Macedonian Journal of Chemistry and Chemical Engineering, Vol. **42**, No. 1, pp. 13–24 (2023)

MJCCA9 – 869

ISSN 1857-5552
e-ISSN 1857-5625

Received: December 31, 2022
Accepted: March 31, 2023

DOI: 10.20450/mjcce.2023.2638
Original scientific paper

DEVELOPMENT OF A SPECTROPHOTOMETRIC METHOD FOR ASSESSMENT OF THE RELATIVE REACTIVITY OF MONOCARBONYL ANALOGS OF CURCUMIN WITH 2-(DIMETHYLAMINO)ETHANETHIOL

Zlatko Lozanovski[1,2], Jasmina Petreska Stanoeva[1], Jane Bogdanov[1*]

[1]*Institute of Chemistry, Faculty of Natural Sciences and Mathematics, Ss. Cyril and Methodius University in Skopje, Arhimedova 5, 1000 Skopje, R. N. Macedonia*
[2]*Higher Medical School, St. Kliment Ohridski University in Bitola, Partizanska bb, 7000 Bitola, R. N. Macedonia*

j_b_bogdanov@yahoo.com; zlatkolozanovski@gmail.com

In order to improve the bioavailability of curcumin, studies have been undertaken to prepare the so-called monocarbonyl analogs of curcumin (MACs) and assess their biological activity. These analogs contain an electrophilic *α,β*-unsaturated carbonyl moiety (Michael acceptor). Several key biological processes are connected/controlled with thiol alkylation (glutathione, cysteine, cysteine peptide residues). The most likely reaction is the Michael addition between the *α,β*-unsaturated acceptor and a corresponding thiol. 2,6-Bisarylidenecyclohexanone and 3,5-bisarylidenepiperidin-4-one scaffolds offer convenient tunability of electrophilicity and redox properties of the Michael acceptor by the introduction of various substituents. In this study, several MACs were prepared by Claisen-Schmidt condensation reaction, and their reactivity with 2-(dimethylamino)ethanethiol was evaluated. For this purpose, based on the UV-Vis spectra of the analogs and thiol(s), a proper method for spectrophotometric evaluation of their reactivity with 2-(dimethylamino)ethanethiol was optimized. The relative reactivity of the analogs was $7 > 2 > 5 > 4 > 1 \approx 6$. The developed method is simple, and it can be extended to assess the reactivity of other MACs.

Keywords: synthesis; monocarbonyl analogs of curcumin; thiols; 2-(dimethylamino)ethanethiol; Michael addition

РАЗРАБОТКА НА СПЕКТРОФОТОМЕТРИСКИ МЕТОД ЗА ПРОЦЕНА НА РЕЛАТИВНАТА РЕАКТИВНОСТ НА МОНОКАРБОНИЛНИ АНАЛОЗИ НА КУРКУМИН СО 2-(ДИМЕТИЛАМИНО)ЕТАНТИОЛ

Со цел да се подобри биорасположливоста на куркуминот, направени се студии за синтеза на таканаречените монокарбонилни аналози на куркумин (МАС) и процена на нивната биолошка активност. Овие аналози содржат електрофилна α,β-незаситена карбонилна група (Мајклов акцептор). Неколку клучни биолошки процеси се поврзани/контролирани преку алкилација на тиоли (глутатион, цистеин, пептиди со цистеински групи). Најверојатно е дека има реакција на Мајклова адиција помеѓу α,β-незаситениот акцептор и соодветниот тиол. 2,6-Бисарилиденциклохексанонските и 3,5-бисарилиден-4-пиперидонските системи нудат можност за приспособување на електрофилноста и редокс-својствата на Мајкловиот акцептор преку воведување различни супституенти. Во оваа студија беа синтетизирани неколку МАС преку Клајзен-Шмит-ова кондензациона реакција и потоа беше проценета нивната реактивност со 2-(диметиламино)етантиол. За таа цел, врз основа на UV-Vis спектрите на аналозите и тиол(ите), оптимизиран е соодветен метод за спектрофотометриска евалуација на нивната реактивност со 2-(диметиламино)етантиол. Релативната реактивност на аналозите беше со редослед

7 > 2 > 5 > 4 > 1 ≈ 6. Развиениот метод е едноставен и може да се примени за процена на реактивноста на други монокарбонилни аналози на куркумин.

Клучни зборови: синтеза; монокарбонилни аналози на куркумин; тиоли; 2-(диметиламино)етантиол; Мајклова адиција

1. INTRODUCTION

Curcumin (diferuloylmethane) is a natural conjugated *β*-diketone isolated from the rhizome of *Curcuma longa* (turmeric), which has been used for a long time in traditional medicine as a cure for diseases of the liver, digestive tract, and urinary tract, and infections, rheumatoid arthritis, and insect bites.[1] Curcumin is a useful phytochemical that has been extensively studied for the last two decades, especially for its anti-cancer, anti-oxidant, anti-inflammatory and anti-microbial properties.[2]

In vivo, curcumin inhibits malignant cell growth. It induces apoptosis in malignant cells by modulating the activity of transcription factors, growth factors, adhesion molecules, and cell signals.[3,4] Although curcumin is non-toxic and shows promising pharmacological properties, numerous studies indicate that it has poor bioavailability, low chemical stability, and rapid metabolism under physiological conditions. According to the literature, its instability is due to the *β*-diketone component, which is strongly reactive at a pH higher than 6.5, resulting in a shortened half-life in the body.[5,6]

Despite the fact that curcumin is recurrently used in research studies for biological activity, one must take into consideration the fact that it has been classified as PAINS (pan-assay interference compounds), as well as IMPS (invalid metabolic panaceas).[7] Curcumin has diverse chemistry, and the role of the auto-oxidation products in its overall activity has been elucidated.[8–10] All in all, curcumin is photolabile and decomposes mainly by solvolysis and oxidative degradation. Most importantly, it does not satisfy the primary criterion for pharmaceutical applications because it is unstable under physiological conditions (pH 7.4 and 37 °C).[7]

Modification of the structure of curcumin implies synthesis of monocarbonyl analogs (MACs) by a crossed aldol condensation between a cycloalkanone and monosubstituted benzaldehyde and removal of the *β*-diketone component as one of the ways to overcome the downsides of curcumin (Fig. 1).[11,12]

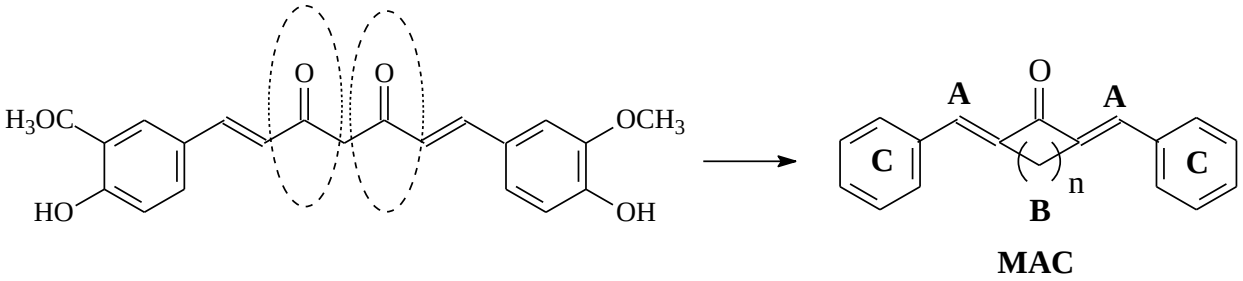


Fig. 1. Transformation of curcumin to monocarbonyl analogs (MACs) in order to improve stability

Many MACs have been synthesized ($n = 0$, $n = 2$ cyclopentanone, $n = 3$ cyclohexanone, 4-piperidone, etc.) and characterized, and their biological properties evaluated. They all contain the 1,5-diaryl-3-oxo-penta-1,4-diene pharmacophore, which is the key to the biological activity (Fig. 2).

