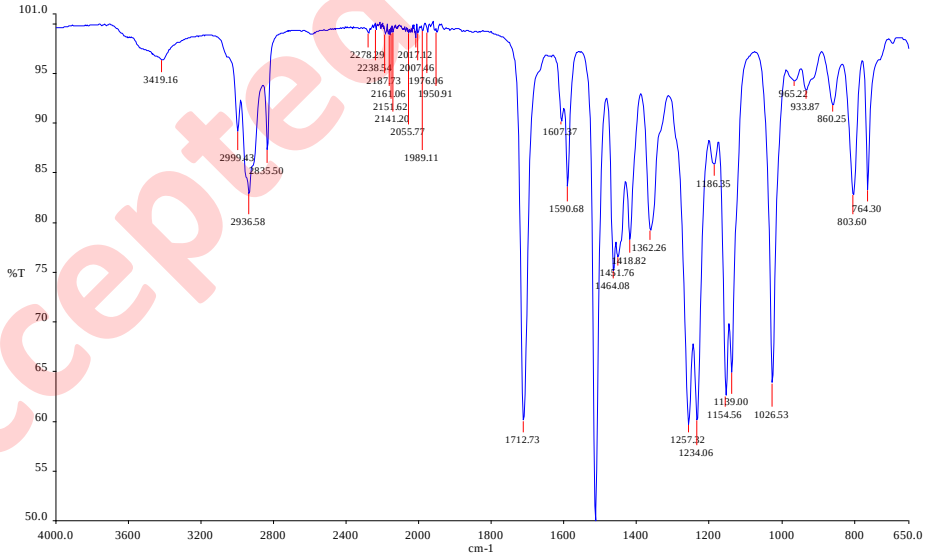


Fig. S-3. IR spectra of4-(3,4-dimethoxyphenyl)butan-2-one (**1a**)

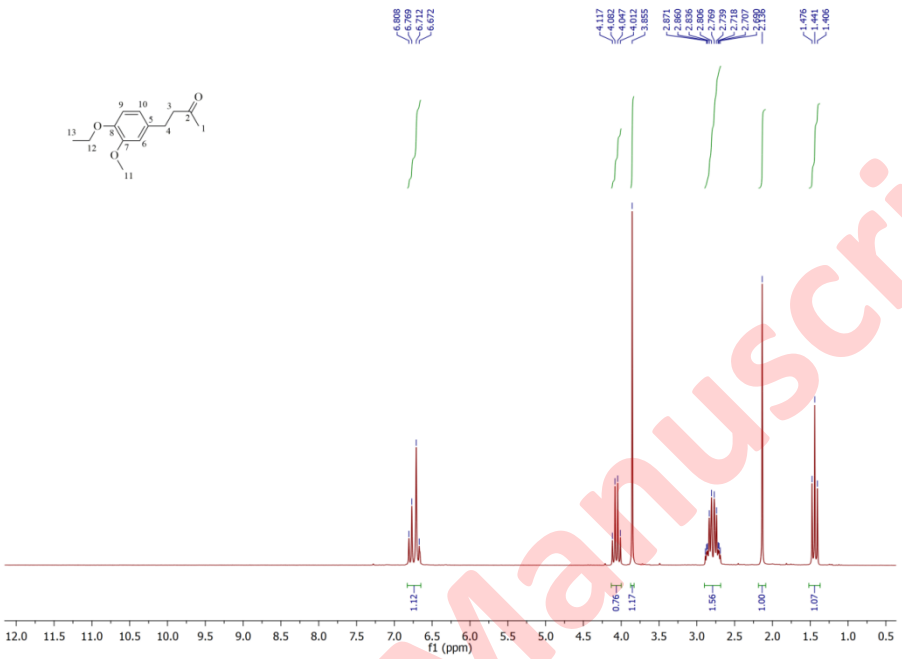


Fig. S-4. ^{1}H-NMR spectra of4-(4-ethoxy-3-methoxyphenyl)butan-2-one (**1b**)

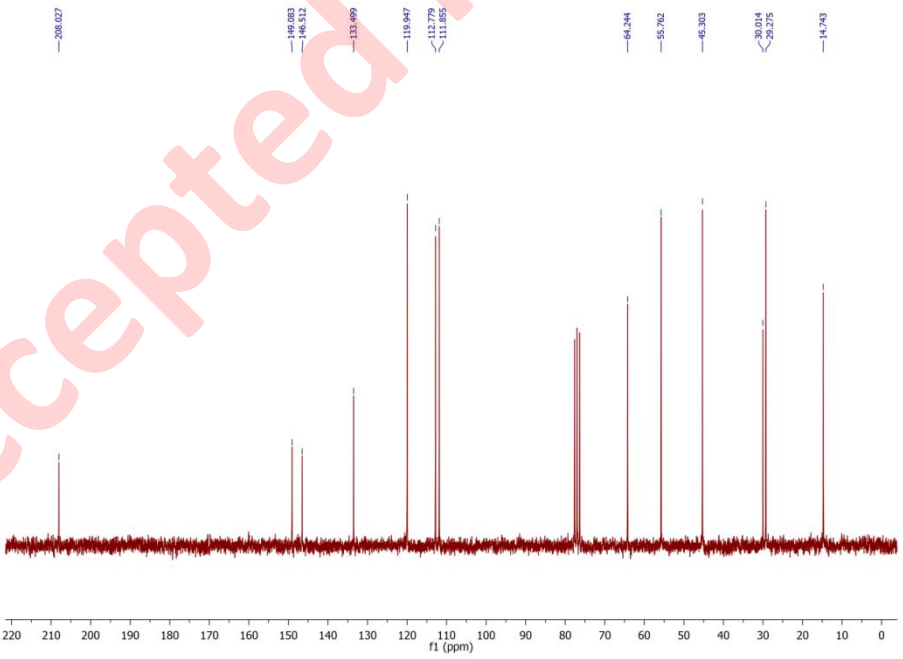


Fig. S-5. ^{13}C-NMR spectra of4-(4-ethoxy-3-methoxyphenyl)butan-2-one (**1b**)

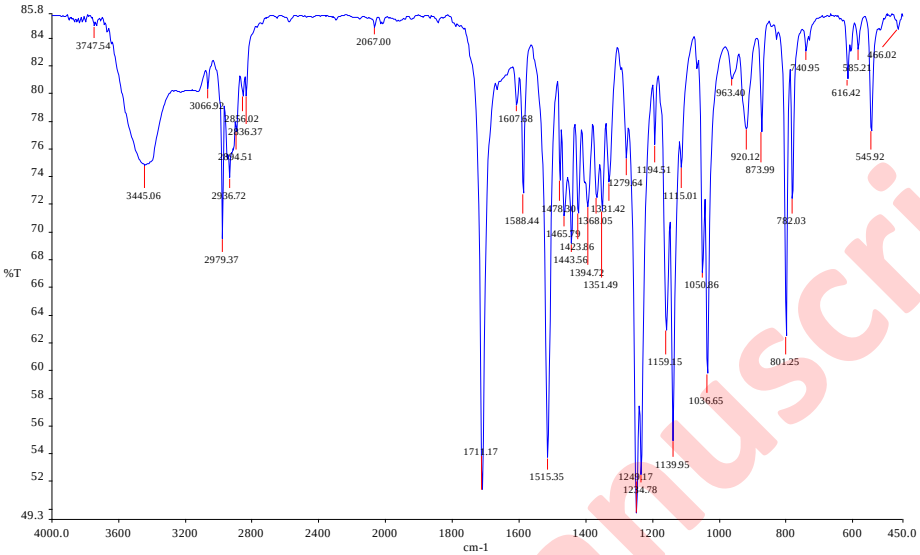


Fig. S-6. IR spectra of4-(4-ethoxy-3-methoxyphenyl)butan-2-one (**1b**)

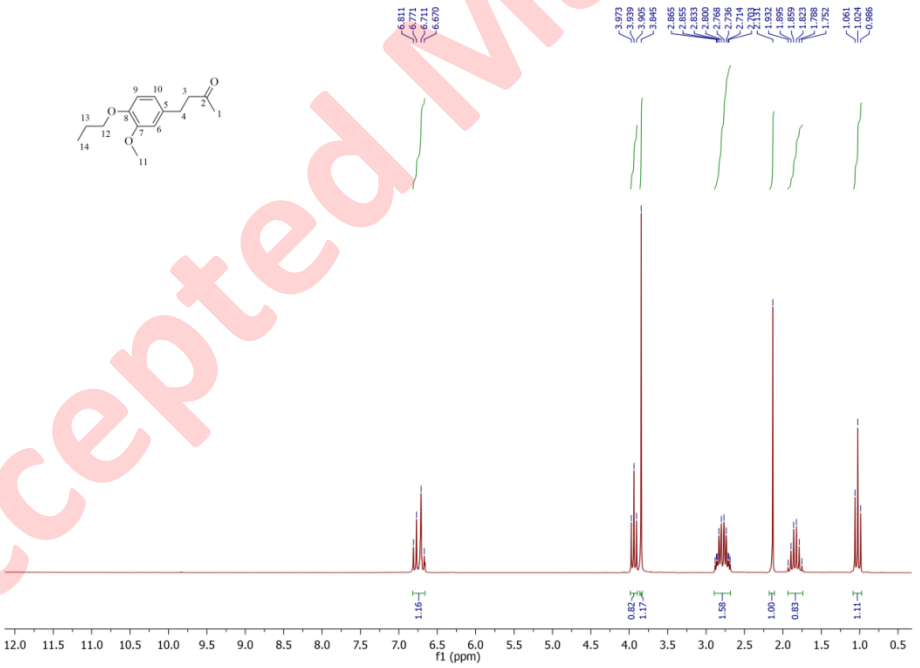


Fig. S-7. [1]H-NMR spectra of 4-(3-methoxy-4-propoxyphenyl)butan-2-one (**1c**)

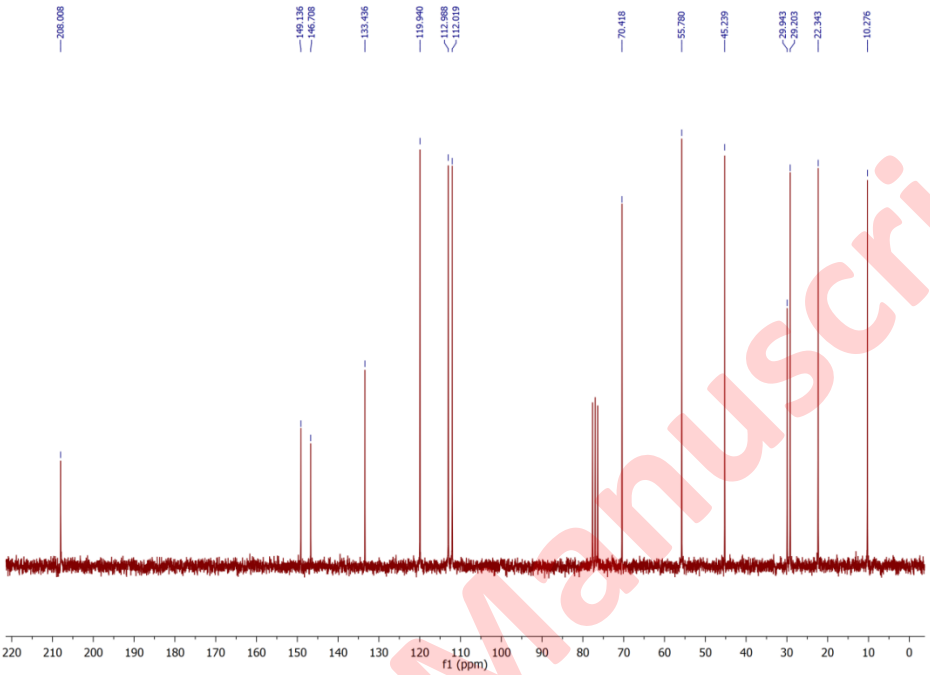


Fig. S-8. ^{13}C-NMR spectra of4-(3-methoxy-4-propoxyphenyl)butan-2-one (**1c**)

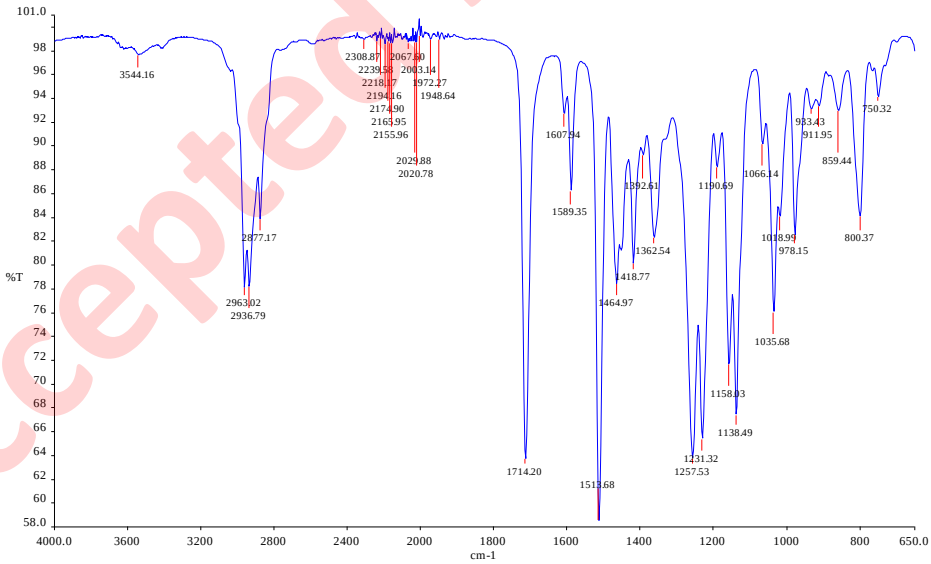


Fig. S-9. IR spectra of4-(3-methoxy-4-propoxyphenyl)butan-2-one (**1c**)

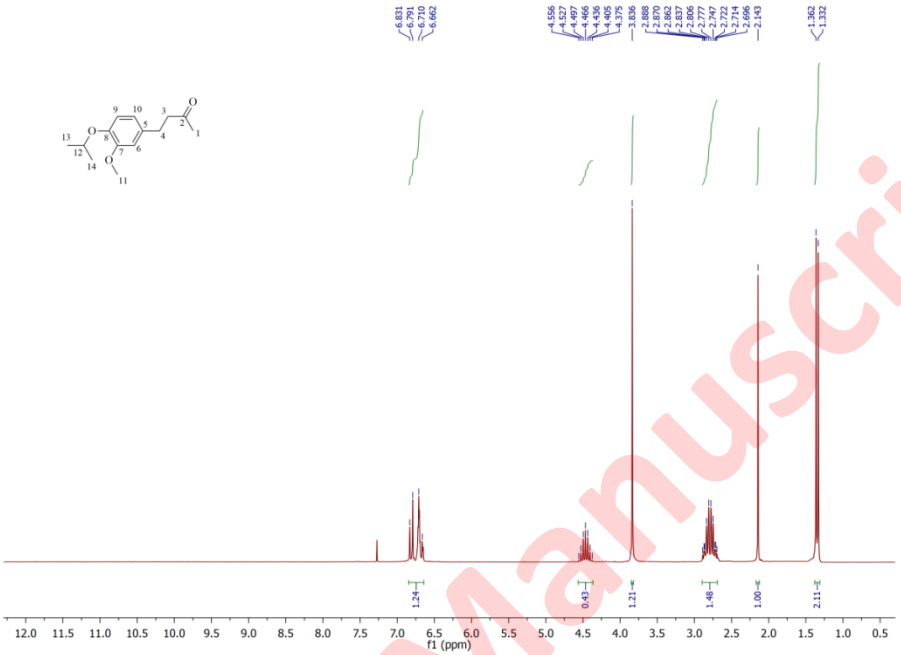


Fig. S-10. ^{1}H-NMR spectra of 4-(4-isopropoxy-3-methoxyphenyl)butan-2-one (**1d**)

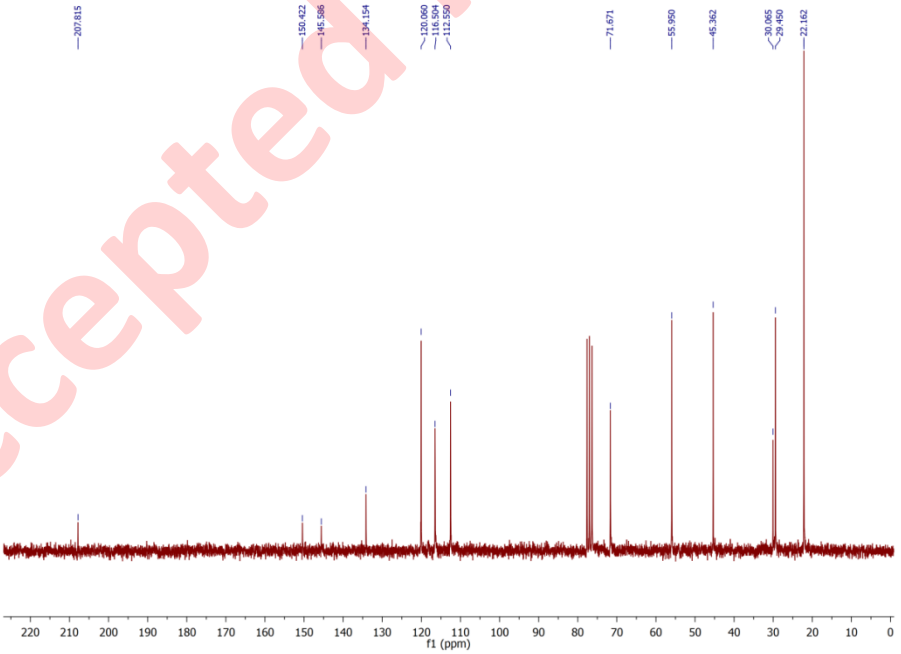


Fig. S-11. ^{13}C-NMR spectra of4-(4-isopropoxy-3-methoxyphenyl)butan-2-one (**1d**)

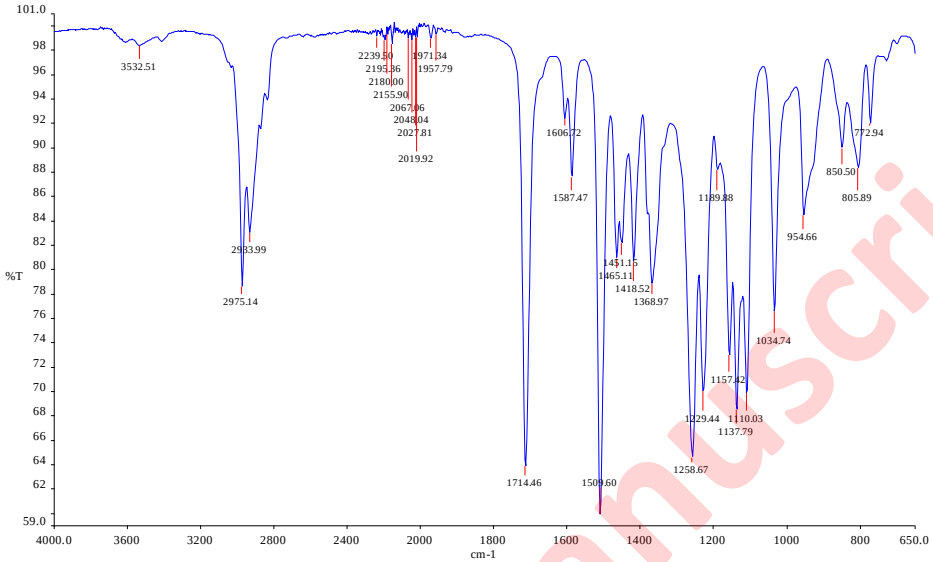


Fig. S-12. IR spectra of4-(4-isopropoxy-3-methoxyphenyl)butan-2-one (**1d**)

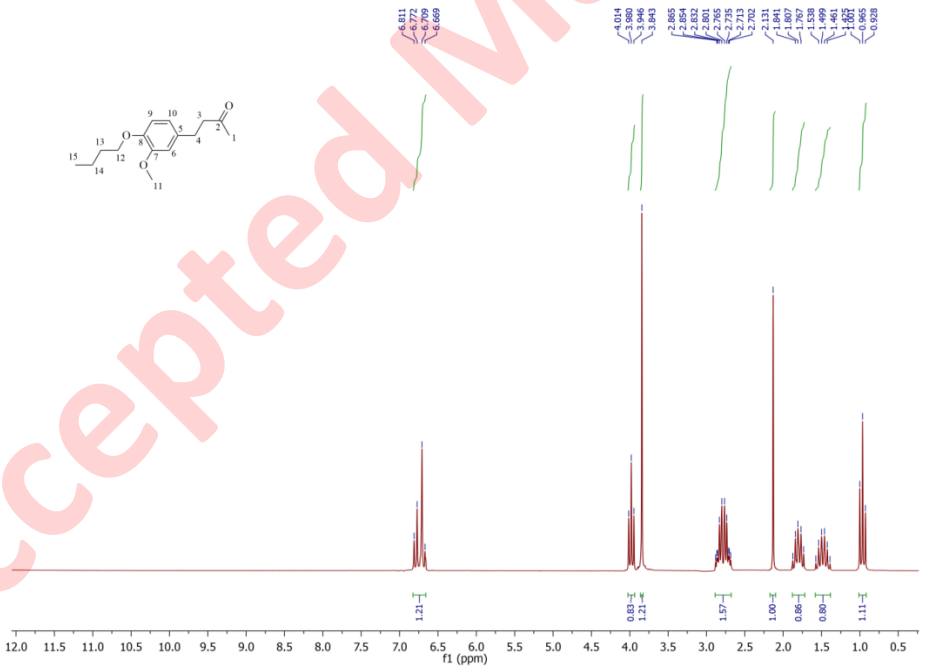


Fig. S-13. ^{1}H-NMR spectra of 4-(4-butoxy-3-methoxyphenyl)butan-2-one (**1e**)