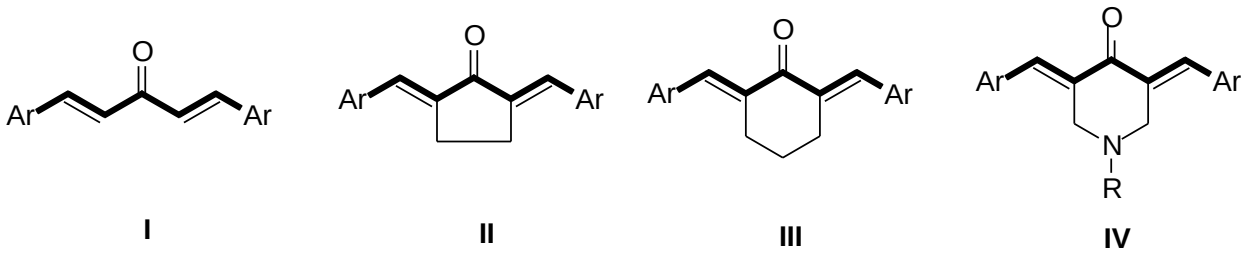


Fig. 2. The general structure of different types of MACs that contain the 1,5-diaryl-3-oxo-penta-1,4-diene pharmacophore

A number of key biological processes are linked/controlled by the thiol alkylation process (glutathione, cysteine, cysteamine, etc.), and MACs and many other natural products with an electrophilic nature are excellent thia-Michael acceptors.[13] Moreover, the properties of MACs can be modified by introducing different central cores, aromatic systems, and the introduction of different substituents. These analogs are involved in highly selective thiol-trapping, which is critical for bioactivity. It is important to stress that these Michael reactions are reversible, but not all thiol-containing systems (R-SH) have the same reactivity. There are other functional groups that can be involved in 1,4-additions, which will result in oxa, carba, and aza-Michael reactions. However, if one considers the respective bond strengths, the new bond formed, the C-S bond (ca. 60 kcal/mol) is the weakest of all the above. This, to the first approximation, makes these thia-Michael reactions reversible.[14] The interesting feature of these cross-conjugated dienones is that they possess two reactive electrophilic centers that can react with 2 equivalents of the respective thiol.

As mentioned above, the sulfides obtained under certain conditions can be "returned" to the initial reactants (conjugated enone and thiol). Knowing the factors that affect the reversibility of this process is the key to controlling biological activity. The rate of the reverse/backward reaction depends on the electronic effects and steric considerations. The reactivity of the –SH functional group in these thia-Michael reactions depends on the pH of the medium, the substrate, and the presence of oxidizing and reducing agents.[15–18] There is not much data in the literature for these types of reactions (MAC as Michael acceptors with thiols).

A key study was performed by Sun et al.,[19] where formation of the adducts of (3*E*,5*E*)-3,5-bis(2-fluorobenzylidene)piperidin-4-one (7) (EF 24, **7**) and (3*E*,5*E*)-3,5-bis(2-pyridin-2-ylmethylydene) piperidin-4-one (EF 31, **8**) and glutathione were studied (Figure 3). Namely, these analogs in acetonitrile (CH_3CN) reacted with 2.1 equivalents of glutathione (GSH) in an aqueous solution at ambient temperature. The yellow color of the unsaturated ketone disappeared as a result of chromophore elimination and the rapid formation of bis adducts with glutathione and the corresponding MAC. According to the authors, it was considered that the formation of the mono-glutathione adduct is an intermediary to the bis-glutathione adduct.[19] Spectroscopic and chromatographic (HPLC-MS) analyses showed that **8** reacted instantly with GSH, while the reaction of **7** and GSH took several hours. The EF 24 analog has been fully characterized. It has diverse biological activity and has been the subject of many detailed studies.[20–29] Related to EF 24 and EF 31 is the analog EF 25 for which the HR-ESI-MS and ^{1}H NMR spectra of the synthesized bis glutathione adduct, EF 25-$(GSH)_2$, have been reported.[30]

In another study, the reactivity of 1,5-bis(3,5-bis(methoxymethoxy)phenyl)-1,4-pentadiene-3-one (GO-Y030), a very potent cytotoxic agent, with cysteamine was assessed by NMR in d_6-DMSO and UV-Vis spectroscopy.[31] In both experiments using the cysteamine assay (^{1}H NMR method)[32] a reversible Michael reaction was observed. Furthermore, various GO-Y030 bis-thiol adducts were synthesized, and the retro-thia-Michael reaction was monitored spectrophotometrically in DMSO at different pH values (3.0, 7.3, and 8.5). In neutral pH, only two adducts with hydrophilic R groups gave back the starting dienone GO-Y030. In slightly basic pH, only the bis thiol adducts with lipophilic groups did not react completely. It was concluded that the MAC, GO-Y030, is a reversible thia-Michael acceptor and that this system may be operable in cells, as indicated by the comparable cytotoxicity of MAC and bis-thiol adducts. All of these adducts can be used as prodrugs.

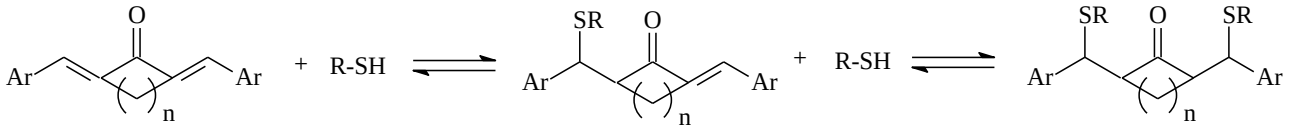


Fig. 3. The thia-Michael reaction of MACs with thiols results in the formation of a mono-adduct and/or bis-adduct. This process is expected to be reversible.

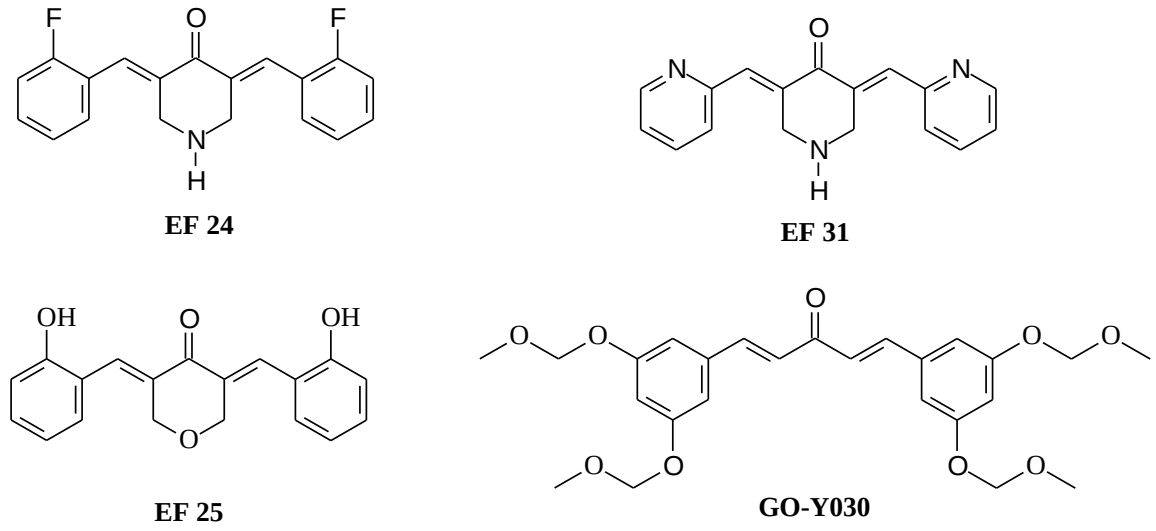


Fig. 4. MACs that had a detectable formation of adducts with relevant thiols (EF 24, EF 31, and EF 25 with glutathione, GO-Y030 with cysteamine)

In the literature, several methods have been reported for the assessment of Michael thiol acceptors. In most cases, they use cysteamine (NMR[14,32], UV-Vis[31,33]) which is suitable for spectrophotometric assays. However, cysteamine has a reactive nucleophilic primary amine that can react in a 1,2-fashion with the ketone to give 1,4-thiazepines (Fig. 5).[14] This can be somewhat problematic for the MACs because this thiazepine intermediate will influence the second addition of thiol.

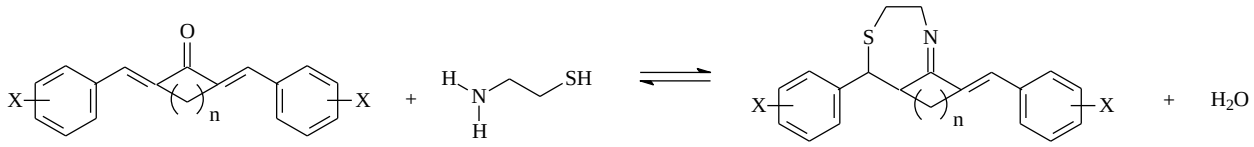


Fig. 5. Reaction of cysteamine with MAC to give a thiazepine derivative

In order to examine the properties of MACs with diverse electrophilicity and redox characteristics, the main purpose of the work presented herein is the synthesis and purification of symmetric analogs with different electron donor/acceptor groups and optimization/improvement of the procedure for monitoring their reaction with the most appropriate thiol (2-(dimethylamino)ethanethiol**)** by UV-Vis spectroscopy.

2. EXPERIMENTAL SECTION

2.1. *Reagents*

Cyclohexanone, 2-bromobenzaldehyde, 2-(trifluoromethyl)benzaldehyde, benzaldehyde, methanol, ethanol, acetonitrile (HPLC gradient grade), ammonium chloride, and sodium hydroxide were obtained from Sigma-Aldrich. Cysteine, glutathione (GSH), cysteamine, 2-mercaptoethanol, 2-(dimethylamino)ethanethiol hydrochloride, and 4-*tert*-butylcyclohexanone were obtained from Merck. Methanol and ethanol were purchased from Alkaloid AD Skopje. All chemicals were used without further purification.

2.2. *Instrumentation*

Melting temperatures were determined on a Mel-Temp apparatus and were not corrected. Infrared spectra were recorded on a Varian Excalibur 3100 FT-IR spectrometer using a KBr pellet. UV-Vis spectra were recorded on a Cary 50 spectrophotometer in a quartz cuvette. The samples were dissolved in acetonitrile or a suitable mixture of acetonitrile and water or acetonitrile/aqueous buffer. The mass spectral measurements were carried out on an Agilent 1100 HPLC system equipped with an ESI interface, and an ion-trap mass analyzer (G2445A Spectrometer) controlled by Chemstation LCMSD software (Agilent, v.4.1) was used to carry out the MS analysis. The measurements were done in APCI positive ion mode.

2.3. *Synthesis method for 2,6-bisarylidencyclohexanones* [34]

The corresponding cyclohexanone (7.5 mmol), the corresponding arylaldehyde (15 mmol), and methanol (10 ml) were added to a round bottom flask. The reaction mixture was stirred for 5 minutes at room temperature, and 20% (w/v) of an aqueous solution of sodium hydroxide (2 ml) was added dropwise over a period of 5 minutes. The reaction mixture was mixed with a magnetic stirrer at room temperature for 60 minutes to give a yellow precipitate. The reaction mixture was then cooled for 10 minutes in an ice bath and filtered through a Büchner funnel. The resulting precipitate was washed with a saturated aqueous solution of ammonium chloride (1 × 10 ml), distilled water (3 × 10 ml), and cold methanol (2 × 5 ml). The resulting product was recrystallized from the appropriate solvent, making sure that the solution was kept in the dark.

(2*E*,6*E*)-2,6-bis[(2-trifluoromethyl)benzylidene] cyclohexanone (**1, C66**): recrystallized from methanol, yellow crystals, yield (79 %) mp 107–110 °C (lit.[35,36] 108–110 °C); **FT-IR** (KBr): 1666 cm^{-1} (C = O); **FT-IR-ATR** (sapphire): 1665 cm^{-1} (C = O); **FT-IR** (CCl_4): 1682 cm^{-1} (C = O); **UV-Vis**: λ_{max} (80:20 CH_3CN/H_2O) = 302 nm (ε = 23675 $l \cdot mol^{-1} \cdot cm^{-1}$); **HPLC-DAD-MS**, t_R = 5.5 min; **MS-APCI+** (*m*/*z*) = 411 (MH^+).

(2*E*,6*E*)-2,6-bis(2-bromobenzylidene)cyclohexanone (2, B2BrBC): recrystallized from 5:2 methanol/ dichloromethane, yellow crystals, yield (52 %), mp 131–133 °C (lit.[36] 120 °C); **FT-IR** (KBr): 1661 cm^{-1} (C=O); **FT-IR-ATR** (sapphire): 1661 cm^{-1} (C=O); **UV-Vis**: λ_{max} (80:20 CH_3CN/H_2O) = 315 nm (ε = 43424 $l \cdot mol^{-1} \cdot cm^{-1}$); **HPLC-DAD-MS**, t_R = 7.1 min; **MS-APCI** (*m*/*z*) = 433 (MH^+).

(2*E*,6*E*)-2,6-dibenzylidenecyclohexanone (3): recrystallized from ethyl acetate, yellow crystals, yield (62 %); mp 115–117 °C (lit.[36] 116–118 °C); **FT-IR** (KBr): 1662 cm^{-1} (C=O); **FT-IR-ATR** (sapphire): 1661 cm^{-1} (C=O); **UV-Vis**: λ_{max} (80:20 $CH_3CN:H_2O$) = 328 nm (ε = 43290 $l \cdot mol^{-1} \cdot cm^{-1}$); **HPLC-DAD-MS**, t_R = 4.9 min; **MS-APCI** (*m*/*z*) = 275 (MH^+).

(2*E*,6*E*)-2,6-bis(4-methoxybenzylidene)cyclohexanone (4): recrystallized from ethyl acetate; yellow needle crystals, yield (54 %); mp 156–158 °C (lit.[37] 160–162 °C, rec. EtOH); **FT-IR** (KBr): 1658 cm^{-1} (C=O); **FT-IR-ATR** (sapphire): 1656 cm^{-1} (C=O); **UV-Vis**: λ_{max} (80/20 $CH_3CN:H_2O$) = 360 nm (ε = 41034 $l \cdot mol^{-1} \cdot cm^{-1}$); **HPLC-DAD-MS**, t_R = 4.3 min; **MS-APCI** (m/z) = 335 (MH^+).

(2*E*,6*E*)-2,6-bis(2-furan-ylmethylidene)cyclohexan-1-one (**5**): recrystallized from 96 % ethanol, yellow-orange crystals, yield (68 %), mp 142–144 °C (lit.[38] 140–141 °C); **FT-IR** (KBr): 1645 cm^{-1} (C=O); **UV-Vis**: λ_{max} (80/20 $CH_3CN:H_2O$) = 370 nm (ε = 34395 $l \cdot mol^{-1} \cdot cm^{-1}$); **HPLC-DAD-MS**, t_R = 3.3 min; **MS-ESI** (*m*/*z*) = 255 (MH^+).

(2*E*,6*E*)-4-*tert*-butyl-2,6-bis(2-furan-2-ylmethylidene)cyclohexan-1-one (**6):** recrystallized from 96 % ethanol, orange crystals, yield (48 %), mp 136–138 °C (lit.[39] 136–138 °C); **FT-IR** (KBr): 1654 cm^{-1} (C = O); **FT-IR-ATR** (sapphire): 1653 cm^{-1} (C = O); **UV-Vis**: λ_{max} (80/20 $CH_3CN:H_2O$) = 370 nm (ε = 31460 $l \cdot mol^{-1} \cdot cm^{-1}$); **HPLC-DAD-MS**, t_R = 5.8 min; **MS-APCI** (m/z) = 311 (MH^+).

(3*E*,5*E*)-3,5-bis(2-fluorobenzylidene)piperidin-4-one (**7, EF24**): recrystallized from 96 % ethanol, yellow crystals, yield 47 %, mp 134–136 °C (lit.[40] 138–142 °C); **FT-IR** (KBr): 3304 cm^{-1} (N-H), 1661 (C = O); **FT-IR-ATR** (sapphire): 3303 cm^{-1} (N-H), 1660 (C = O); **UV-Vis**: λ_{max} (80/20 $CH_3CN:H_2O$) = 327 nm (ε = 26688 $l \cdot mol^{-1} \cdot cm^{-1}$); **HPLC-DAD-MS**, t_R = 2.8 min; **MS-APCI** (*m*/*z*) = 312 (MH^+).