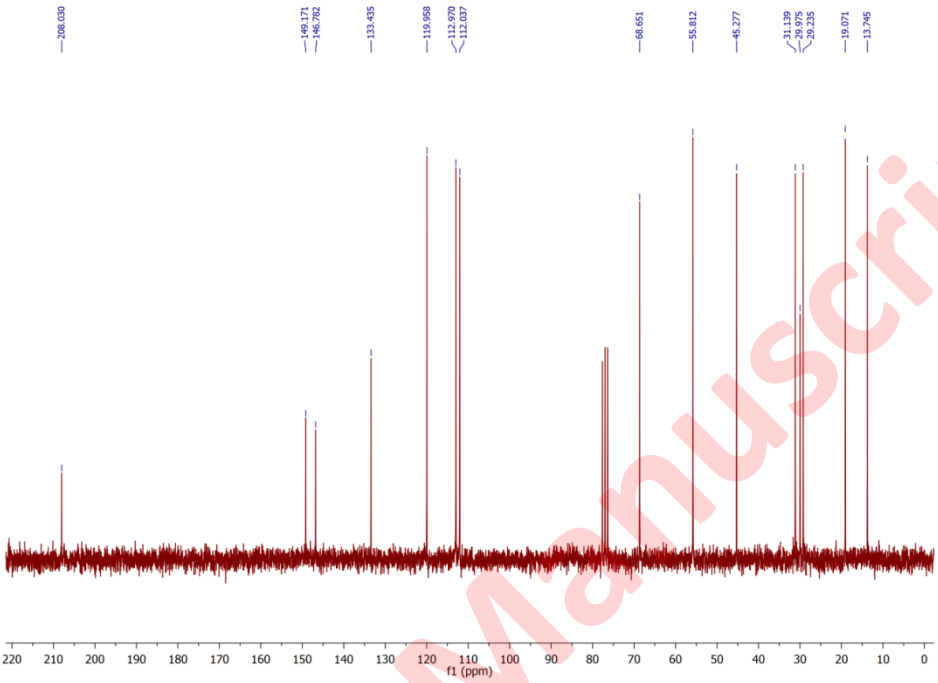


Fig. S-14. ^{13}C-NMR spectra of4-(4-butoxy-3-methoxyphenyl)butan-2-one (**1e**)

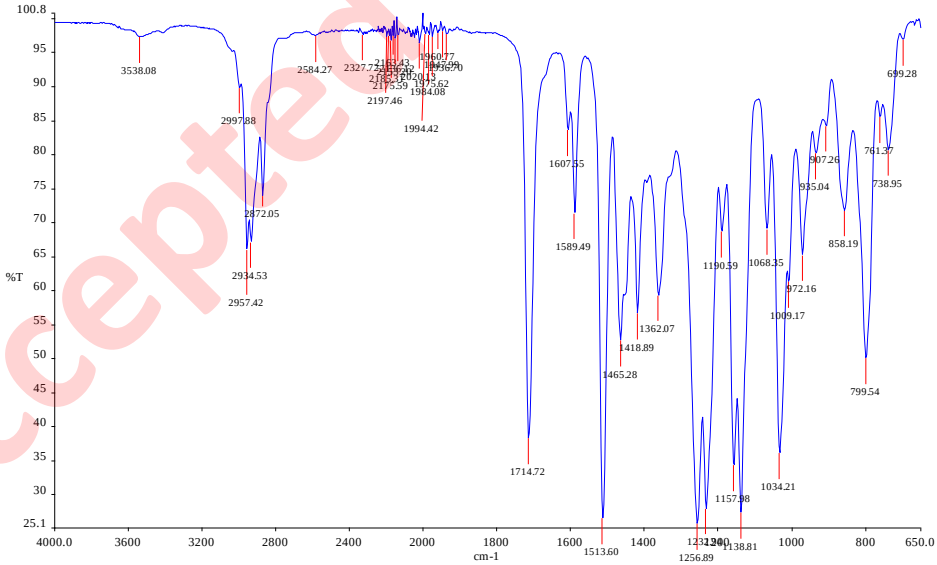


Fig. S-15. IR spectra of4-(4-butoxy-3-methoxyphenyl)butan-2-one (**1e**)

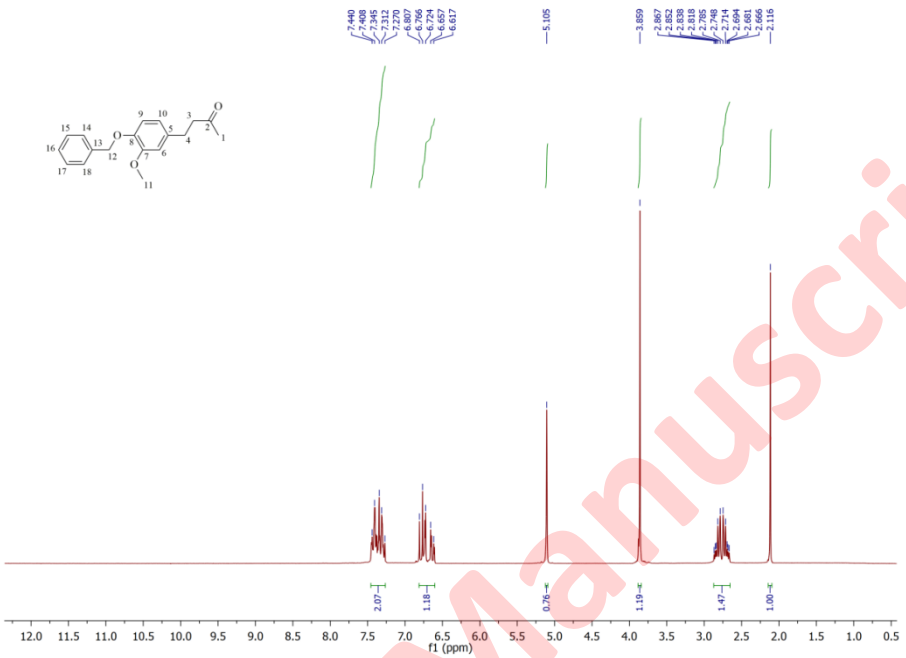


Fig. S-16. ^{1}H-NMR spectra of 4-(4-(benzyloxy)-3-methoxyphenyl)butan-2-one (**1f**)

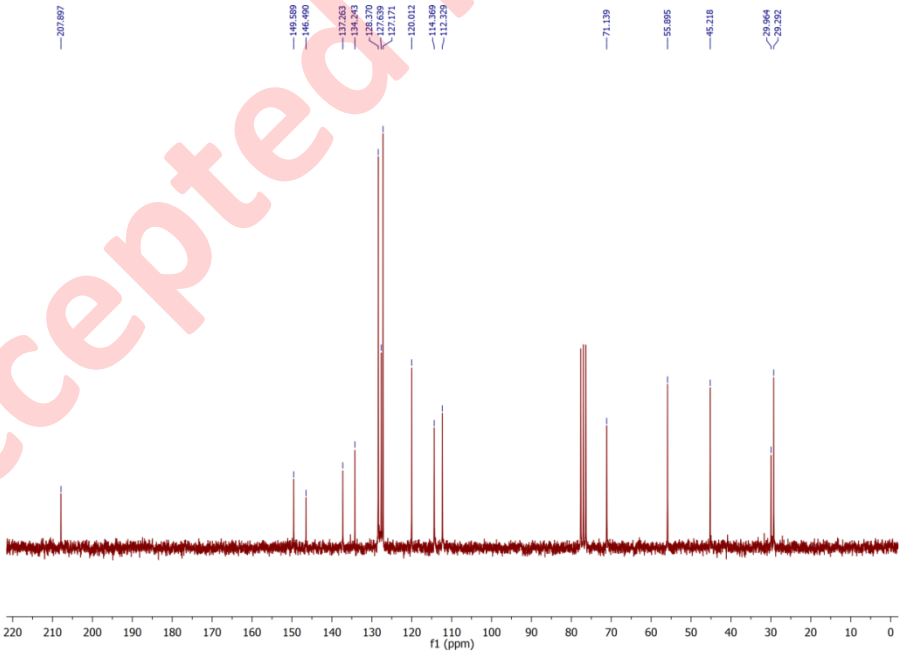


Fig. S-17. ^{13}C-NMR spectra of4-(4-(benzyloxy)-3-methoxyphenyl)butan-2-one (**1f**)

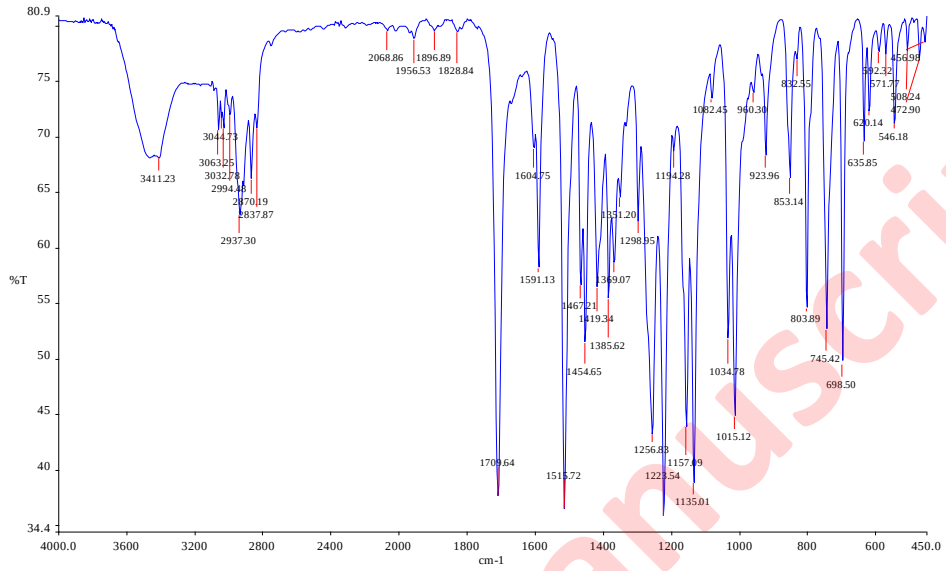


Fig. S-18. IR spectra of4-(4-(benzyloxy)-3-methoxyphenyl)butan-2-one (**1f**)

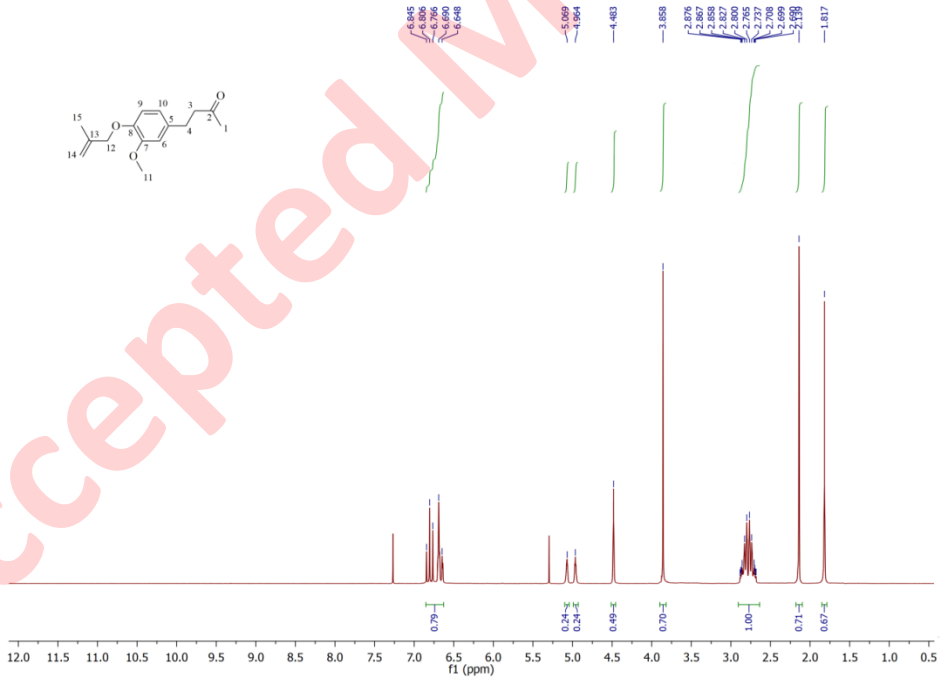


Fig. S-19. ^{1}H-NMR spectra of 4-(3-methoxy-4-((2-methylallyl)oxy)phenyl)butan-2-one (**1g**)

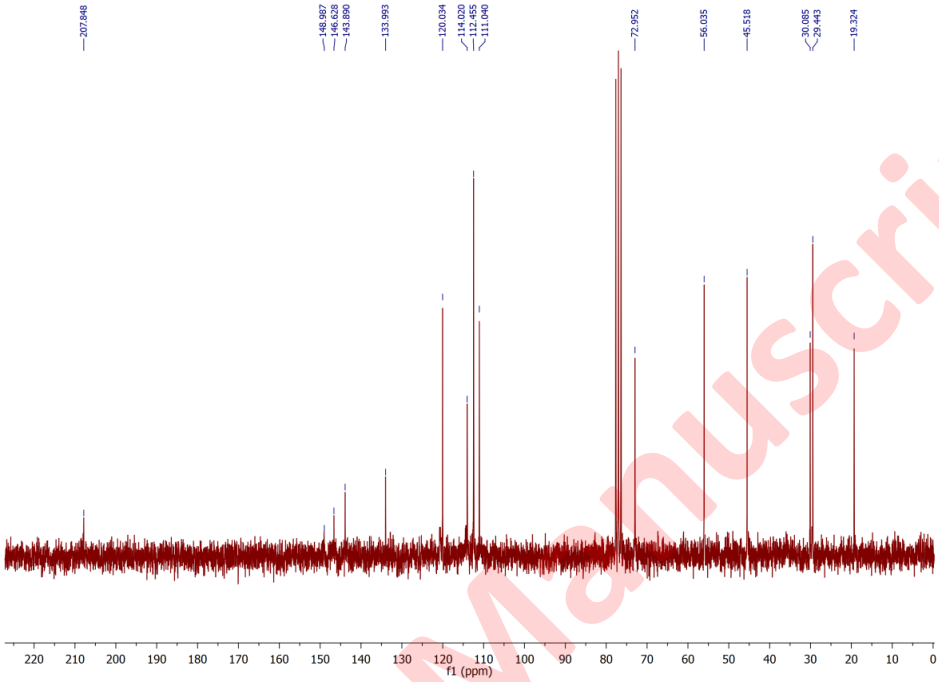


Fig. S-20. ^{13}C-NMR spectra of4-(3-methoxy-4-((2-methylallyl)oxy)phenyl)butan-2-one (**1g**)

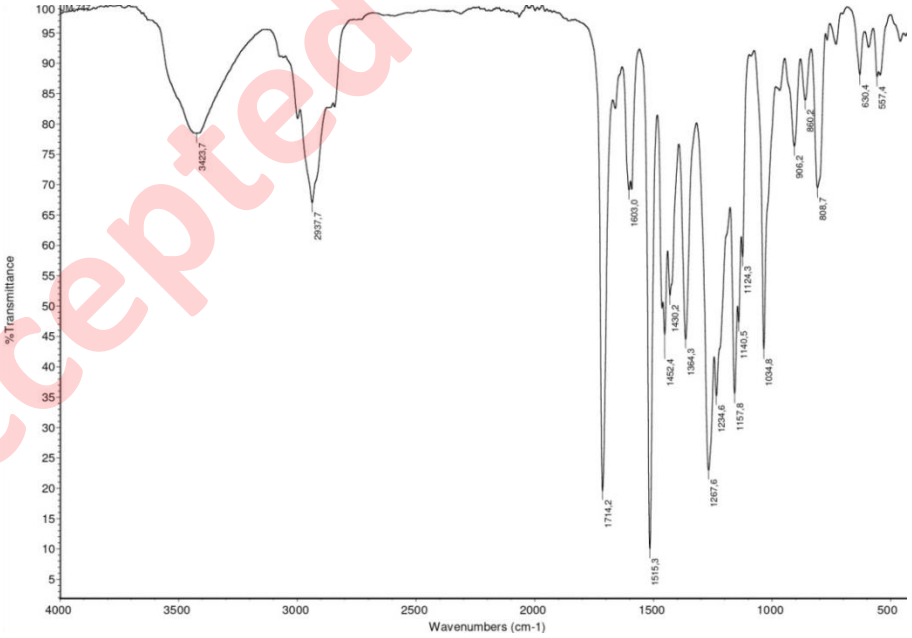


Fig. S-21. IR spectra of4-(3-methoxy-4-((2-methylallyl)oxy)phenyl)butan-2-one (**1g**)

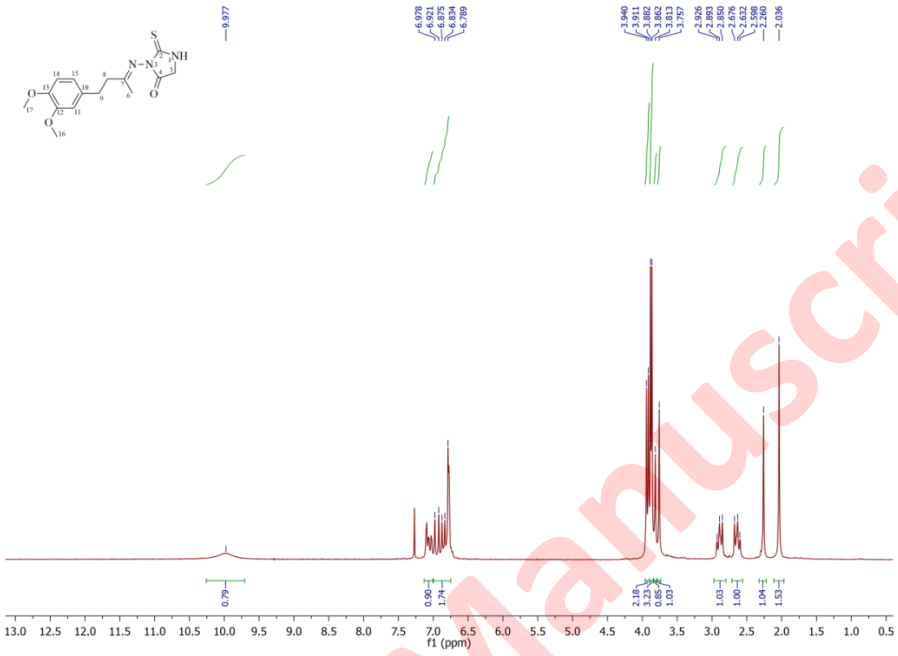


Fig. S-22. ^{1}H-NMR spectra of 3-((4-(3,4-dimethoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2a**)

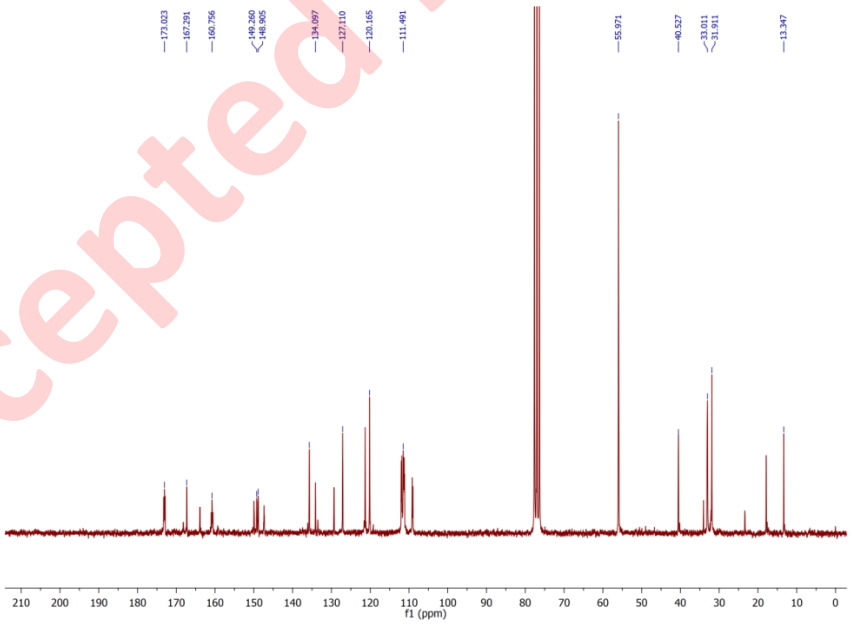


Fig. S-23. ^{13}C-NMR spectra of 3-((4-(3,4-dimethoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2a**)

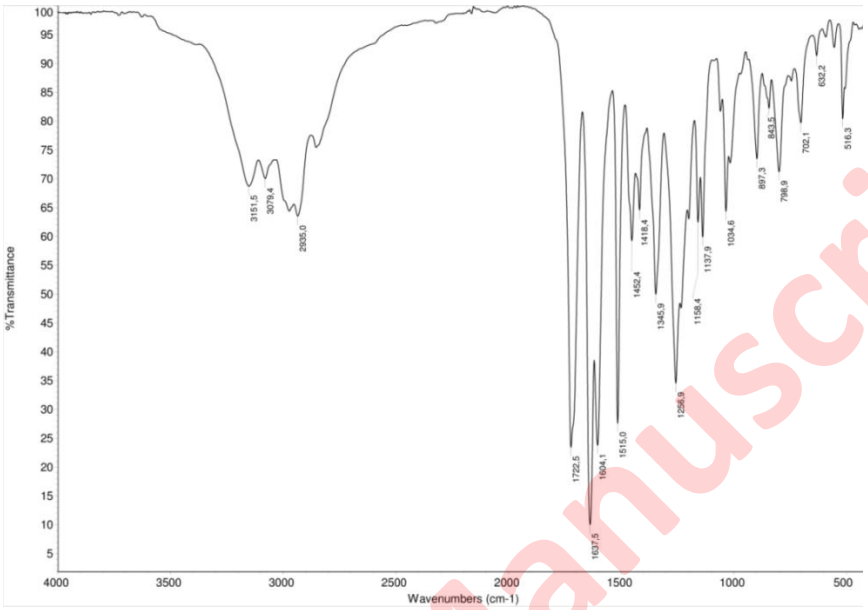


Fig. S-24. IR spectra of3-((4-(3,4-dimethoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2a**)

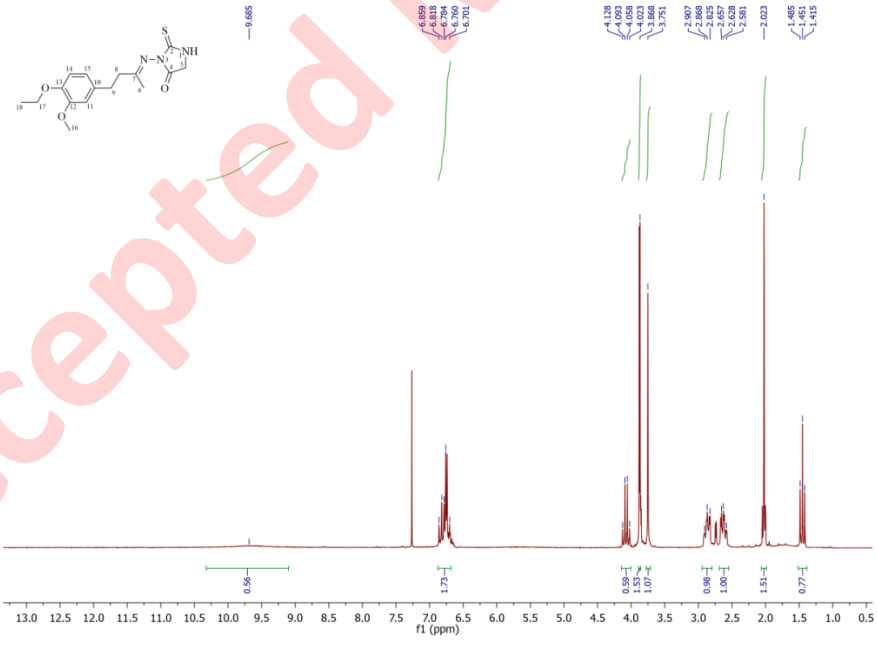


Fig. S-25. ^{1}H-NMR spectra of *3*-((4-(4-ethoxy-3-methoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2b**)