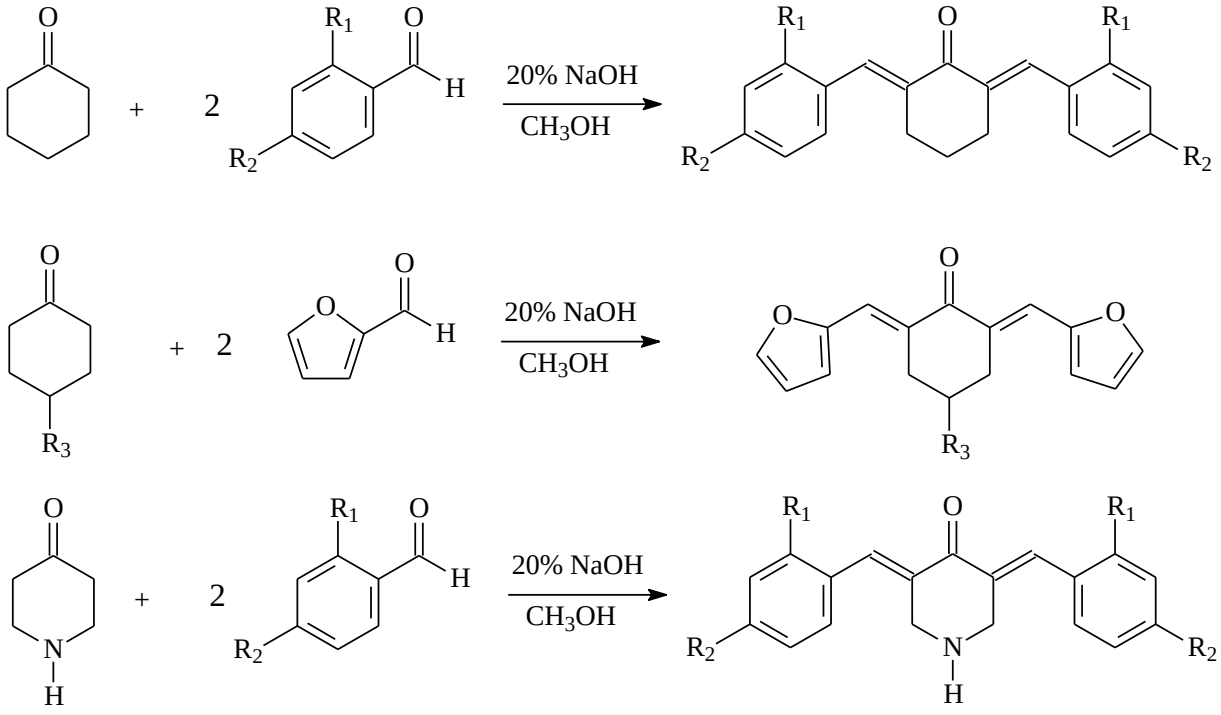
2.4. *Spectrophotometric assay*

Stock solutions of compounds (**1**–**7**) with concentrations of 0.2–0.4 mg/ml in acetonitrile were prepared in amber volumetric flasks. Also, a solution of 2-(dimethylamino)ethanethiol with a concentration of 1.2 mg/ml was prepared prior to the UV-Vis studies to avoid oxidation to the disulfide. The blank was recorded in 80/20 acetonitrile/ distilled water solvent. UV-Vis spectra of the compounds of interest were recorded in 80/20 acetonitrile/distilled water. A 2.9 ml sample of 2-(dimethylamino)ethanethiol solution was placed in a quartz cuvette, and the UV-Vis spectrum was recorded. This solution has significant absorbance up to 280 nm. Directly into the quartz cuvette containing the 2-(dimethylamino)ethanethiol solution, 50–100 μl of the stock solution of compounds (**1**–**7**) were added, rapidly mixed, and the UV spectrum recorded at 25 ºC. Afterward, a UV spectrum was recorded every 5 minutes, depending on the rate of change, while maintaining the temperature at 25 ºC. For example, the final concentrations in the quartz cuvette for 2-(dimethylamino)ethanethiol and **2** were 0.008 mmol/ml and $2 \cdot 10^{-5}$ mol/ml, respectively.

3. RESULTS AND DISCUSSION

Several curcumin monocarbonyl analogs (2,6-diarylidenecyclohexanones and 3,5-bis(2-fluorobenzylidene)piperidin-4-one) were prepared by Claisen-Schmidt condensation between cyclohexanone and two equivalents of the corresponding benzaldehyde or furfural[34] (Figure 6). All synthesized compounds are known, and their characterization was performed based on melting points, IR spectroscopy, mass spectrometry, and a comparison of available data in the literature (Table 1). The compounds were carefully purified by recrystallization in the dark and kept in amber vials throughout the study.

The next step was to investigate the solubility of the synthesized compounds in solvents suitable for UV-Vis spectroscopy (acetonitrile, dichloromethane, dimethylsulfoxide, ethanol, methanol, ethylene glycol, etc.). We also had in mind a solvent that may be compatible for subsequent HPLC analysis. Even though several studies of the assessments were done in DMSO[30,31] we found that it may interfere with the compounds with lower values for λ_{max} such as **1** and **2** (302 nm and 315 nm, respectively). Another criterium was for the solvent to be miscible with water so that a buffer or other water-soluble reagent could be added. The UV spectra of all compounds (**1**–**7**) in acetonitrile/H_2O exhibit two maxima, one at a long wavelength (302–370 nm) that corresponds to n→π* transition and another at lower wavelengths, ranging from 226–250 nm, that originates from a π→π* transition (Table 1).



1: $R_1 = CF_3$; R_2 = H **(C66)**
2 : R_1 = Br; R_2 = H (**B2BrBC)**
3 : R_1 = H; R_2 = H
4 : R_1 = H; $R_2 = OCH_3$

5 : R_3 = H (2-furfurylidene)
6 : R_3 = *tert*-Bu (2-furfurylidene)
7: R_1 = F; R_2 = H **(EF 24)** (4-piperidone)

Fig. 6. Synthesis of monocarbonyl curcumin analogues (MACs): (**1**) (2*E*,6*E*)-2,6-bis[(2-trifluoromethyl)benzylidene]cyclohexanone, (**2**) (2*E*,6*E*)-2,6-bis(2-bromobenzylidene)cyclohexanone, (**3**) 2,6-bisbenzylidencyclohexanone, (**4**) (2*E*,6*E*)-2,6-bis(4-methoxybenzylidene)cyclohexanone, (**5**) (2*E*,6*E*)-2,6-bis(2-furan-2-ylmethylidene)cyclohexan-1-one, (**6**) (2*E*,6*E*)-4-*tert*-butyl-2,6-bis(2-furan-2-ylmethylidene)cyclohexan-1-one, (**7**) (3*E*,5*E*)-3,5-bis(2-fluorobenzylidene)piperidin-4-one

T a b l e 1

Melting points and key spectroscopic/chromatographic data of the synthesized monocarbonyl analogs of curcumin

Comp.	**mp (°C)**	**FT-IR** (KBr) cm^{-1}	**FT-IR-ATR** (sapphire) cm^{-1}	**UV-Vis** λ_{max1} (80/20 $CH_3CN:H_2O$)	**UV-Vis** λ_{max2} (80/20 $CH_3CN:H_2O$)	**HPLC-DAD-MS,** t_R (min)	**MS-APCI** *(m/z)*
1	107–110 lit.[35,36] 108–110	1666 (C = O)	1665 (C = O)	302 nm	226 nm	5.5 min	411 (MH^+)
2	131–133 lit.[36] 120	1661 (C = O)	1661 (C = O)	315 nm	237 nm	7.1 min	433 (MH^+)
3	115–117 lit.[36] 116-118	1662 (C = O)	1661 (C = O)	328 nm	232 nm	4.9 min	275 (MH^+)
4	156–158 lit.[37]160–162	1658 (C = O)	1656 (C = O)	361 nm	243 nm	4.3 min	335 (MH^+)
5	142–144 lit.[38] 140–141	1645 (C = O)	/	370 nm	250 nm	3.3 min	255 (MH^+)
6	136–138 lit.[39]136–138	1654 (C = O)	1653 (C = O)	370 nm	250 nm	5.8 min	311 (MH^+)
7	134–136 lit.[40] 138–142	1661 (C = O)	1660 (C = O)	327 nm	233 nm	2.9 min	312 (MH^+)