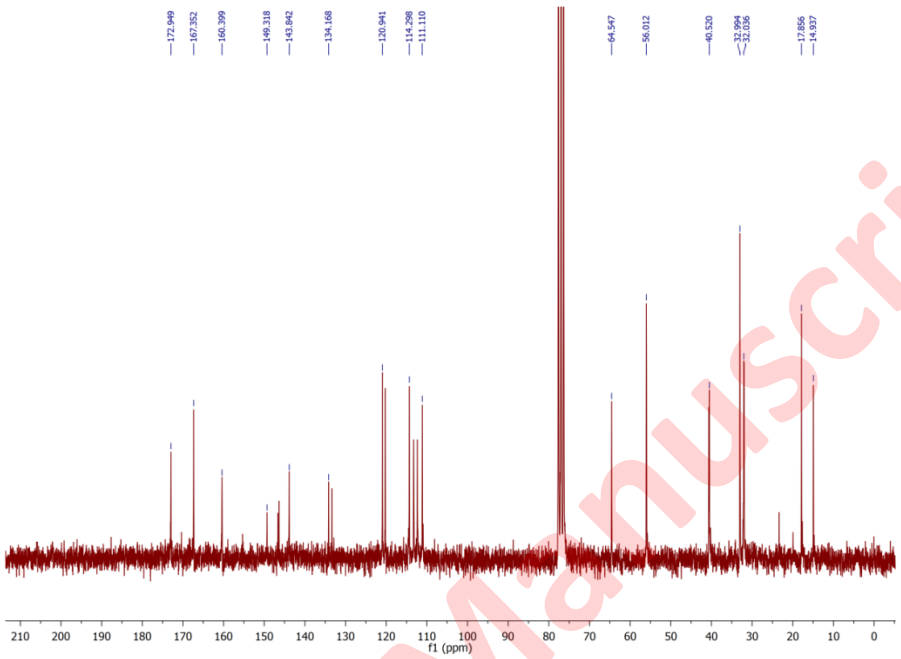


Fig. S-26. ^{13}C-NMR spectra of *3*-((4-(4-ethoxy-3-methoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2b**)

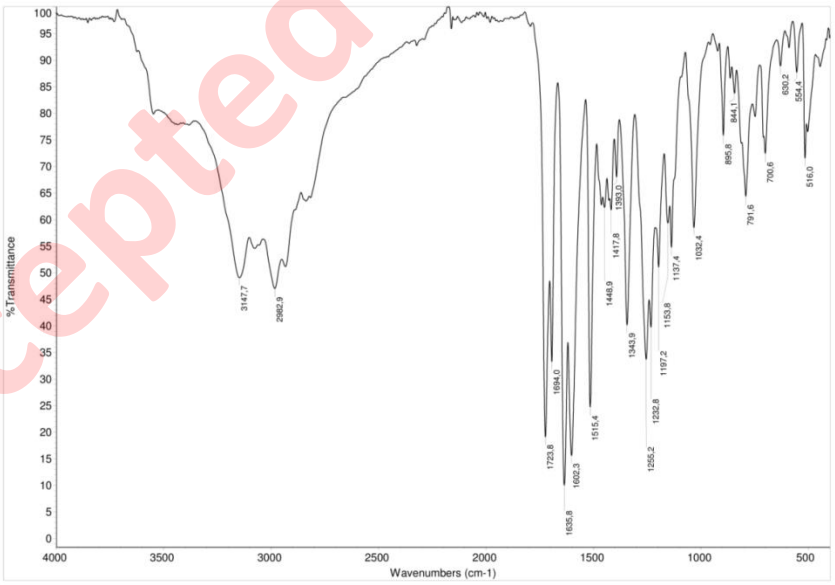


Fig. S-27. IR spectra of *3*-((4-(4-ethoxy-3-methoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2b**)

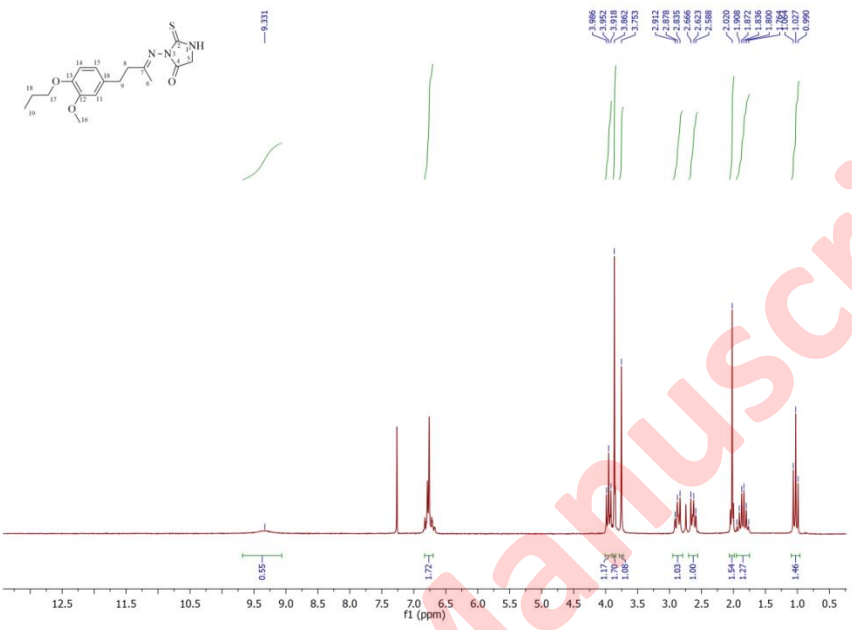


Fig. S-28. ^{1}H-NMR spectra of
3-((4-(3-methoxy-4-propoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2c**)

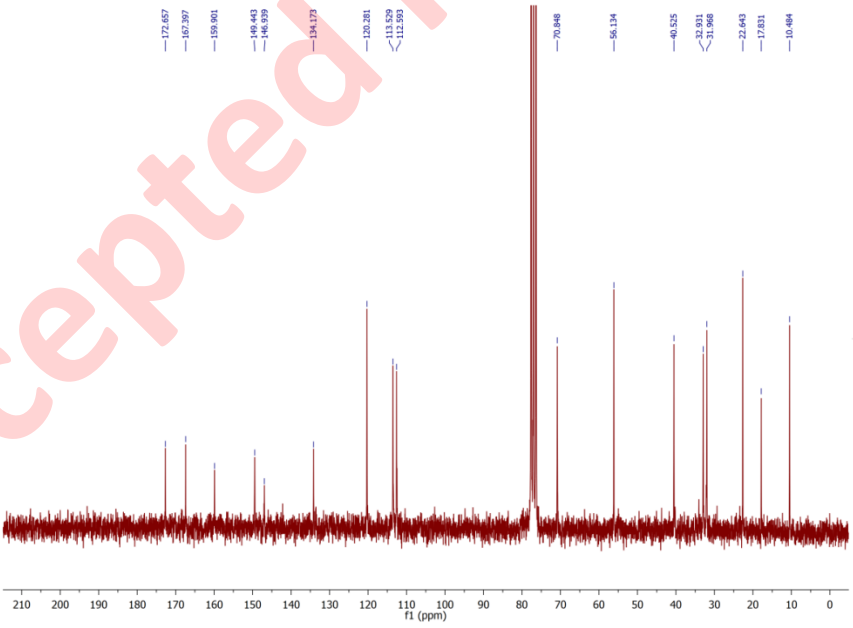


Fig. S-29. ^{13}C -NMR spectra of
3-((4-(3-methoxy-4-propoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2c**)

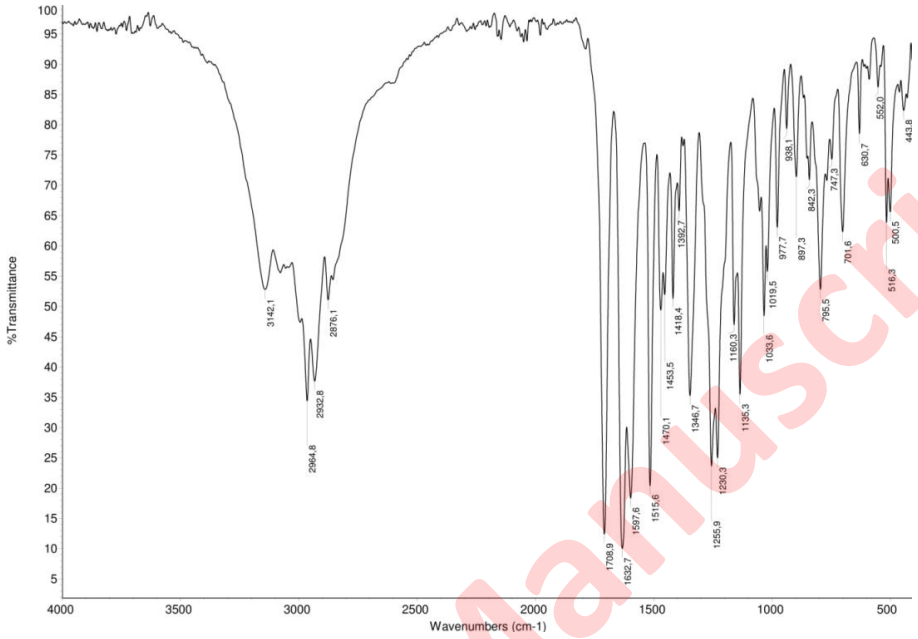


Fig. S-30. IR spectra of
3-((4-(3-methoxy-4-propoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2c**)

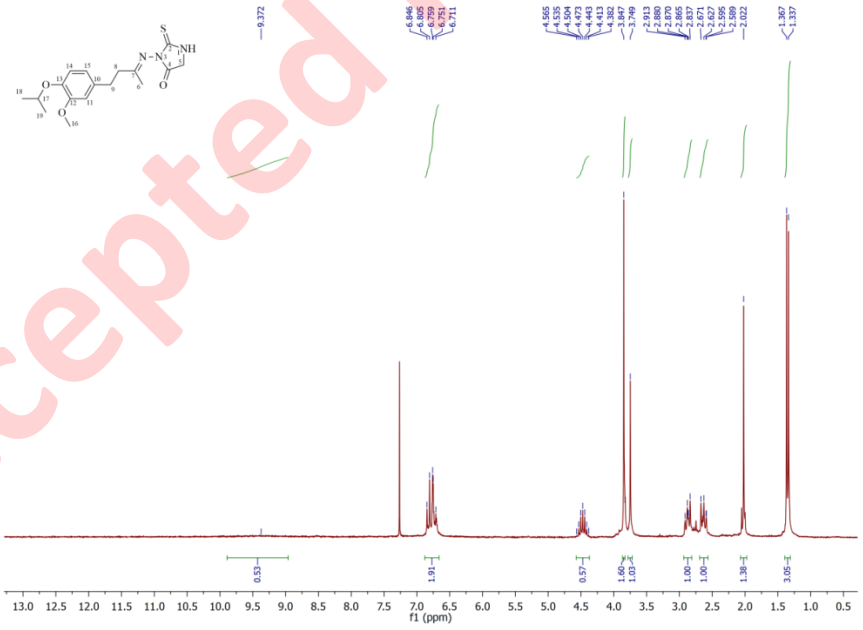


Fig. S-31. ^{1}H-NMR spectra of3-((4-(4-isopropoxy-3-methoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2d**)

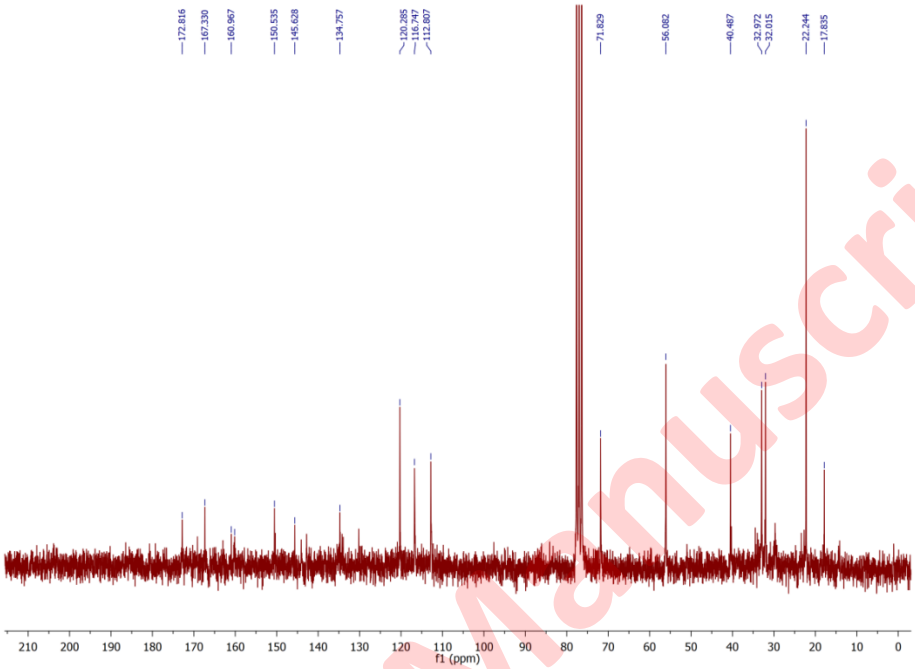


Fig. S-32. ^{13}C -NMR spectra of3-((4-(4-isopropoxy-3-methoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2d**)

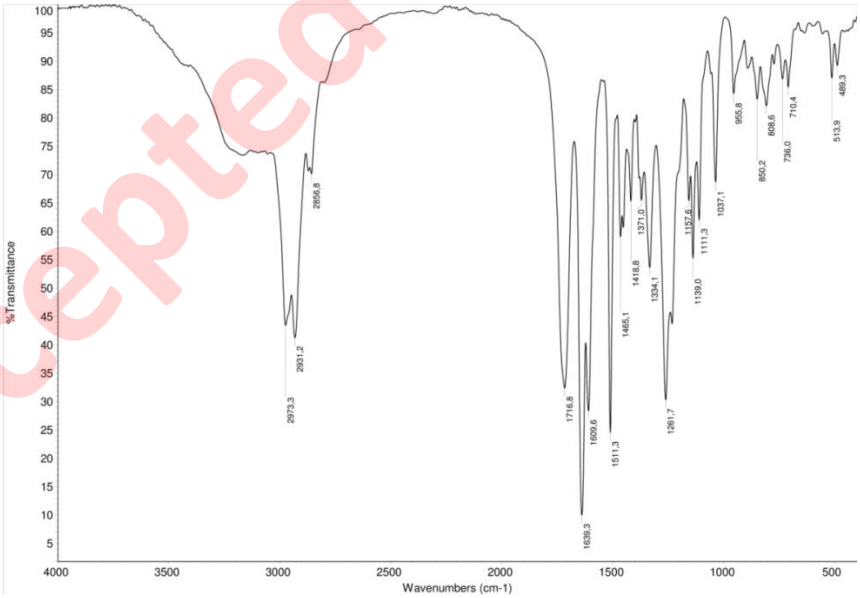


Fig. S-33. IR spectra of3-((4-(4-isopropoxy-3-methoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2d**)

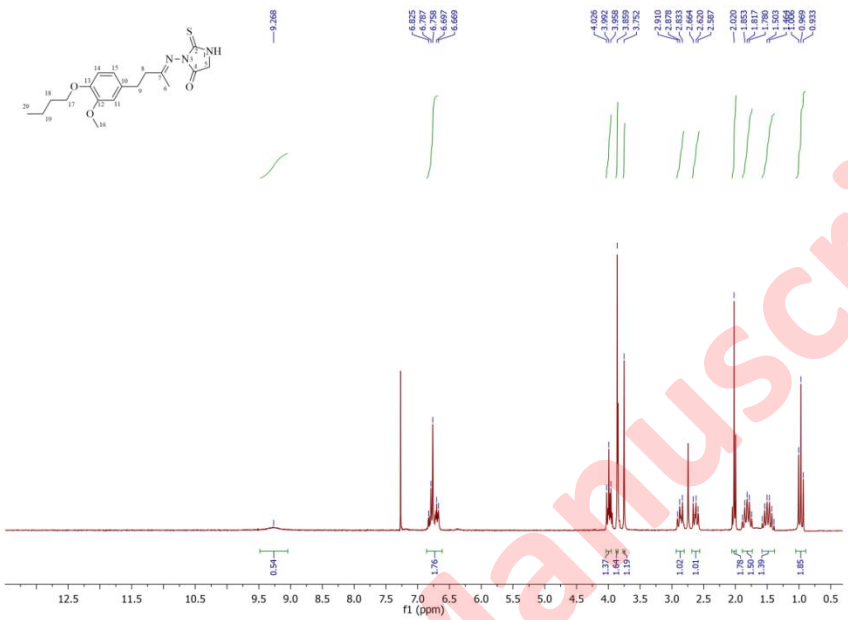


Fig. S-34. ^{1}H-NMR spectra of 3-((4-(4-butoxy-3-methoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2e**)

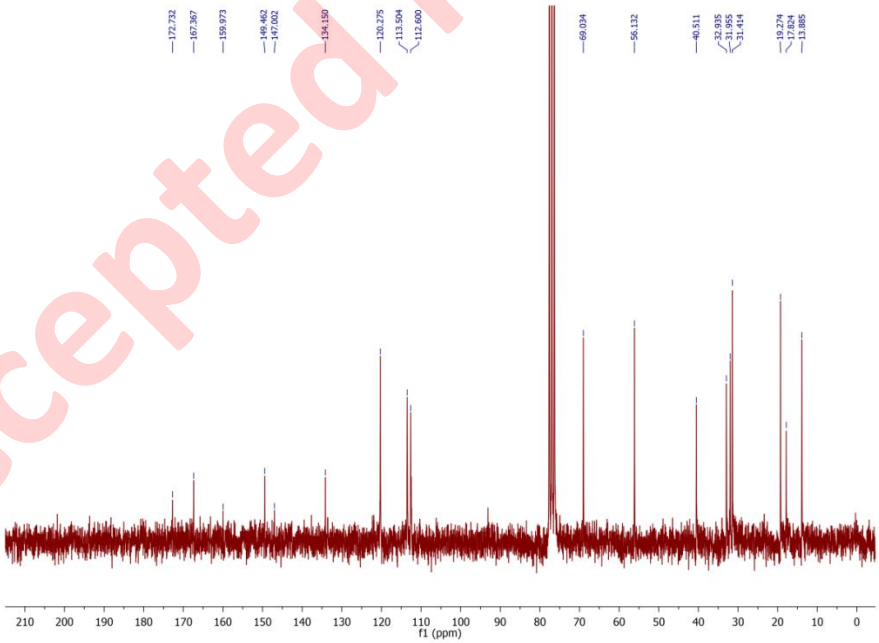


Fig. S-35. ^{13}C -NMR spectra of 3-((4-(4-butoxy-3-methoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2e**)

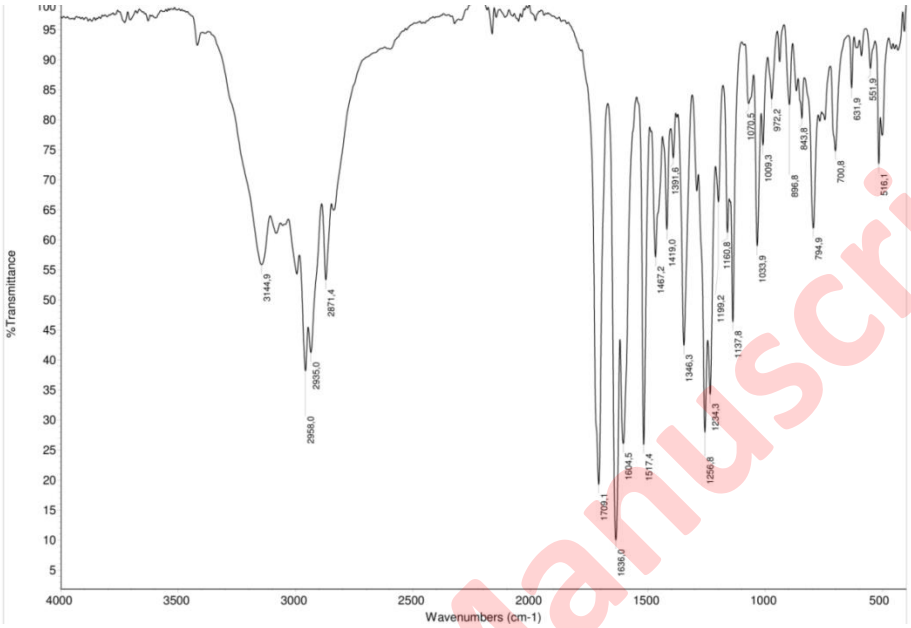


Fig. S-36. IR spectra of 3-((4-(4-butoxy-3-methoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2e**)

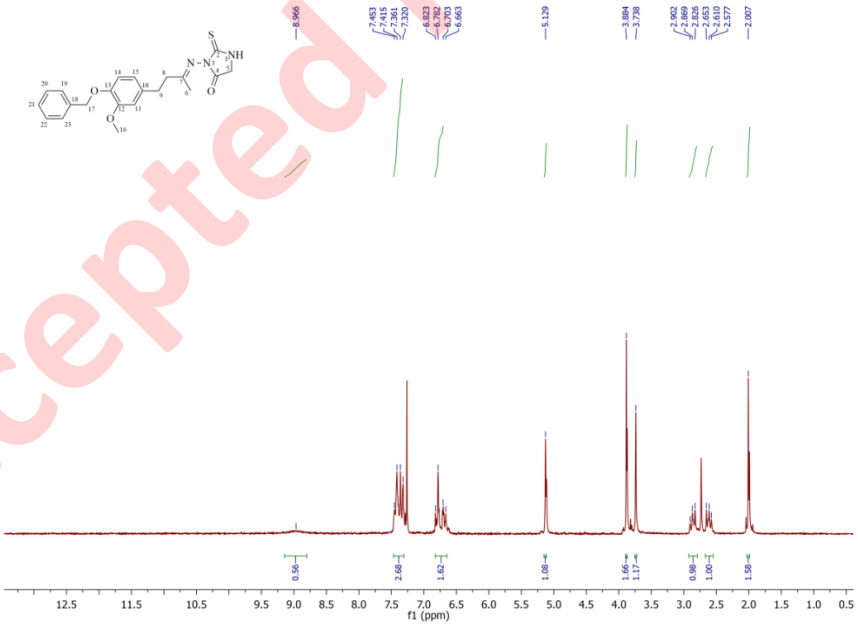


Fig. S-37. ^{1}H-NMR spectra of3-((4-(4-(benzyloxy)-3-methoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2f**)