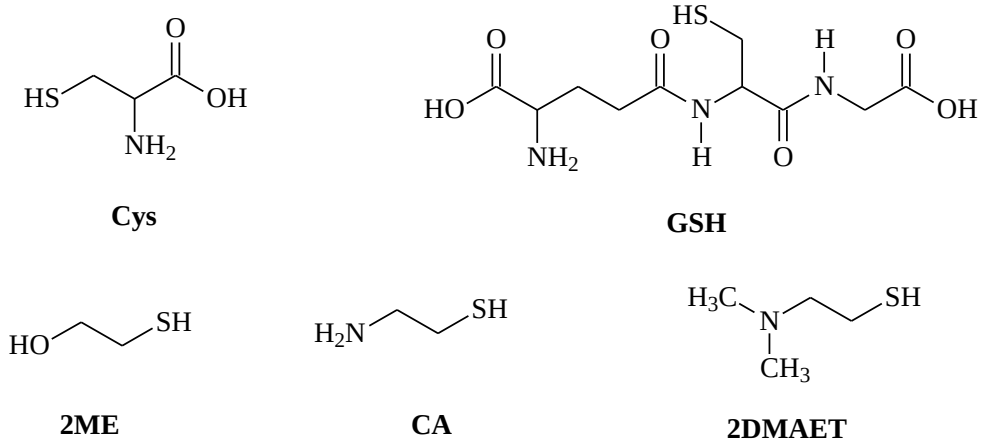
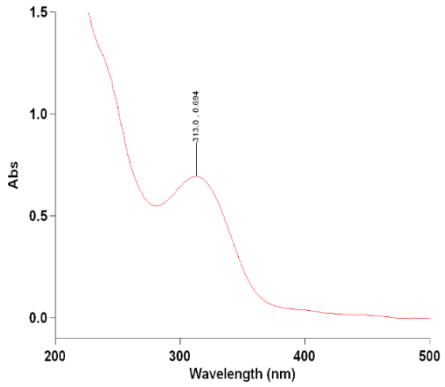


Fig. 7. Structural formulae and appropriate abbreviations for the relevant thiols. (Cys–Cysteine, GSH–glutathione (reduced), 2ME–2-mercaptoethanol, CA–cysteamine, 2DMAET–2-(dimethylamino)ethanethiol)

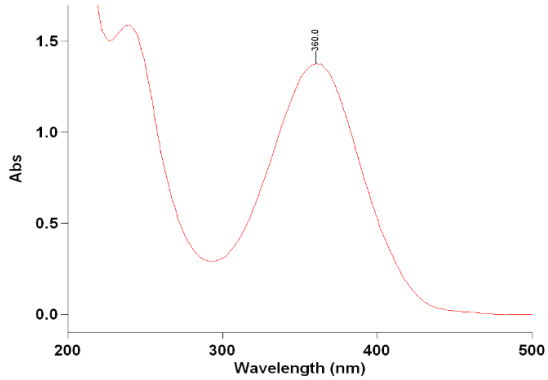
Secondly, we selected the most appropriate thiol (cysteine (Cys), glutathione (GSH), cysteamine (CA), 2-(dimethylamino) ethanethiol (2DMAET), 2-mercaptoethanol (2ME), etc.). This was followed by reaction optimization using the appropriate thiol, pH optimization, solvent(s), mixing procedure, and avoidance of thiol oxidation, as well as determination of concentrations, as key to the reaction with the curcumin monocarbonyl analogs.

Driven by the study of Sun et al.[19] and the simplicity of the spectrophotometric procedure for monitoring the reaction between *α,β*-unsaturated chalcone acceptors and thiols described in the study by Amslinger et al.,[33] we decided to adapt it and apply it to the previously synthesized MACs (**1–7**). For that purpose, it was necessary to optimize the UV-Vis method for monitoring the process. We used a buffer with a pH of 7.4 and evaluated the combinations with a number of solvents such as acetone, acetonitrile, ethanol, methanol, and dimethylsulfoxide.

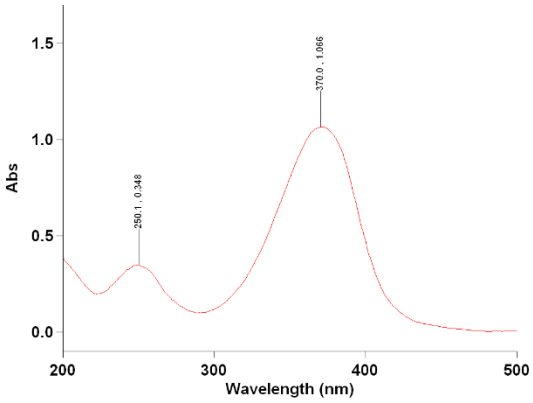
We found differences in the solubility and stability of MACs with the appropriate solvents. The best solvent combination proved to be acetonitrile with 100 mM TRIS-HCl at pH 7.4. It was necessary to avoid thiol oxidation. So, according to the literature, we added a 2 mM EDTA to the buffer.[41] Representative UV-Vis spectra of certain synthesized MACs (40 μM) in 100 mM TRIS-HCl (pH 7.4) with 2 mM EDTA/acetonitrile in a ratio of 20:80 are shown in Figure 8.



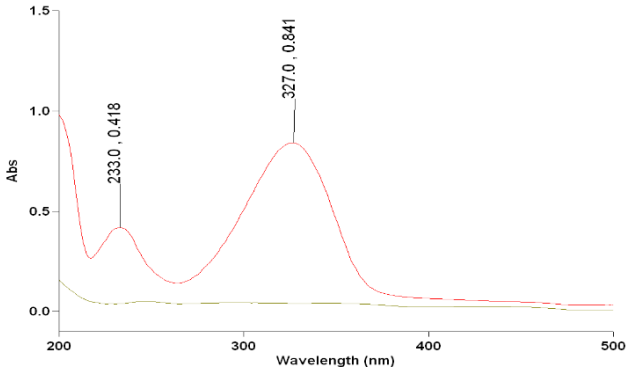
a) (2*E*,6*E*)-2,6-bis(2-bromobenzylidene)cyclohexanone (**2**)



b) (2*E*,6*E*)-2,6-bis(4-methoxybenzylidene)cyclohexanone (**4**)



c) (2*E*,6*E*)-2,6-bis(2-furan-2-ylmethyylidene)cyclohexanone (**5**)



d) (3*E*,5*E*)-3,5-bis(2-fluorobenzylidene)piperidin-4-one (**7**)

Fig. 8. Representative UV-Vis spectra of MACs (40 μM) in 100 mM TRIS-HCl (pH 7.4) and 2 mM EDTA/acetonitrile at a ratio of 20 : 80

It was crucial to determine the appropriate thiol concentration to obtain appropriate MACs according to the literature. Thiol was added in an excess of 20 to 500 times,[33] usually in the range of a 200 fold excess. We used the following thiols: 2-(dimethylamino)ethanol hydrochloride, cysteine, glutathione (GSH), cysteamine, and 2-mercapto-ethanol. We noticed that cysteine gave a yellow product that was not suitable for analysis by UV-Vis spectroscopy, and we eliminated it from further analysis. From the study by Amslinger et al.[33] we found that cysteamine reacts rapidly with the Michael acceptor and makes small or no interference in UV-Vis assays, but cyclization reactions are possible after the first addition. As a logical choice, 2-(dimethylamino)ethanol (2DMAET), which is commercially available in high purity as the hydrochloride salt, was introduced as a nucleophile (with reactivity similar to cysteamine) which would not react in cyclization reactions with the keto group of MACs (see Figure 5). In principle, this is possible after the first addition of the thiol. Solutions of 2DMAET in 80:20 v/v acetonitrile/water had minimal absorption above 290 nm and could be used in the assessment studies.

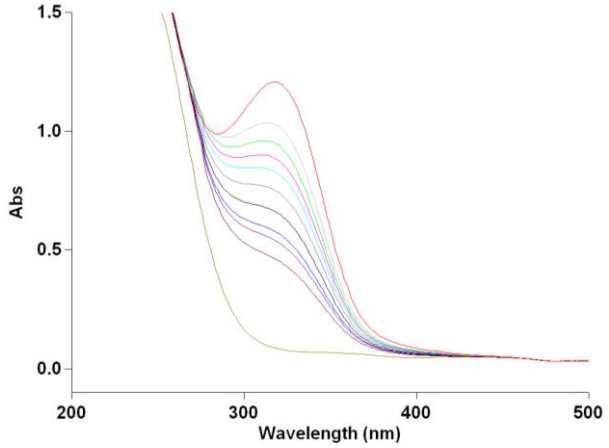
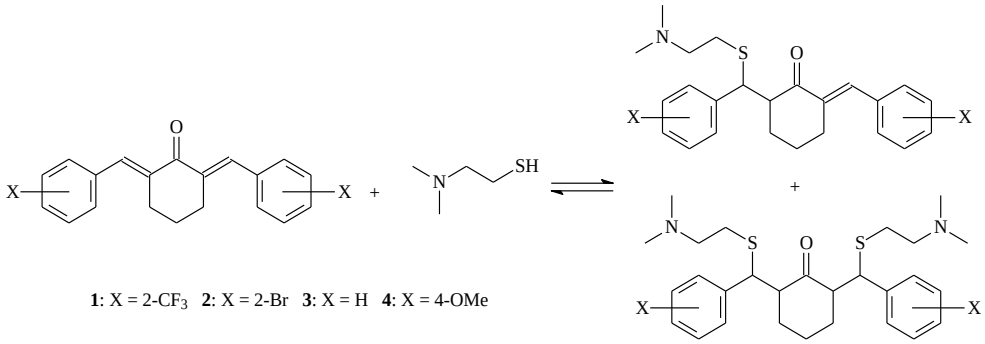


Fig. 9. Representative UV-Vis spectra of EF 24 (40 μM) added to 2-(dimethylamino)ethanethiol (1.2 mg/ml) in 80 : 20 acetonitrile/H_2O (0 to 90 minutes: red trace 0 min; the beige trace on the bottom is just from a solution of 2-(dimethylamino)ethanethiol (1.2 mg/ml)).The traces below 0 min (red trace) correspond consecutively to reaction times of 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 45 min, 60 min, and 90 min (brown trace).

The substrate that we used for optimization was (3*E*,5*E*)-3,5-bis(2-fluorobenzylidene)-4-piperidone (EF 24) because it is known to form mono and bi-thiol adducts with glutathione.[19,28]

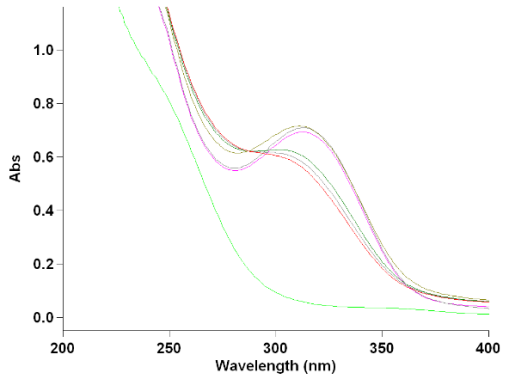
We found that **7 (EF 24)** had the fastest reactions with 2-(dimethylamino)ethanethiol hydrochloride. The second fastest reaction (based on the decrease of absorbance at λ_{max}) was with **2 (B2BrBC)**, then with **5,** and with **4**. The reaction was performed in 100 mM TRIS-HCl at pH 7.4 with 2 mM EDTA/acetonitrile in a ratio of 20:80 and at a concentration of 40 µM B2BrBC and an excess of 2-(dimethylamino)ethanthiol hydrochloride of 400 times. From the UV spectra, it can be noticed that there is a decrease of the peak at around 327 nm and a shift towards lower wavelengths. It is likely that after the first addition of 2-(dimethylamino)ethanethiol, a mono adduct is obtained which still contains conjugated enone. In principle, if a bis adduct is obtained, the peak around 320 nm should disappear. The identity of all products should be established by alternative methods. The corresponding expected reactions of certain synthesized derivatives are given in Figure 10.



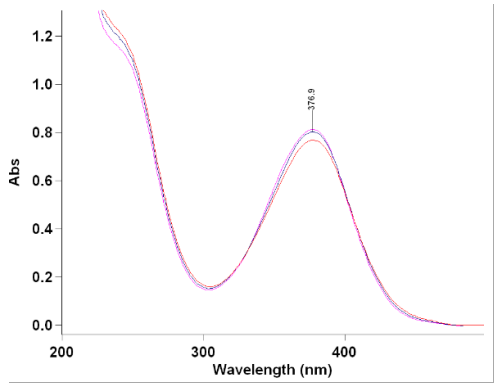
1: X = 2-CF_3 **2**: X = 2-Br **3**: X = H **4**: X = 4-OMe

Fig. 10. Reaction of 2,6-bisarylidenecyclohexanones and 2-(dimethylamino)ethanethiol and formation of mono and bis adducts

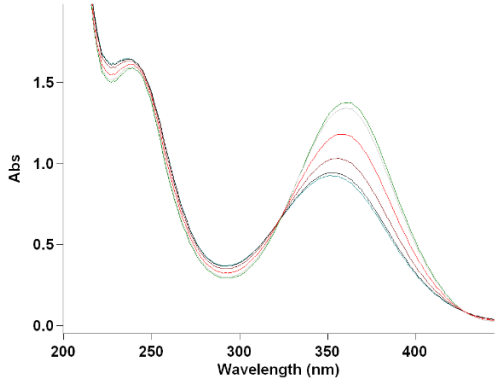
The reaction between the corresponding MACs and 2-(dimethylamino)ethanthiol hydrochloride was monitored by UV-Vis spectroscopy for about 3 hours, where the amine was added in an excess of 20 to 500 times according to the literature[13] (Figure 11).



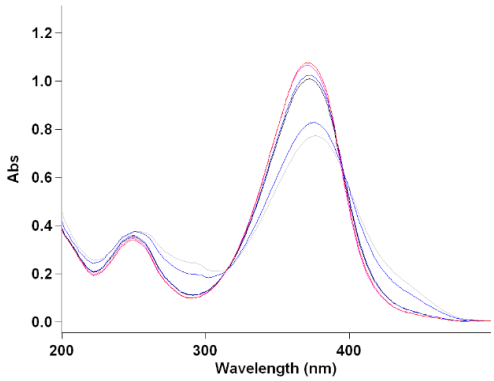
a) Reaction of **2** with 2-(dimethylamino)ethanethiol



b) Reaction of **6** with 2-(dimethylamino)ethanethiol



c) Reaction of **4** with 2-(dimethylamino)ethanethiol



d) Reaction of **5** with 2-(dimethylamino)ethanethiol

Fig. 11. (a–d). Representative UV-Vis spectra of MACs (40 µM) in 100 mM TRIS-HCl (pH 7.4) and 2 mM EDTA/acetonitrile, at a ratio of 20:80, and 2-(dimethylamino)ethanethiol

Depending on the benzene ring substituents as well as the aromatic ring, the λ_{max} absorbance ranges from 299 nm to 370 nm in the examined analogs. To avoid interference from the buffer, thiol, and other additives, it is most appropriate to monitor the reaction by UV-Vis spectroscopy with compounds having a λ_{max} above 310 nm. With optimized conditions of spectrophotometric analysis (preventing thiol oxidation), slow processes can be monitored (reaction time over 12 hours). From the tested compounds **(1–7)**, the fastest changes were observed in the reaction of 2-(dimethylamino) ethanethiol with **7** (**EF 24**) followed by **2 (B2BrBC)**, then with **5** and with **4**. From the spectroscopic analysis of **1 (C66)** and **6,** only minor changes were observed after 3 hours of reaction time. As a future direction, the developed method can be extended to HPLC-DAD monitoring, as well as the characterization of mono and bis adducts by HPLC-MS. Furthermore, this method can be carried out without buffers and additives in CD_3CN and use NMR spectroscopy to identify and classify thiol-trapping agents.