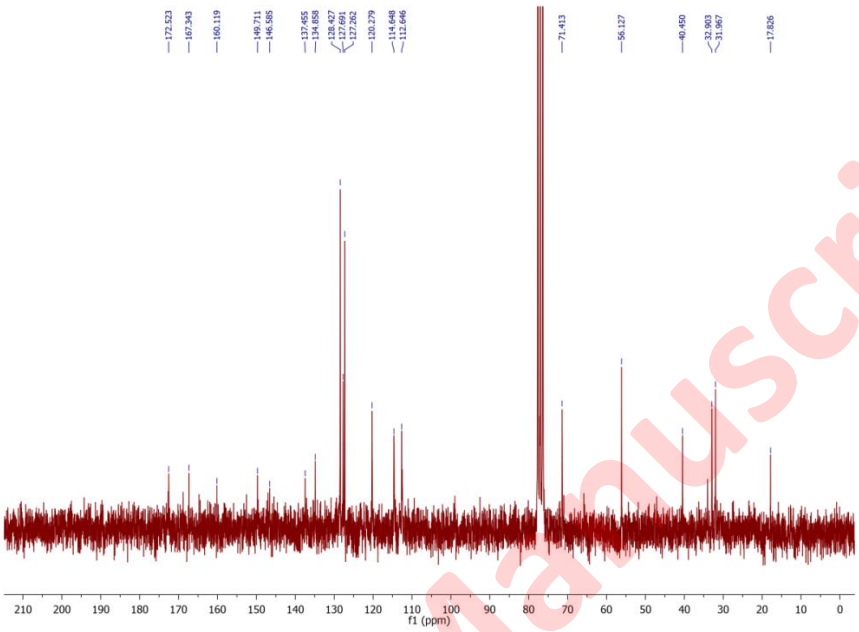


Fig. S-38. ^{13}C -NMR spectra of3-((4-(4-(benzyloxy)-3-methoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2f**)

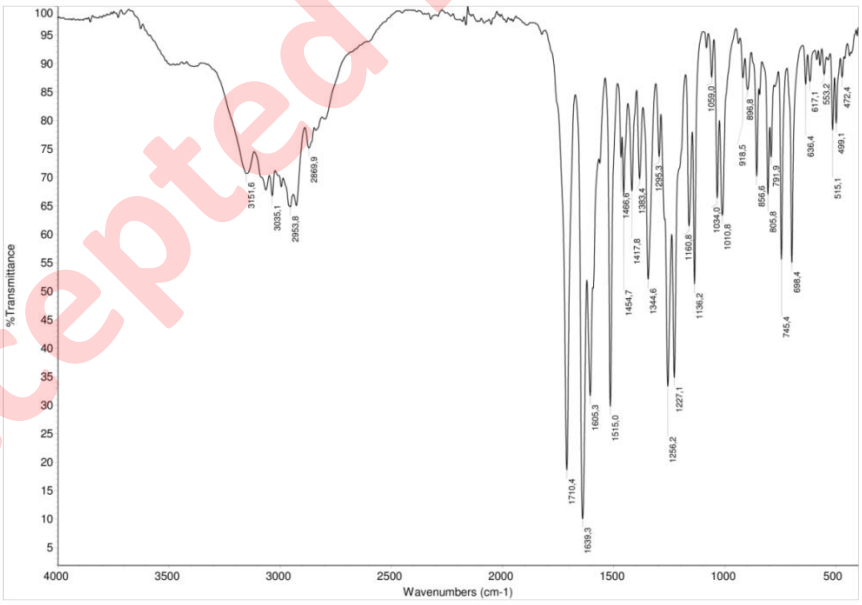


Fig. S-39. IR spectra of3-((4-(4-(benzyloxy)-3-methoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2f**)

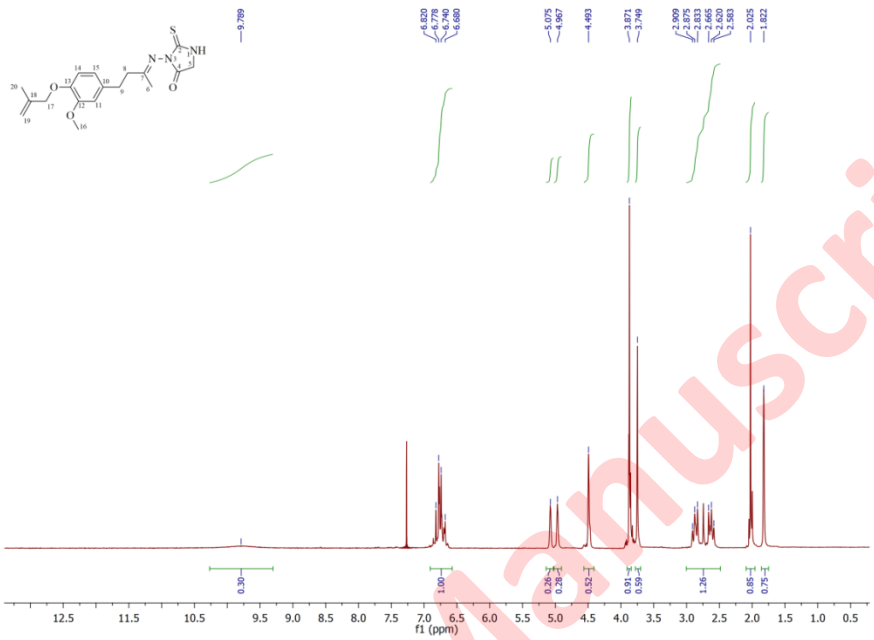


Fig. S-40. ^{1}H-NMR spectra of3-((4-(3-methoxy-4-((2-methylallyl)oxy)phenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2g**)

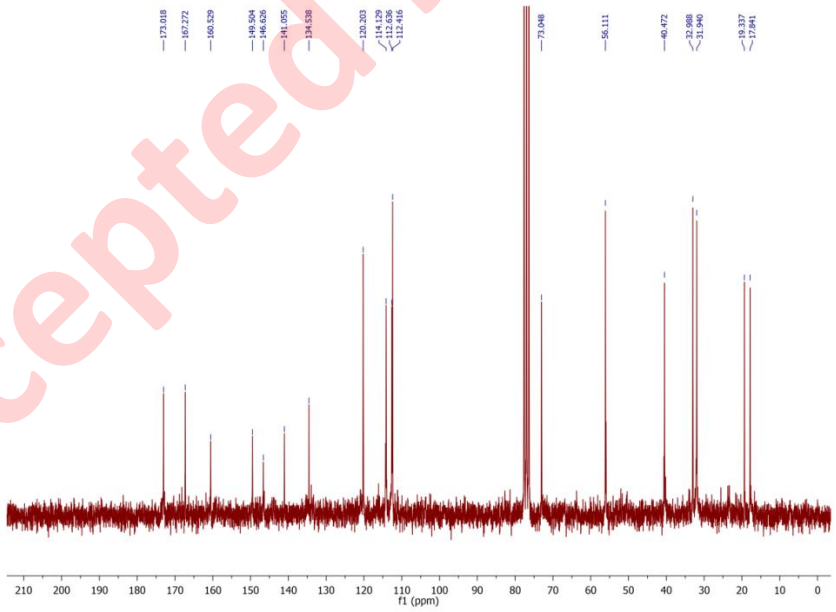


Fig. S-41. ^{13}C-NMR spectra of3-((4-(3-methoxy-4-((2-methylallyl)oxy)phenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2g**)

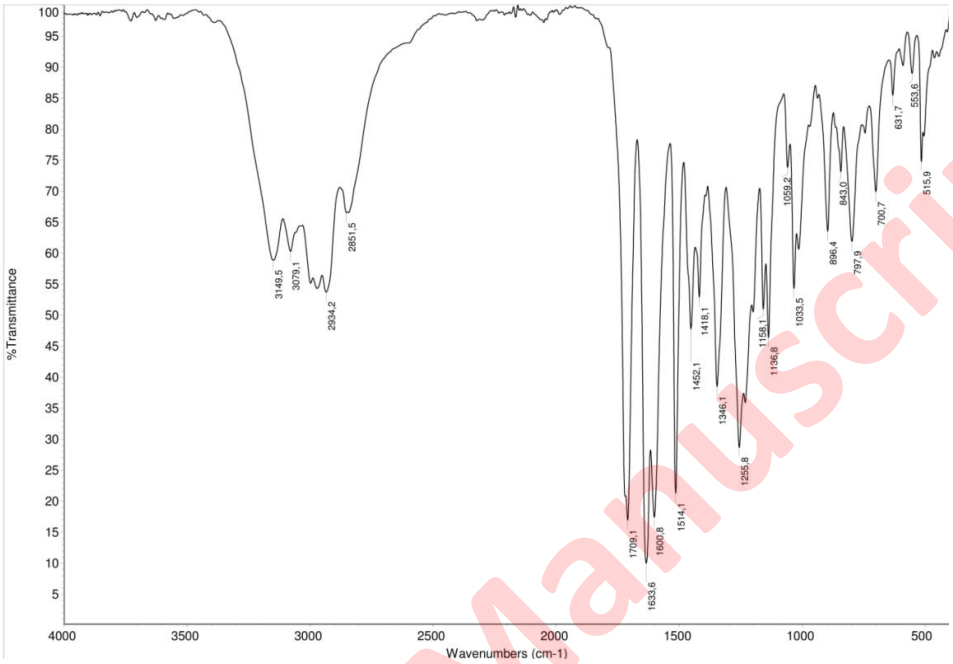


Fig. S-42. IR spectra of3-((4-(3-methoxy-4-((2-methylallyl)oxy)phenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2g**)

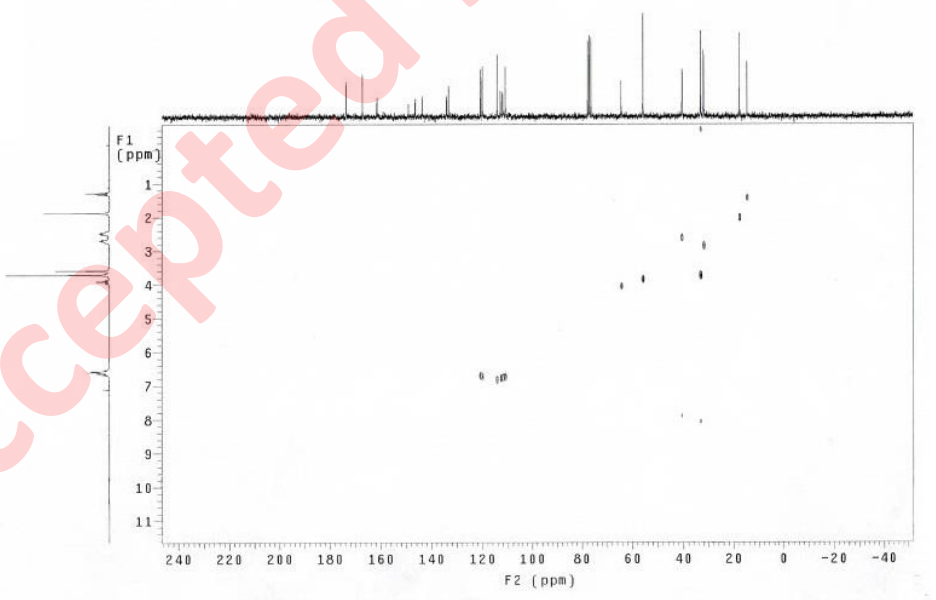


Fig. S-43. 2D HETCOR spectra of *3*-((4-(4-ethoxy-3-methoxyphenyl)butan-2-ylidene)amino)-2-thioxoimidazolidin-4-one (**2b**)