4. CONCLUSION

Several monocarbonyl curcumin analogs (2,6-diarylidenecyclohexanones and 3,5-dirylidenepiperidin-4-one) were synthesized in medium to high yields via a Claisen-Schmidt condensation reaction between cyclohexanone, 4-*tert*-butylcyclohexanon, or 4-piperidone and the corresponding arylaldehyde. The obtained solid MACs were carefully purified by recrystallization and characterized. An optimized method suitable for spectrophotometric analysis of the reaction between MACs and the most suitable thiol (2-(dimethylamino)ethanethiol) was established. Analysis conditions prevented thiol oxidation and provided the possibility to observe slow processes (reaction time over 12 hours). From the tested compounds **(1–7)**, the fastest changes were observed in the reaction of 2-(dimethylamino) ethanthiol with **7** (**EF 24**), followed by **2 (B2BrBC)**, then with **5** and with **4**. From the spectroscopic analysis of **1 (C66)** and **6,** only minor changes were observed after 3 hours of reaction time. Acetonitrile and water are suitable solvents, and they are also suitable for subsequent HPLC-DAD-MS assays. The reactivity is most likely governed by the electrophilicity of the Michael acceptor. The developed method is simple and inexpensive, and it can be extended to assess the reactivity of other MACs.

REFERENCES

(1) Goel, A.; Kunnumakkara, A. B.; Aggarwal, B. B., Curcumin as “Curecumin”: from kitchen to clinic. *Biochem. Pharmacol.* **2008**, *75* (4), 787–809. https://doi.org/10.1016/j.bcp.2007.08.016

(2) Wilken, R.; Veena, M. S.; Wang, M. B.; Srivatsan, E. S., Curcumin: A review of anti-cancer properties and therapeutic activity in head and neck squamous cell carcinoma. *Mol. Cancer* **2011**, *10*, 12. https://doi.org/10.1186/1476-4598-10-12

(3) Chen, B.; Zhang, Y.; Wang, Y.; Rao, J.; Jiang, X.; Xu, Z., Curcumin inhibits proliferation of breast cancer cells through Nrf2-mediated down-regulation of Fen 1 expression. *J. Steroid Biochem. Mol. Biol.* **2014**, *143*, 11–18. https://doi.org/10.1016/j.jsbmb.2014.01.009

(4) Wang, K.; Fan, H.; Chen, Q.; Ma, G.; Zhu, M.; Zhang, X.; Zhang, Y.; Yu, J., Curcumin inhibits aerobic glycolysis and induces mitochondrial-mediated apoptosis through hexokinase II in human colorectal cancer cells in vitro. *Anticancer Drugs* **2015**, *26* (1), 15–24 https://doi.org/10.1097/CAD.0000000000000132

(5) Shetty, D.; Kim, Y. J.; Shim, H.; Snyder, J. P., Eliminating the heart from the curcumin molecule: Monocarbonyl Curcumin Mimics (MACs). *Molecules* **2014**, *20* (1), 249–292. https://doi.org/10.3390/molecules20010249

(6) Das, U.; Sharma, R. K.; Dimmock, J. R., 1,5-Diaryl-3-oxo-1,4-pentadienes: a case for antineoplastics with multiple targets. *Curr. Med. Chem.* **2009**, *16* (16), 2001–2020. https://doi.org/10.2174/092986709788682218

(7) Nelson, K. M.; Dahlin, J. L.; Bisson, J.; Graham, J.; Pauli, G. F.; Walters, M. A., The essential medicinal chemistry of curcumin. *J. Med. Chem.* **2017**, *60* (5), 1620–1637. https://doi.org/10.1021/acs.jmedchem.6b00975

(8) Gordon, O. N.; Luis, P. B.; Sintim, H. O.; Schneider, C., Unraveling curcumin degradation: autoxidation proceeds through spiroepoxide and vinylether intermediates en route to the main bicyclopentadione. *J. Biol. Chem.* **2015**, *290* (8), 4817–4828. https://doi.org/10.1074/jbc.M114.618785

(9) Gordon, O. N.; Luis, P. B.; Ashley, R. E.; Osheroff, N.; Schneider, C., Oxidative transformation of demethoxy- and bisdemethoxycurcumin: products, mechanism of formation, and poisoning of human topoisomerase IIα. *Chem. Res. Toxicol.* **2015**, *28* (5), 989–996. https://doi.org/10.1021/acs.chemrestox.5b00009

(10) Schneider, C.; Gordon, O. N.; Edwards, R. L.; Luis, P. B., Degradation of curcumin: from mechanism to biological implications. *J. Agric. Food Chem.* **2015**, *63* (35), 7606–7614. https://doi.org/10.1021/acs.jafc.5b00244

(11) Hsu, C. H.; Cheng, A. L. Clinical studies with curcumin. *Adv. Exp. Med. Biol.* **2007**, *595*, 471–480. https://doi.org/10.1007/978-0-387-46401-5

(12) Zhao, C.; Liu, Z.; Liang, G., Promising curcumin-based drug design: mono-carbonyl analogues of curcumin (MACs). *Curr. Pharm. Des.* **2013**, *19* (11), 2114–2135. https://doi.org/10.2174/138161213805289282

(13) Al-Rifai, N.; Rücker, H.; Amslinger, S., Opening or closing the lock? When reactivity is the key to biological activity. *Chem. Eur. J.* **2013**, *19* (45), 15384–15395. https://doi.org/10.1002/chem.201302117

(14) Caprioglio, D.; Minassi, A.; Avonto, C.; Taglialatela-Scafati, O.; Appendino, G., Thiol-trapping natural products under the lens of the cysteamine assay: friends, foes, or simply alternatively reversible ligands? *Phytochem. Rev.* **2020**, *19* (6), 1307–1321. https://doi.org/10.1007/s11101-020-09700-w

(15) Grynkiewicz, G.; Slifirski, P., Curcumin and curcuminoids in quest for medicinal status. *Acta Biochim. Pol.* **2012**, *59* (2), 201–212.

(16) Gupta, S. C.; Prasad, S.; Kim, J. H.; Patchva, S.; Webb, L. J.; Priyadarsini, I. K.; Aggarwal, B. B., Multitargeting by curcumin as revealed by molecular interaction studies. *Nat. Prod. Rep.* **2011**, *28* (12), 1937–1955. https://doi.org/10.1039/C1NP00051A

(17) Priyadarsini, K. I., Chemical and structural features influencing the biological activity of curcumin. *Curr. Pharm. Des.* **2013**, *19* (11), 2093–2100.

(18) Iersel, M. L.; Ploemen, J. P.; Struik, I.; Van Amersfoort, C.; Keyzer, A. E.; Schefferlie, J. G.; Van Bladeren, P. J., Inhibition of glutathione s-transferase activity in human melanoma cells by alpha,beta-unsaturated carbonyl derivatives. effects of acrolein, cinnamaldehyde, citral, crotonaldehyde, curcumin, ethacrynic acid, and trans-2-hexenal. *Chem. Biol. Interact.* **1996**, *102* (2), 117–132

(19) Sun, A.; Lu, Y. J.; Hu, H.; Shoji, M.; Liotta, D. C.; Snyder, J. P., Curcumin analog cytotoxicity against breast cancer cells: exploitation of a redox-dependent mechanism. *Bioorg. Med. Chem. Lett.* **2009**, *19* (23), 6627–6631. https://doi.org/10.1016/j.bmcl.2009.10.023

(20) Adams, B. K.; Cai, J.; Armstrong, J.; Herold, M.; Lu, Y. J.; Sun, A.; Snyder, J. P.; Liotta, D. C.; Jones, D. P.; Shoji, M., EF 24, a novel synthetic curcumin analog, induces apoptosis in cancer cells via a redox-dependent mechanism. *Anticancer Drugs* **2005**, *16* (3), 263–275. https://doi.org/10.1097/00001813-200503000-00005