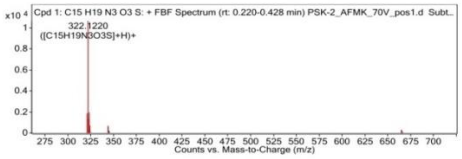
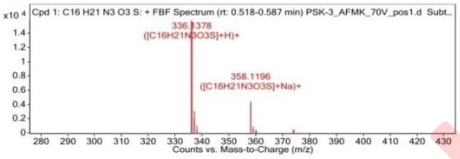
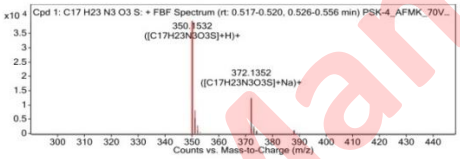
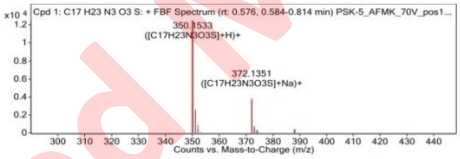
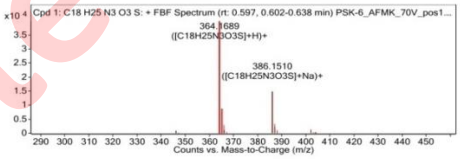
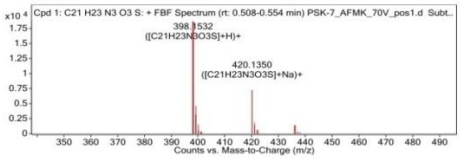
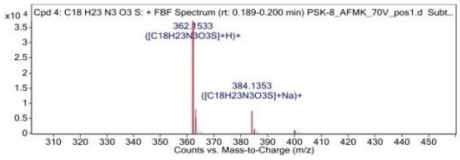
DFT calculation

All calculations were conducted using Gaussian 09[1] with the B3LYP functional[2,3] and the split-valence triple-zeta basis set 6-311+G.[4,5] To attain better description of the delocalization effects that are crucial for the geometry and electronic structure of the investigated molecules, diffuse functions were added to the heavy atoms. The p and d polarization functions were also used. Full geometry optimizations, without any symmetry constraints, and frequency calculations were performed for all species in gas phase. Frequency calculations were performed to confirm that the optimized structures are energetic minima (no imaginary frequencies).

LC-HRMS analysis

Samples dissolved in the methanol (c @ 0.1 mg mL^{-1}) were directly, without separation, injected into analysing system including liquid chromatograph (1290 Infinity LC system; Agilent Technologies, Waldbronn, Germany) with a quarternary pump, a column oven, and an autosampler, connected to the Quadrupole Time-of-Flight mass detector (6550 iFunnel QTOF MS, Agilent Technologies; Santa Clara, CA, USA) equipped with a dual spray Agilent Jet Stream (AJS) electrospray ion source. Mobile phase was composed of a solvents A (water containing both 0.1 % formic acid and 5 mM ammonium formate) and B (ACN containing 0.1 % formic acid), 1:1 (v/v). The mobile phase flow rate was 0.20 mL min^{-1}, the column oven temperature was 25 °C and the injection volumes of samples were 0.2 μL. The compounds were analysed using a mass detector. Positive ion mode was recorded, and the instrument was operated in accurate TOF/MS scanning mode in the *m/z* range of 100 – 1,500, under following conditions: capillary voltage, 3,500 V, fragmentor voltage, 70 V, nozzle voltage, 1,000 V, skimmer 1, 65 V, octupole RF peak, 750 V, desolvatation gas (nitrogen) temperature, 200 °C, desolvatation gas (nitrogen) flow, 14 L min^{-1}, nebulizer, 241.32 kPa, sheat gas (nitrogen) temperature, 350 °C, sheat gas (nitrogen) flow, 11 L min^{-1}. Ions *m/z* 121.05087300 and 922.00979800 were used as a lock mass for accurate mass measurements. A personal computer system running Agilent MassHunter software (revisions B.06.01 and B.07.00) was used for data acquisition and processing, respectively.

Table S-I. LC-HRMS analysis of **2a-g**

Sample code	Molecular formula	Molecular mass calculated	Molecular mass measured	$[M+H]^+$ *m/z* calculated	$[M+H]^+$ *m/z* measured	Difference (ppm)
2a	$C_{15}H_{19}N_3O_3S$	321.1147	321.1138	320.1074	322.1220	-0.04
						
2b	$C_{16}H_{21}N_3O_3S$	335.1304	335.1305	336.1376	336.1378	-0.48
						
2c	$C_{17}H_{23}N_3O_3S$	349.1460	349.1459	350.1533	350.1532	+0.25
						
2d	$C_{17}H_{23}N_3O_3S$	349.1460	349.1459	350.1533	350.1533	-0.03
						
2e	$C_{18}H_{25}N_3O_3S$	363.1617	363.1616	364.1689	364.1689	+0.11
						
2f	$C_{21}H_{23}N_3O_3S$	397.1460	397.1459	398.1533	398.1532	-0.03
						
2g	$C_{18}H_{23}N_3O_3S$	361.1460	361.1460	362.1533	362.1533	-0.03
						

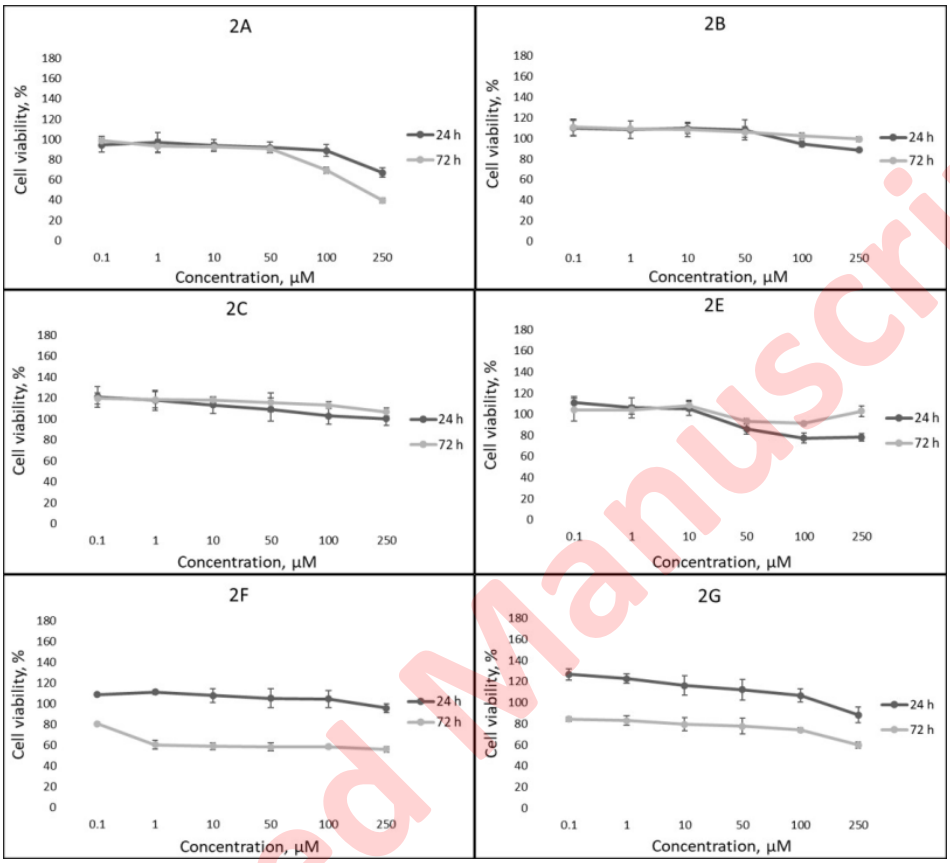


Fig. S-44. The effects of zingerone-thiohydantoin derivatives on MRC-5 cell viability

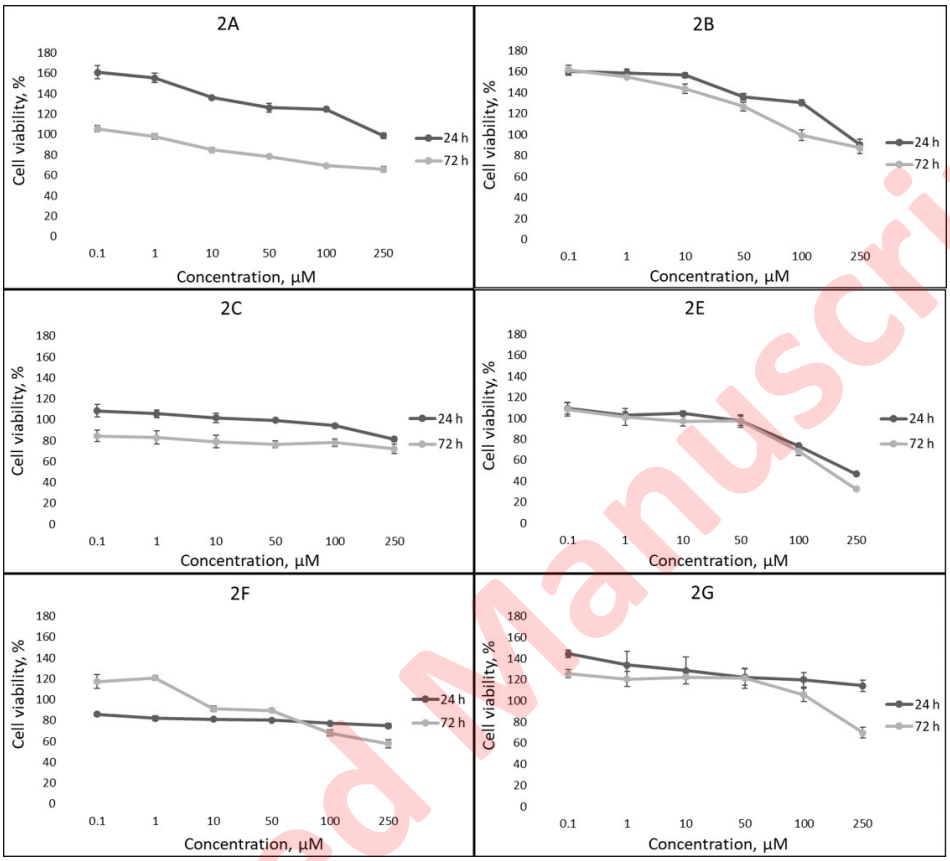


Fig. S-45. The effects of zingerone-thiohydantoin derivatives on HCT-116 cell viability

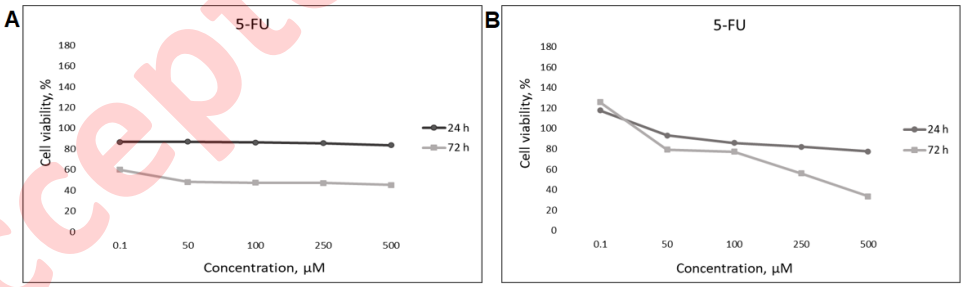


Fig. S-46. The effects of reference control 5-FU on MRC-5 (A) and HCT-116 (B) cell viability

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