(21) Mosley, C. A.; Liotta, D. C.; Snyder, J. P., In: *The Molecular Targets and Therapeutic Uses of Curcumin in Health and Disease*; Springer US: Boston, MA, 2007; pp 77–103. https://doi.org/10.1007/978-0-387-46401-5_2

(22) Tan, X.; Sidell, N.; Mancini, A.; Huang, R.-P.; Wang, S.; Horowitz, I. R.; Liotta, D. C.; Taylor, R. N.; Wieser, F., Multiple anticancer activities of EF 24, a novel curcumin analog, on human ovarian carcinoma cells. *Reprod. Sci.* **2010**, *17* (10), 931–940. https://doi.org/10.1177/1933719110374239

(23) Moore, T. W.; Zhu, S.; Randolph, R.; Shoji, M.; Snyder, J. P., Liver S9 fraction-derived metabolites of curcumin analogue UBS109. *ACS Med. Chem. Lett.* **2014**, *5* (4), 288–292. https://doi.org/10.1021/ml4002453

(24) Yin, D.-L.; Liang, Y.-J.; Zheng, T.-S.; Song, R.-P.; Wang, J.-B.; Sun, B.-S.; Pan, S.-H.; Qu, L.-D.; Liu, J.-R.; Jiang, H.-C.; Liu, L.-X., EF 24 inhibits tumor growth and metastasis via suppressing NF-kappaB dependent pathways in human cholangiocarcinoma. *Sci. Rep.* **2016**, *6*, 32167. https://doi.org/10.1038/srep32167

(25) Chen, W.; Zou, P.; Zhao, Z.; Chen, X.; Fan, X.; Vinothkumar, R.; Cui, R.; Wu, F.; Zhang, Q.; Liang, G.; Ji, J., Synergistic antitumor activity of rapamycin and EF 24 via increasing ROS for the treatment of gastric cancer. *Redox Biol* **2016**, *10*, 78–89. https://doi.org/10.1016/j.redox.2016.09.006

(26) Skoupa, N.; Dolezel, P.; Ruzickova, E.; Mlejnek, P., Apoptosis induced by the curcumin analogue EF-24 is neither mediated by oxidative stress-related mechanisms nor affected by expression of main drug transporters ABCB1 and ABCG2 in human leukemia cells. *Int. J. Mol. Sci.* **2017**, *18* (11). https://doi.org/10.3390/ijms18112289

(27) Lin, C.; Tu, C.; Ma, Y.; Ye, P.; Shao, X.; Yang, Z.; Fang, Y., Curcumin analog EF 24 induces apoptosis and downregulates the mitogen activated protein kinase/extracellular signal-regulated signaling pathway in oral squamous cell carcinoma. *Mol. Med. Rep.* **2017**, *16* (4), 4927–4933. https://doi.org/10.3892/mmr.2017.7189

(28) He, Y.; Li, W.; Hu, G.; Sun, H.; Kong, Q., Bioactivities of EF 24, a novel curcumin analog: A review. *Front. Oncol.* **2018**, *8*, 614. https://doi.org/10.3389/fonc.2018.00614

(29) Bertazza, L.; Barollo, S.; Mari, M. E.; Faccio, I.; Zorzan, M.; Redaelli, M.; Rubin, B.; Armanini, D.; Mian, C.; Pezzani, R., Biological effects of EF 24, a curcumin derivative, alone or combined with mitotane in adrenocortical tumor cell lines. *Molecules* **2019**, *24* (12), 2202. https://doi.org/10.3390/molecules24122202

(30) Zhou, T.; Ye, L.; Bai, Y.; Sun, A.; Cox, B.; Liu, D.; Li, Y.; Liotta, D.; Snyder, J. P.; Fu, H.; Huang, B., Autophagy and apoptosis in hepatocellular carcinoma induced by EF25-$(GSH)_2$: a novel curcumin analog. *PLoS One* **2014**, *9* (9), e107876. https://doi.org/10.1371/journal.pone.0107876

(31) Kohyama, A.; Fukuda, M.; Sugiyama, S.; Yamakoshi, H.; Kanoh, N.; Ishioka, C.; Shibata, H.; Iwabuchi, Y., Reversibility of the thia-Michael reaction of cytotoxic C_5-curcuminoid and structure-activity relationship of bis-thiol-adducts thereof. *Org. Biomol. Chem.* **2016**, *14* (45), 10683–10687. https://doi.org/10.1039/C6OB01771A

(32) Avonto, C.; Taglialatela-Scafati, O.; Pollastro, F.; Minassi, A.; Di Marzo, V.; De Petrocellis, L.; Appendino, G., An NMR spectroscopic method to identify and classify thiol-trapping agents: revival of Michael acceptors for drug discovery? *Angew. Chem. Int. Ed.* **2011**, *50* (2), 467−471. https://doi.org/10.1002/anie.201005959

(33) Amslinger, S.; Al-Rifai, N.; Winter, K.; Wormann, K.; Scholz, R.; Baumeister, P.; Wild, M., Reactivity assessment of chalcones by a kinetic thiol assay. *Org. Biomol. Chem.* **2013**, *11* (4), 549–554. https://doi.org/10.1039/C2OB27163J

(34) Qian, Y.; Zhong, P.; Liang, D.; Xu, Z.; Skibba, M.; Zeng, C.; Li, X.; Wei, T.; Wu, L.; Liang, G. A., Newly designed curcumin analog Y20 mitigates cardiac injury via anti-inflammatory and anti-oxidant actions in obese rats. *PLoS One* **2015**, *10* (3), e0120215. https://doi.org/10.1371/journal.pone.0120215

(35) Zhang, Y.; Pan, K.-L.; He, F.; Chen, L.-F.; Liu, Z.-G.; Liang, G., Crystal structure of (2E,6E)-2,6-bis[2-(trifluoromethyl)benzylidene]cyclohexanone, C22H16F6O. *Zeitschrift für Kristallograhie – New Crystral Stuctures* **2015**, *230* (3), 271–272. https://doi.org/10.1515/ncrs-2015-0009

(36) Liang, G.; Shao, L.; Wang, Y.; Zhao, C.; Chu, Y.; Xiao, J.; Zhao, Y.; Li, X.; Yang, S., Exploration and synthesis of curcumin analogues with improved structural stability both in vitro and in vivo as cytotoxic agents. *Bioorganic and Medicinal Chemistry* **2009**, *17* (6), 2623–2631. https://doi.org/ 10.1016/j.bmc.2008.10.044

(37) Kar, S.; Ramamoorthy, G.; Sinha, S.; Ramanan, M.; Pola, J. K.; Golakoti, N. R.; Nanubolu, J. B.; Sahoo, S. K.; Dandamudi, R. B.; Doble, M., Synthesis of diarylidenecyclohexanone derivatives as potential anti-inflammatory leads against COX-2/MPGES1 and 5-LOX. *New J. Chem.* **2019**, *43* (23), 9012–9020. https://doi.org/10.1039/C9NJ00726A

(38) Ma, S. Y.; Zheng, Z. B.; Sun, Y. F.; Wang, Z. Y., (2E,6E)-2,6-difurfurylidenecyclohexanone. *Acta Crystallogr. Sect. E Struct. Rep. Online* **2009**, *65* (12), o3085. https://doi.org/10.1107/S160053680904728X

(39) Pinto, N.; Retailleau, P.; Voituriez, A.; Marinetti, A., Organocatalytic enantioselective desymmetrization of cyclic enonesviaphosphine promoted [3+2] annulations. *Chem. Commun.* **2011**, *47* (3), 1015–1017. https://doi.org/10.1039/C0CC03164J

(40) Liu, G.-Y.; Jia, C.-C.; Han, P.-R.; Yang, J., 3,5-Bis(2-fluorobenzylidene)-4-piperidone induce reactive oxygen species-mediated apoptosis in A549 cells. *Med. Chem. Res.* **2018**, *27* (1), 128–136. https://doi.org/10.1007/s00044-017-2056-x

(41) Bagiyan, G. A.; Koroleva, I. K.; Soroka, N. V.; Ufimtsev, A. V., Oxidation of Thiol compounds by molecular oxygen in aqueous solutions. *Russ Chem Bull* **2003**, *52* (5), 1135–1141. https://doi.org/10.1023/A:1024761324710